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EVALUATION OF THE METABOLIC
RESPONSE, BLOOD CHEMISTRY PROFILE,
AND CARDIAC MORPHOLOGY OF BEEF
HEIFERS SUPPLEMENTED WITH
ZILPATEROL HYDROCHLORIDE
FOLLOWED BY AN ENDOCRINE INDUCED
STRESS CHALLENGE

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EVALUATION OF THE METABOLIC RESPONSE, BLOOD CHEMISTRY
PROFILE, AND CARDIAC MORPHOLOGY OF BEEF HEIFERS SUPPLEMENTED
WITH ZILPATEROL HYDROCHLORIDE FOLLOWED BY AN ENDOCRINE
INDUCED STRESS CHALLENGE

by

Joe O. Buntyn

A DISSERTATION

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Major: Animal Science (Meat Science and Muscle Biology)

Under the Supervision of Professor Ty B. Schmidt

Lincoln, Nebraska

August, 2016

EVALUATION OF THE METABOLIC RESPONSE, BLOOD CHEMISTRY
PROFILE, AND CARDIAC MORPHOLOGY OF BEEF HEIFERS SUPPLEMENTED
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Joe Oscar Buntyn, Ph.D.

University of Nebraska, 2016

Advisor: Ty B. Schmidt

The objective of this study was to determine the metabolic, stress, hematology, organ weights, histology and cardiac anatomical features of beef heifers supplemented with zilpaterol hydrochloride (ZH) when exposed to a corticotropin-releasing hormone (CRH) and arginine vasopressin (VP) challenge. Crossbred heifers ($n = 20$; 556 ± 7 kg BW) were randomized into two treatment groups: 1) Control (CON): no ZH, and 2) Zilpaterol (ZIL): supplemented with ZH at 8.33 mg / kg (DM basis). The ZIL group was supplemented ZH for 20 d, with a 3-d withdrawal period. On d 24, heifers received an intravenous bolus of CRH ($0.3 \mu\text{g} / \text{kg BW}$) and VP ($1.0 \mu\text{g} / \text{kg BW}$). Blood samples were collected at 30- and 60-min intervals for serum, and 60-min intervals for plasma and whole blood, from -2 to 8 h relative to the challenge. Heifers were harvested to collect organ weights and histology samples on d 25, 26, and 27. There was a treatment ($P \leq 0.001$) effect for vaginal temperature (VT), with ZIL having a 0.1°C decrease in VT. A treatment x time effect ($P = 0.002$) was observed for non-esterified fatty acid such that concentrations were increased in CON heifers at 4, 6, 7, 7.5, and 8 hrs. There was a treatment effect for cortisol ($P \leq 0.01$) and epinephrine ($P = 0.003$) with ZIL having

decreased concentrations. Glucose concentrations within the *longissimus* muscle were greater ($P = 0.03$) in CON heifers. Lactate concentrations and glycolytic potential within the *biceps femoris* were greater in CON heifers ($P = 0.05$). A treatment effect ($P \leq 0.02$) was observed for calcium and potassium, with ZIL heifers having decreased concentrations. Zilpaterol heifers had increased concentrations of creatinine ($P = 0.02$). A treatment x time interaction ($P = 0.02$) was observed for phosphorus. Raw liver weights tended ($P = 0.08$) to be less in ZIL heifers. These data suggest some variations between treatments in terms of response to ZH supplementation and challenge; however, in the environmental conditions of this study, the data suggests that the supplementation of ZH does not negatively alter the status of homeostasis in cattle.

DEDICATION

I dedicate this dissertation to my mother; Lorraine Buntyn; and my beautiful wife, Whitney Buntyn. My mother has always believed in me and given me unconditional love and support with inspiration to always pursue my dreams. She is the reason I developed a passion for cattle and even pursued a college degree. And to the love of my life Whitney, there is no telling where I would be if I had not meet her. Her love and support has made every endeavor we take on possible. I know she will always be right beside me no matter where this journey takes us.

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Chapter I

Introduction

The United States feedlot industry operates with small profit margins yearly but is able to increase profit potential by marketing more pounds of product. Therefore, repartitioning agents are beneficial in beef production to increase pounds of marketable product and improve growth efficiencies. The first reported repartitioning beta-adrenergic agonist in livestock species was clenbuterol (Ricks et al., 1984). To date, two β -AA are approved to be fed to cattle in the United States. Increases in ADG have been observed (Montgomery et al., 2009a) with slight decreases in DMI with β -AA supplementation resulting in increased feed efficiency (Mersmann, 2002) and an increase in gain to feed ratio (Avenidaño-Reyes et al., 2006). β -adrenergic receptor agonists are associated with an increase in lean tissue deposition in carcass components (HCW increase 13 and 11 kg in steers and heifers, respectively, (Montgomery et al., 2009a) and a decrease in adipose tissue (Mersmann, 2002; Mersmann, 1998).

Although the first approved β -AA has been on the market for over 25 years, little is actually known about how β -AA interact with endogenous stress hormones during a stress response. Anecdotal claims have implemented zilpaterol hydrochloride (ZH) as a potential cause of lame, stiff moving cattle at abattoirs. Furthermore, an epidemiological risk of increased mortality associated with ZH supplementation during the late feeding period has been reported; however, no cause of the mortality was reported (Loneragan et al., 2014). Therefore, the following literature review evaluates the overall status of homeostasis of animals' supplemented β -AA.

CHAPTER II

LITERATURE REVIEW

History of beta-adrenergic agonist

The cost of livestock products directly depends on the efficiency of production; which is categorized into three segments: female production, reproduction, and growth of the young (Dickerson, 1970). An issue that affects the production and efficiency of growth of livestock is the ratio of adipose tissue to lean muscle mass (Ricks et al., 1984). Two metabolic modifier technologies have been utilized in the cattle industry to increase the accretion of lean muscle mass. These metabolic modifiers are anabolic steroids and β -adrenergic agonists (β -AA; (Schmidt and Olson, 2007). This literature review will focus on the history, mode of action, and production effects of β -AA with an emphasis on changes to blood metabolites, stress response, and organ effects of β -AA.

The use of β -AA as a growth promotion tool in livestock has been investigated for over 50 years. Beta adrenergic agonists are phenethanolamines with a similar structure to the naturally occurring catecholamines (dopamine, norepinephrine, and epinephrine). Catecholamines and β -AA elicit a response by binding to β adrenergic receptors (β -AR; (Scramlin et al., 2010). Phenethanolamine adrenergic agents share common structural components; however, not all phenethanolamines elicit β -AA activity. In fact, some phenethanolamine compounds actually elicit α and β adrenergic antagonist effects (Smith, 1998). Furthermore, phenethanolamines are further divided into subcategories depending on activation of different α and β receptor families (Smith, 1998). These adrenergic receptors were first described in 1948 when two distinct classes of receptors

where distinguished and classified as α and β adrenotropic receptors (Ahlquist, 1948). Ahlquist, (1948) suggested that α receptors were associated with excitatory functions such as vasoconstriction and uterine contractions, while β receptors were associated with inhibitory functions such as vasodilation and inhibition of uterine and bronchial muscle contraction. Ahlquist's suggestion were approximately 47 years after Takamine and Aldrich isolated adrenalin from adrenal glands in 1901 (Dakin, 1905). However, the idea of potential receptors mediating the response of a stimulus was first introduced by Lewandowsky in 1899 and Langley in 1901 when suprarenal extract was able to elicit a response after the removal of the superior cervical ganglion (Langley, 1905). These results led to the conclusion that adrenal gland extracts acted directly on the tissue of interest and not nerve endings (Langley, 1905). These discoveries allowed for the development of pharmaceuticals that are able to elicit a response through β - adrenergic receptors.

β – adrenergic agonist

In livestock, two β -AA have received approval by the United States Food and Drug Administration, ractopamine hydrochloride and zilpaterol hydrochloride. Ractopamine hydrochloride received approval for the use in swine to increase rate of weight gain, improve feed efficiency, and increase carcass leanness in 1999 (NADA, 1999). In 2003, ractopamine hydrochloride was approved for use in beef cattle fed in confinement the last 28 - 42 d on feed to increase rate of weight gain, improve feed efficiency, and increase carcass leanness (NADA, 2003). In 2006, zilpaterol hydrochloride was approved to be fed to cattle in confinement during the last 20 – 40 d

on feed to increase rate of weight gain, improve feed efficiency and increase carcass leanness (NADA, 2006). Lastly, ractopamine hydrochloride was approved in 2009 to be fed to turkeys for the last 7 – 14 d before harvest in hen turkeys and the last 14 d before harvest in tom turkeys to increase rate of weight gain and improve feed efficiency (NADA, 2009). To date, ractopamine hydrochloride is approved for use in swine, cattle and turkey production in the United States. Zilpaterol hydrochloride is only approved for use in confined cattle finishing in the United States.

Mode of action

The binding of either a natural or synthetic ligand to a β -AR results in the activation of the receptor and is required to initiate a cellular response (Mersmann, 1998). β - adrenergic receptors are G stimulatory protein coupled receptors that initiate a signal response of cascading events when bound to naturally occurring catecholamines or β -AA (Mersmann, 1998; Scramlin et al., 2010). β - adrenergic receptors are located on the plasma membrane of most mammalian cells and are physiologically stimulated by natural catecholamines (Mersmann, 1998). When β -AAs are orally ingested, binding and activation to β -ARs can increase protein accretion while decreasing lipogenesis (Mersmann, 1998). After oral ingestion and absorption, peak plasma concentrations are observed in approximately 1 to 3 h in humans (Morgan, 1990) with a similar response observed in livestock (Smith, 1998). β - adrenergic agonist are virtually insoluble in non-polar solvents and are not generally considered lipophilic (Smith, 1998).

Upon absorption, β -AA are transported through the body to different cells via the circulation system. At the designated cell, β -AA bind to β -AR to form an agonist-

receptor complex which stimulates the G stimulatory protein (Mersmann, 1998).

Activation of the α -subunit of the G protein couple seven-transmembrane receptor results in the production of the enzyme adenylyl cyclase (Mersmann, 1998). Adenylyl cyclase produces cyclic adenosine monophosphate (cAMP) which is a major intracellular signaling compound. To elicit a response, cAMP binds the regulatory subunit of protein kinase A which results in the release of the catalytic subunit of protein kinase A (Liggett and Raymond, 1993; Mersmann, 1998). The catalytic subunit phosphorylates intracellular proteins which activates the targeted protein. Protein kinase A also phosphorylates the cAMP response element binding protein (CREB; (Mersmann, 1998). Once phosphorylated, CREB can bind to a cAMP response element located in the regulatory portion of a specific gene which enables transcription of that particular gene (Mersmann, 1998). With the phosphorylation of CREB, transcriptional activity of the gene increases allowing β -AA to elicit a response in gene transcription of mammalian cells (Mersmann, 1998). The use of caffeine, theophylline, nicotine, and epinephrine in a pig model displayed an increase in cAMP resulting in an increase in muscle mass (Cunningham, 1965). This discovery led to the utilization of β -AA as potential growth promotion technology in livestock production.

β – adrenergic agonist do not elicit similar responses across all species. These effects tend to be less pronounced in broilers when compared to sheep and cattle; swine displaying an intermediate response (Mersmann, 1998). One potential explanation to the observed results could be due to genetic selection for maximum growth potential in a vertically integrated system such as the poultry industry (Mersmann, 1998). However,

another potential explanation of the species difference could potentially be explained by different quantities and subtypes of β -AR.

Cattle performance

The magnitude of the response observed in cattle administered a β -AA varies depending on which β -AA is utilized. Furthermore, the age, gender, breed, plane of nutrition, and duration of β -AA administration can affect the results observed in cattle (Bell et al., 1998b; Lean et al., 2014; Mersmann, 1998). The utilization of ZH at the end of the feeding period resulted in a 15 kg increase in hot carcass weight (HCW) with increased dressing percentages and decreased USDA yield grade consistently (Hilscher et al., 2015; Lean et al., 2014; Vasconcelos et al., 2008). There was no observed difference in final live BW when control cattle were compared with ZH supplementation for 20, 30, and 40 d of feeding. However, HCW was increased approximately 17 kg in ZH treatments when compared to with the control cattle (Vasconcelos et al., 2008). Furthermore, there was no observed increase in final live BW or ADG for cattle fed ZH for 20 d when compared with control steers (Hilscher et al., 2015). In contrast, an approximate 9 kg increase in final BW and 14 kg increase in HCW was reported for steers supplemented with ZH, when compared with control steers (Elam et al., 2009).

Zilpaterol hydrochloride has been reported to increase ADG and G:F when supplemented to cattle. Steers supplemented with ZH for 20 d resulted in an increase of 0.47 kg / d and 0.056 kg / kg for ADG and G:F, respectively, with a tendency for decreased DMI (Montgomery et al., 2009a). Furthermore, steers supplemented with ZH for 21 d resulted in an increase of 0.8 kg / d and 0.016 kg / kg for ADG and G:F

respectively; however, no decrease in DMI was observed over the duration of the feeding period (Hales et al., 2014). No difference were observed for DMI or ADG in steers supplemented with ZH for 20 d when compared with control steers; however, there was an increase observed for G:F over the duration of the feeding period for steers supplemented with ZH when compared with the negative control (Hilscher et al., 2015). Lastly, there was no reported difference in DMI for steers fed ZH for 30 d (Baxa et al., 2010). However, a meta-analysis of studies evaluating the effect of supplementing cattle with ZH revealed an increase of 8 kg in live BW with a 0.12 kg / d reduction in DMI and an increase of 0.15 kg / d in ADG (Lean et al., 2014). Overall, decreases in DMI and increases in ADG and final live BW have been variable across studies evaluating the effects of supplementing cattle with ZH regardless of duration.

In steers supplemented with ZH for 20, 30, or 40 d, a 14 kg increase was observed for HCW when compared with control steers, and an approximate 2 kg increase in HCW between all ZH treatments (Elam et al., 2009). A 13 kg increase in HCW with a 1.3 percent increase in dressing percentage with a 0.38 unit decrease in USDA yield grade and a 7.2 cm² increase in loin muscle area was reported for steers supplemented with ZH for 20 d (Montgomery et al., 2009a). Furthermore, steers supplemented with ZH for 30 days resulted in an average 8.8 cm² increase in loin muscle area and an average of 0.70 unit decrease in USDA yield grade (Hilton et al., 2009). Hilscher et al. (2015) reported similar results; 13 kg increase in HCW and a 7.3 cm² increase in loin muscle area, and a 0.67 unit decrease in USDA yield grade when ZH was fed for 20 d. Overall, ZH has been reported to increase loin muscle area and decrease USDA yield grade in cattle regardless of feeding duration.

Effects on body temperature

Maintaining body temperature requires internal thermoregulatory mechanism of the animal and a resistance of energy exchange from the environment (Finch, 1986). The feeding of ZH has been reported to decrease rumen temperature when compared with control steers (Boyd et al., 2015). As reported by Boyd et al (2015) the decreased body temperature may be attributed to increased respiration rates, allowing ZH supplemented steers the ability to dissipate excess body heat. Another biological response typically observed following stimulation of the β -AR is vasodilation of interior tissues (e.g., skeletal muscle, heart, lungs, liver, and gastrointestinal tract; (Dawes et al., 1997). An increase in vasodilation could potentially allow dissipation of heat from the body surface at a faster rate. However, it would be expected to see a decline in vasodilation with continued treatment of β -AA due to a down-regulation of the β -AR (Smith et al., 1994). Understanding blood flow changes as a result of β -AA supplementation, and particularly ZH, would help explain differences observed in body temperature, and also nutrient delivery and blood cell population changes.

Blood metabolites and beta-adrenergic agonist

Glucose: Glucose is a sugar molecule that is the primary source of energy for life processes of mammalian cells. Historically, glucose has not been considered a prominent metabolic intermediate in the rumen due to irrelevant concentrations (Owens et al., 1998). However, in engorgement studies, rumen glucose concentrations have exceeded 160 mg / dL which exceeds normal blood reference ranges for cattle (Horn et al., 1979).

Reported reference ranges for blood glucose concentrations in cattle are 45-75 mg / dL (Kaneko et al., 2008).

In rainbow trout supplemented RH at 5, 10, 20, and 40 ppm for 12 wks, serum glucose concentrations were increased (Vandenberg et al., 1998). Furthermore, rainbow trout supplemented RH at 40 ppm for 8 wk had increased glucose concentrations when compared to controls (Vandenberg et al., 1998). Acute stimulation of β -AR increases the release of glucose from the liver and muscles (Mersmann et al., 1987). However, this initial increase in glucose concentration is thought to diminish over time due to decreased tissue sensitivity and return to concentrations observed in control calves following acute β -AA ingestion (Zimmerli and Blum, 1990). Steers fed clenbuterol (β_2 -AA) over a 9 d period had increased plasma glucose concentrations on d 1 of supplementation. However, on d 9 of clenbuterol supplementation, plasma glucose concentrations were not different when compared to control steers (Eisemann et al., 1988a).

In steers supplemented with ZH for 23 d, whole blood glucose decreased over time compared with control steers (Van Bibber-Krueger et al., 2015). β - adrenergic receptors were determined to be located on spleen and lung tissue samples (Hasegawa and Townley, 1983). Van Bibber-Krueger et al. (2015) hypothesized that β -AA could potentially activate β -AR located on the spleen to increase secretion of red blood cells which could potentially increase cells available to metabolize glucose. Steers supplemented with cimaterol (β_2 -AA), at a rate of 0, 10, or 500 mg / d for 98 d had no effect on plasma glucose concentrations (Ricks et al., 1984). Similar results of no change in plasma glucose concentrations were observed in steers supplemented with cimaterol at

rates of 0, 33, 49.5, or 66 mg / d (Quirke et al., 1988). Furthermore, no difference was observed in blood glucose concentrations of lambs fed cimaterol at 0 or 10 mg / kg for 7 or 12 wks (Beermann et al., 1987). Lastly, no difference was observed in serum glucose concentrations of feedlot lambs supplemented with either RH or ZH at 0.35, 0.70, or 1.05 mg / kg of BW / d; or 0.10, 0.20, or 0.30 mg / kg of BW / d, respectively, when compared to control lambs (López-Carlos et al., 2010).

Insulin: Insulin is a peptide hormone secreted by β cells in the islets of Langerhans located in the pancreas. Insulin inhibits the release of glucagon and stimulates the uptake of glucose into muscle and adipose tissue (Reece et al., 2015). Three-week treatment of growing lambs with cimaterol resulted in a 50% decrease in insulin concentrations (O'Connor et al., 1991). It has been hypothesized that the repartitioning effects of phenethanolamines could be partially due to opposing effects of insulin sensitivity in adipose tissue compared to muscle tissue (Anderson et al., 1991). In rats and pigs supplemented RH, reduced insulin sensitivity in adipocytes has been observed (Hausman et al., 1989; Liu and Mills, 1990). In rats chronically treated with adrenaline, an increase in insulin sensitivity was observed in the soleus muscle (Budohoski et al., 1987). Cattle supplemented with RH at 80 mg / kg of feed had no change in insulin secretion or uptake when compared with controls utilizing the hyperinsulinemic, euglycemic clamp approach to conduct measurement (Eisemann and Bristol, 1998). Lastly, in postpartum beef cows supplemented with ZH at 0.15 mg / kg of BW for 33 d had decreased insulin concentrations starting on d 14 of supplementation when compared with controls (Guzman et al., 2012). The conflicting reports in the

literature on β -AA effects on insulin concentrations could potentially be due to the activation of different subclasses of β -AR and diets fed during these research trials.

Blood urea nitrogen: Blood urea nitrogen (BUN) is a measurement of urea concentrations produced by the urea cycle as a waste product of protein breakdown and is utilized as a measurement of renal function (Kaneko et al., 2008). Blood urea nitrogen concentrations can increase in healthy animals due to increased dietary protein (White et al., 1984). In cattle, reported reference ranges for BUN are 20-30 mg / dL (Kaneko et al., 2008). Steers supplemented with clenbuterol at 500 mg / d for 98 days had decreased BUN concentrations at harvest when compared with control cattle that did not receive clenbuterol. Furthermore, steers supplemented with clenbuterol at 10 mg / dL for 98 d tended to have decreased BUN concentrations when compared with controls (Ricks et al., 1984). Decreased BUN concentrations are associated with an increase in nitrogen retention (Davis et al., 1970; Preston, 1968). Van Bibber-Krueger et al. (2015) reported decreased plasma urea concentrations in steers supplemented with ZH at 8.33 mg / kg of the diet for 23 d; suggesting decreased protein catabolism in skeletal muscle. A decrease in BUN concentrations would be expected in cattle administered β -AA due to increased nitrogen retention associated with the increased deposition of muscle mass (increased HCW (Hilscher et al., 2015; Montgomery et al., 2009b).

Non-esterified fatty acid: Non-esterified fatty acids (NEFAs) are fatty acids released from stored triacylglycerols in adipose tissue through actions of hormone-sensitive lipase (Reece et al., 2015). Once released, NEFAs are transported by albumin to target tissues where oxidation takes place releasing energy (Reece et al., 2015). A

common effect of β -AA supplementation is decreased adipose tissue which could possibly be due to increased fat mobilization, decreased lipogenesis, or a combination of both of these biological activities (Yang and McElligott, 1989). In an attempt to estimate lipolysis, plasma NEFA concentrations can be utilized due to the strong correlation between lipolysis and NEFA turnover (Beermann et al., 1987). Beermann et al. (1987) attributed a 60% increase in circulating NEFA concentrations in lambs to fasting for 1 d before sampling instead of cimaterol supplementation. In steers administered cimaterol at rates of 0, 33, 49.5, or 66 mg / d, a quadratic increase was observed for NEFA concentrations with steers receiving 33 mg / d having the least NEFA concentrations and steers receiving 66 mg / d having the greatest NEFA concentrations between treatments (Quirke et al., 1988). Steers supplemented with cimaterol at 0.09 mg / kg of BW for 13 d had an increase in NEFA concentrations on d 1 and 13 when compared to steers not receiving cimaterol (Chikhou et al., 1991a). Chikhou et al. (1991) attributed increased NEFA concentrations of steers receiving cimaterol to increased lipolysis and reduced free fatty acid uptake. In a closed arterial infusion trial utilizing steers, cimaterol administration increased plasma NEFA concentrations at 6 h following initial infusion (Byrem et al., 1996). In a following closed arterial infusion trial, cimaterol administration increased plasma NEFA concentrations of steers at 6 h of infusion; however, at 24 h of infusion, NEFA concentrations had returned to concentrations observed during basal sampling (Byrem et al., 1998). In steers supplemented with ZH for 23 d, no difference in NEFA concentrations were observed when compared with control steers (Van Bibber-Krueger et al., 2015).

β - hydroxybutyrate: Metabolism of NEFAs in the liver produces β - hydroxybutyrate which is a ketone body that can be increased during times of negative energy balance (Ospina et al., 2010). In cattle, the average range for β - hydroxybutyrate are reported as 0.41 ± 0.03 mmol / liter (Kaneko et al., 2008). No difference was observed for β - hydroxybutyrate in steers supplemented with 8 mg / d of clenbuterol in a balanced, single reversal trial (Eisemann et al., 1988a). In steers supplemented with ZH for 23 d, no difference was observed in β - hydroxybutyrate concentrations when compared with control steers (Van Bibber-Krueger et al., 2015). It appears cattle supplemented β-AA are capable of metabolizing β - hydroxybutyrate sufficiently which prevents a detectable increase in circulating concentrations (Van Bibber-Krueger et al., 2015).

Creatinine: Reported reference values for serum creatinine in cattle are 1.0-2.0 mg / dL (Kaneko et al., 2008). During normal muscle metabolism, creatine is broken down into creatinine which has been utilized as an indicator of muscle mass in serum (Russell and Roussel, 2007). Furthermore, serum creatinine is positively correlated with carcass weight, dressing percentage, and proportion of lean meat (Istasse et al., 1990). Cimaterol supplementation , increased plasma creatinine concentrations in Friesian steers during long term supplementation (Chikhou et al., 1993; Signorile et al., 1995). Furthermore, increased creatinine concentrations have been observed in sheep and cattle during heat stress (Koubkova et al., 2002; Srikandakumar et al., 2003). Peripheral vasodilation increases during heat stress, reducing blood flow to organs in an attempt to dissipate more heat (Srikandakumar et al., 2003). This decrease in blood flow during

vasodilation could potentially decrease renal filtration of creatinine resulting in increased circulating concentrations of creatinine (Scharf et al., 2010). Since β -AA act as vasodilators, an increase in creatinine concentrations could potentially be due to decreased renal blood flow and increases in total muscle mass in animals supplemented with β -AA.

Creatine kinase: Creatine kinase is a key enzyme for cellular energetics as a catalyst for the reversible reaction of creatine to phosphocreatine (Wallimann et al., 1992). Reported normal reference ranges for creatine kinase in cattle are 4.8-12.1 U / L (Kaneko et al., 2008). During FDA approval, creatine kinase concentrations were reported to be increased in cattle fed ZH during approval trials (NADA, 2006). No increase in creatine kinase activity was observed in steers under normal feedlot conditions supplemented with ZH or RH when compared with a control group. However, creatine kinase concentrations were increased above normal reference ranges in two downer steers euthanized at a commercial abattoir that were reportedly fed ZH (Thomson et al., 2015). Creatine kinase has been reported to increase when steers are transported due to an increase in muscle cell membrane permeability due to increased muscle activity (Warriss et al., 1995). Furthermore, increased creatine kinase concentrations were observed in steers on d 14 and 21 of ZH supplementation (Fuller, 2014). Increased creatine kinase concentrations were also observed in swine supplemented with RH (Athayde et al., 2013). In three horses supplemented with ZH at 0.17 mg / kg of BW, increased creatine kinase concentrations were observed after 1 d of supplementation (Wagner et al., 2008). The authors attributed this increase in creatine kinase to the

experimental design in which the horses consumed the entire dose of ZH at one time; thus potentially causing muscle damage (Wagner et al., 2008).

Lactate and lactate dehydrogenase: Cells with increased glucose demand produce lactate during glucose utilization. Pyruvate is produced when glucose is broken down during glycolysis. Pyruvate concentrations increases intracellular absorbing two protons allowing the enzymatic production of lactate (Reece et al., 2015). Thomson et al. (2015) and Van Bibber-Krueger et al. (2015) reported no difference in lactate concentrations of steers supplemented with ZH. Van Bibber-Krueger et al. (2015) observed a numerical decrease in plasma lactate concentrations as d on ZH increased. The inclusion of P-5369 and Q-2636 β -AA within milk replacer increased lactate concentrations in calves (Blum and Flueckiger, 1988). Eisemann et al. (1988) observed increased lactate concentrations with the inclusion of clenbuterol in steer diets. Furthermore, arterial cimaterol infusion increased lactate concentrations in a dose-dependent manner (Byrem et al., 1996). The reported increases in lactate concentrations suggest that cattle supplemented with β -AA have an increase in peripheral glycolysis (Eisemann et al., 1988a) in anaerobic conditions.

The glycolytic enzyme lactate dehydrogenase (LDH) catalyzes the conversion of lactate to pyruvic acid (Doornenbal et al., 1988). The reported reference range for LDH in cattle is 692 – 1445 U / liter (Kaneko et al., 2008). When ZH was administered to horses at a rate of 0.17 mg / kg of BW, an increase in LDH was observed (Wagner et al., 2008). In cattle, the effects of supplementation with β -AA on LDH concentrations have not been reported in controlled studies.

Serum protein profiles: There are two major proteins (i.e., albumin and globulin) that contribute to the total serum proteins concentrations evaluated during a serum chemistry profile test (Russell and Roussel, 2007). Albumin and other globulin fractions are indicators of protein status (Evans and Duncan, 2003). Albumin is primarily responsible for the sequestering of H₂O into the circulatory system during oncotic pressure and is synthesized in the liver (Russell and Roussel, 2007). Immunoglobulins synthesized in lymphoid cells make up a majority of the total globulins located in circulation, with other globulins being synthesized in the liver (Russell and Roussel, 2007). The reported reference concentrations for albumin and globulin in cattle are 30.3-35.5 and 32.9 ± 1.3 g / L, respectively (Kaneko et al., 2008). An increase in albumin and/or globulin results in a condition known as hyperproteinemia. Dehydration is the major known cause of an increase in albumin resulting in hyperalbuminemia (Russell and Roussel, 2007). Cases of hyperproteinemia without dehydration are usually caused by increased globulin concentrations. One common cause of hyperglobulinemia is chronic inflammatory diseases and hepatic disease (Russell and Roussel, 2007). Lopez-Carlos et al. (2010) reported that ZH supplementation at either 0.10, 0.20, or 0.30 mg / kg of BW/d for the last 42 d of feeding had no impact on serum total protein (López-Carlos et al., 2010). Furthermore, the feeding of clenbuterol to Hereford steers did not impact serum total protein or albumin (Ricks et al., 1984).

Minerals

The mineral calcium is vital for vertebrate animals to maintain homeostasis and functions in muscle contraction, blood coagulation, enzyme activity, hormone secretion,

and is a major component of bone structure (Capen and Rosol, 1993). The two primary functions of calcium in the body are to maintain bone structure and serve as a messenger or regulatory ion in the body (Ebashi, 1985). Ninety-nine percent of calcium in the body is located in the inorganic matrix of bone and hydroxyapatite with the remaining 1% of calcium being located in plasma membranes, endoplasmic reticulum of cells, and extracellular fluids (Kaneko et al., 2008). Calcium is absorbed from the diet with the majority of absorption taking place in the duodenum (McDowell, 2003). Ricks et al. (1984) reported no difference in blood calcium concentrations at harvest of steers receiving 0, 10, or 500 mg / hd / d supplemented with clenbuterol for 98 d. Supplementation of ZH for 20 d resulted in a decrease in calcium concentrations in steers and heifers when compared with cattle not receiving ZH. Furthermore, a greater reduction of calcium concentrations was observed in steers receiving ZH supplementation when compared with heifers receiving ZH supplementation (Burson, 2014). However, in steers receiving 90 mg / hd / d of ZH, there was no difference in calcium⁺ concentration when compared with control steers (Fuller, 2014).

In the mammalian body, 90% of phosphorus is located in the mineralized matrix of bone in the body as hydroxyapatite. The remaining 10% of phosphorus is located in soft tissues and serves as a major intracellular anion (Dennis, 1996). Approximately 60 – 70% percent of dietary phosphorus is absorbed by active transport coupled to H⁺ ion in ruminants and by passive diffusion (Shirazi-Beechey et al., 1996). In ruminants, phosphorus adsorption primarily takes place in the forestomachs (Care, 1994). Furthermore, salivary secretions supply endogenous phosphorus to the gastrointestinal tract in ruminants (Shirazi-Beechey et al., 1996). Ricks et al. (1984) reported no

difference in phosphorus concentrations at harvest in steers receiving 0, 10, or 500 mg / hd / d of clenbuterol for 98 d. In steers supplemented with 90 mg / hd / d of ZH, there was no difference in phosphorus concentrations when compared with steers that received no β -AA (Fuller, 2014).

The major cation maintaining osmotic pressure and water content of extracellular fluid is sodium (Reece et al., 2015). Sodium functions to maintain acid-base balance and is crucial in the transmission of nervous impulses by determining electrical potential of nerve tissues. The major cation maintaining osmotic pressure and water content of intracellular fluid is potassium (Reece et al., 2015). Potassium is present in extracellular fluid but is highly regulated due to major effects on organs that could become life threatening (Russell and Roussel, 2007). Lastly, chloride is the major anion of extracellular fluid that is a component of bodily secretions such as sweat, saliva, and gastric secretions (Russell and Roussel, 2007). Normally, sodium and chloride concentrations are parallel due to simultaneous reabsorption in the kidneys. Together, these three minerals are classified as electrolytes which can be utilized with the inclusion measurement of carbon dioxide or bicarbonate to calculate the anion gap (Russell and Roussel, 2007). The anion gap is the calculated difference of the major cations (sodium and potassium) and the major anions (chloride and bicarbonate) that is utilized to determine acid-base disorders. Bicarbonate is a buffer that neutralizes H^+ that is produced during metabolism (Kaneko et al., 2008).

Due to a significant amount of mineral storage in bone, serum circulating concentrations of minerals may be an unreliable measurement on total body mineral

status of an animal. However, in steers and heifers, supplementation of ZH resulted in an increase in blood potassium concentrations when compared with steers and heifers that received no β -AA (Burson, 2014). Furthermore, Fuller (2014) reported an increase in potassium and chloride in steers receiving either 300 mg / d of RH or 90 mg / d of ZH when compared with steers receiving no β -AA. Ramirez et al. (1992) reported a decrease in bicarbonate concentrations of Holstein cows reared at sea level when compared with Holstein cows reared at 3,000 m. Cows reared in high altitude are exposed to ambient hypoxia resulting in the development of decreased bicarbonate concentrations to compensate abnormal acid-base concentrations (Ramirez et al., 1992). Clenbuterol has been reported to increase blood flow and oxygen uptake in the hind-quarters of cattle during early supplementation (Eisemann and Huntington, 1987). Due to an increase in oxygen uptake observed in β -AA supplemented cattle, greater bicarbonate concentrations could suggest an increase in oxygen availability with β -AA supplementation.

Liver enzymes

Alkaline phosphatase (ALP) catalyzes the cleavage of inorganic phosphate from phosphate esters with production of ALP originating in the liver of mature animals (Doornenbal et al., 1988). Furthermore, in normal animals, serum ALP originates from liver and bone tissue (Rogers, 1976). The reference values for ALP in the cow are 78 – 132 U / L (Kaneko et al., 2008). Alkaline phosphatase concentrations will increase with acute or chronic liver disease; however, cholestasis conditions result in significant ALP concentration increases (Kaneko et al., 2008). Ricks et al. (1984) reported no difference in ALP in Hereford steers supplemented with clenbuterol. In dogs with increased

corticosteroid concentrations, ALP concentrations are increased due to the formation of a more heat stable isoenzyme of ALP (Teske et al., 1986).

Aspartate transaminase (AST) is an enzyme that catalyzes the transfer of an α -amino group from an amino acid to an α -keto acid (Doornenbal et al., 1988). The most important tissues for AST production are liver and muscle (Russell and Roussel, 2007). During acute or chronic liver injury, serum AST concentrations may increase; however, AST activity can also be elevated in kidney, pancreas, and erythrocytes which can increase circulating concentrations if cells are damaged (Kaneko et al., 2008). An increase in AST concentrations was observed in Friesian calves administered cimaterol (Chikhou et al., 1993). Ricks et al. (1984) also reported an increase in AST concentrations in steers supplemented with clenbuterol at 10 and 500 mg / hd / d for 98 d when compared to steers not receiving clenbuterol. Furthermore, AST concentrations were increased above normal reference ranges in two downer steers euthanized at a commercial abattoir that were reportedly fed ZH (Thomson et al., 2015).

Gamma glutamyltransferase (GGT) is a membrane bound enzyme that catalyzes the transfer of gamma glutamyl groups from gamma glutamylpeptides to other peptides or amino acids with a normal reference range in cattle of 6.1 – 17.4 U / L (Kaneko et al., 2008). In ruminants, GGT is considered to be a more appropriate indication of liver disease than ALP (Russell and Roussel, 2007). Serum GGT predominately originates from cellular membranes of the bile and liver and is indicative of liver disease (Russell and Roussel, 2007). In canines, glucocorticoids have been associated with an increase in GGT concentrations (Shull and Hornbuckle, 1979).

Sorbitol dehydrogenase (SDH) can be utilized as a specific indicator of acute liver damage in ruminants due to a dramatic increase in serum concentrations followed by a drastic decrease in activity after liver damage (Russell and Roussel, 2007). Reported SDH reference ranges for the cow are 4.3 – 15.3 U / L (Kaneko et al., 2008). Feed restriction in llamas resulted in an increase in hepatic lipidosis which resulted in elevated SDH concentrations (Tornquist et al., 2001).

Complete blood cell count (CBC)

A complete blood cell count (CBC) is a hematology test that evaluates the leukocytes and other constituents of blood with the primary function of screening for hemic system abnormalities due to disease or determining hematologic disorders (Stockham and Scott, 2013). Red blood cells (RBCs) or erythrocytes are evaluated in a CBC to determine size and abnormalities. Erythropoietin production in the kidneys results in the production of RBCs in bone marrow (Jones and Allison, 2007). The function of RBCs is gas exchange which takes place when RBCs are in the heme structure allowing for the transportation of oxygen and carbon dioxide (Jones and Allison, 2007). Hematocrit or packed cell volume (PCV) in conjunction with total plasma protein can be utilized to evaluate changes in fluid and electrolyte balance. A change in hematocrit values results from a plasma volume alteration if no change in total protein is assumed (Kaneko et al., 2008). In steers and heifers, ZH supplementation for 20 days did not affect hematocrit concentrations when compared with control cattle not receiving ZH (Burson, 2014). The effects of clenbuterol supplementation and treadmill exercise were evaluated utilizing male Simmental calves with an average BW of 88 ± 4

kg over a 6 wk period. Calves were supplemented 10 μg of clenbuterol / kg of BW daily except on treadmill exercise days which were d 0, 1, and 14 relative to clenbuterol supplementation (Bruckmaier and Blum, 1992). On treadmill exercise days, calves were supplemented 25 μg of clenbuterol / kg of BW. Pack cell volume increased from baseline concentrations during exercise; however, PCV values did not differ between clenbuterol and control calves except on exercise d 14 when clenbuterol steers had increased PCV during exercise when compared with control calves (Bruckmaier and Blum, 1992). However, PCV values returned to observed pre-exercise baseline values following exercise regime in both clenbuterol and control calves (Bruckmaier and Blum, 1992). The increase in PCV were suggested to be caused by a decrease in blood water content and the increase of RBC release from the spleen (Bruckmaier and Blum, 1992).

Hemoglobin is a heme-containing protein transported in RBCs and accounts for 95 percent of the total protein located in RBCs (Kaneko et al., 2008). The transportation of hemoglobin inside RBCs allows for a longer half-life which would only be a few hours if not bound inside RBCs. The overall function of hemoglobin is the transportation of oxygen and carbon dioxide in the body (Kaneko et al., 2008). Bruckmaier and Blum (1992) observed an increase in hemoglobin in both calves supplemented 10 μg of clenbuterol / kg of BW daily and control calves during treadmill exercise with no clenbuterol effect on hemoglobin concentrations.

Blood platelets are cellular particles with growth regulatory factors produced by megakaryocytes (Kaneko et al., 2008). The approximate life span of a platelet is 10 days with new platelet production taking place daily (Kaushansky, 2005). Platelets are a key

component of hemostasis, thrombosis, and also produce proteins, lipids, carbohydrates, and nucleotides (Kaneko et al., 2008). In a horse supplemented with ZH at 0.17 mg / kg of BW for 1 d and approximately 0.02 mg / kg of BW on d 2 and last day of supplementation, platelet concentrations were increased above normal reference ranges 8 and 14 days following ZH supplementation (Wagner et al., 2008). No conclusion was drawn on why platelets were increased approximately 6 and 12 d following ZH supplementation.

Lastly, leukocytes or white blood cells (WBC) are measured as part of a CBC and are divided into granulocytes and mononuclear cells. Granulocytes consist of neutrophils, eosinophils, and basophils while mononuclear cells consist of lymphocytes and monocytes (Jones and Allison, 2007). Neutrophils, eosinophils, basophils, and monocytes are produced and mature in bone marrow while lymphocytes that originate from bone marrow stem cells mature and proliferate in lymph tissue (Jones and Allison, 2007). Within 2 h of a foreign object or bacterial insult, neutrophils migrate to the site of damage in an attempt to neutralize the threat through phagocytosis (Morris and Large, 1990). In one horse supplemented with ZH, damaged neutrophils were observed; however, two other horses supplemented with ZH did not have damaged neutrophils (Wagner et al., 2008). Clenbuterol had no effect on total WBC counts when supplemented to rats; however, clenbuterol treatment did increase neutrophils and monocytes (Shirato et al., 2007). Monocytes in circulation are able to phagocytose infectious organisms or cellular debris and become macrophages (Jones and Allison, 2007). In steers supplemented with ZH and RH, neutrophils increased on d 1 and 3

during ZH supplementation and on d 7 of RH supplementation when compared to control steers not supplemented a β -AA (Fuller, 2014).

The function of eosinophils is to initiate an immune response when exposed to parasites, allergens, or other inflammatory reagents by releasing cytotoxic proteins (Jones and Allison, 2007). Eosinophil concentrations can fluctuate in cattle, but may be increased during inflammation or during a stress response (Jones and Allison, 2007). Basophils are usually in small concentrations in ruminants and respond to allergic and inflammatory processes by releasing inflammatory mediators such as histamine (Jones and Allison, 2007). Two classes of lymphocytes exist which are B cells that produce antibodies and T cells which function in the regulation of the immune response and cytotoxic immunity (Jones and Allison, 2007). In rats supplemented with clenbuterol, eosinophil and lymphocyte concentrations decreased when compared with rats that did not receive clenbuterol (Shirato et al., 2007). In pigs, supplementation of clenbuterol resulted in an increase in apoptosis of T cells (Blanco et al., 2003). This cell death of T cells was attributed to decreased thymus cell size which could potentially result in a decrease in interleukin-6 concentrations during β -AA supplementation (Blanco et al., 2003). In lambs supplemented the β -AA L-646, 969, no difference in WBCs, lymphocytes, or neutrophils were observed between treatments. An increase in lymphocyte proliferation was observed in lambs supplemented with L-646, 969 when compared with the control treatment; however, no difference in overall immune function was observed between treatment groups (Li et al., 2000).

Organ and tissue affects

In human patients with failing hearts, β_2 -AR have been reported to increase allowing for increased stimulation by β_2 -AA when utilized (Bristow et al., 1986). Therefore, the effects of ZH supplementation on heart weight and anatomy would be beneficial when evaluating bovine hearts during supplementation. No difference was observed in combined heart and lung weights of beef steers fed ZH for 20 d (Holland et al., 2010). However, the feeding of clenbuterol in normal mice decreased heart weights by 8% when compared with control mice (Sharma et al., 1997). Furthermore, a tendency for a decrease in heart weights of Holstein steers supplemented with ZH for 20 d was reported with no difference in lung weights between ZH and control steers (May et al., 2014). Also, May et al. (2014) reported a decrease in kidney and liver mass of ZH supplemented steers when compared with control steers. The feeding of salbutamol (β_2 -AA) to pigs has been reported to decrease kidney and liver weights when compared with control pigs (Hansen et al., 1994). The decrease in renal and liver mass could be a result of decreased gut fill. The repartitioning of energy to skeletal muscle growth and a decrease in feed intake resulting in less gut fill could explain the decrease in renal and liver mass of ZH supplemented cattle (Montgomery et al., 2009b). The comparing of organ weight ratios to BW can result in misleading interpretation of data due to differences in animal adipose and muscle tissues (Joseph, 1908), as well as the previously reported effect of ZH on HCW (Montgomery et al., 2009a; Montgomery et al., 2009b). Reported percentages for bovine heart weight ratios to BW are 0.26, 0.52, and 0.38 for steers, bulls, and females respectively (Joseph, 1908).

Glycolytic potential (GP) is a measurement of glucose, lactate, glycogen, and glucose-6-phosphatase that is present in muscle that ultimately can be converted to lactic acid. This index can be used as a measurement of the capacity for postmortem glycolysis in muscle resulting in a decrease of muscle pH in the conversion of muscle to meat (Monin and Sellier, 1985). Glycolytic potential has been evaluated as a potential measurement of meat quality in beef and pork (Hamilton et al., 2003; Wulf et al., 2002). In cattle, GP has been reported to have a curvilinear response with ultimate *longissimus* muscle (LM) pH after harvest. Muscle samples with a GP less than 100 $\mu\text{mol} / \text{g}$ are associated with a greater postmortem pH that results in decreased lactate production and can cause dark, firm, and dry beef (Wulf et al., 2002). In previous ZH studies, Hilton et al. (2009) reported ZH supplementation increased beef color resulting in a brighter cherry red beef color during a 5 d shelf-life study. Furthermore, Montgomery et al. (2009a) reported ZH supplementation increased LM cherry red color and resulted in a decrease of dark colored LM when compared with control carcasses. The increase in bright cherry red color was attributed to an increase in myoglobin content that resulted in a favorable improvement in muscle color (Montgomery et al., 2009a). In the rat model, it has been reported that the activity of protein kinases on glycogen synthase in skeletal muscle predominantly take place in the liver (Imazu et al., 1984). If blood glucose concentrations are decreased, glucagon would be released from the α -cells of the pancreas to raise blood glucose concentrations (Navarro et al., 1999).

Stress

Stress is a term that has been utilized in the literature to explain a condition resulting in a forfeiture of homeostasis of a biological system. Distress is described as an event caused by some form of stressor resulting in a biological response due to a threat to homeostasis. However, not all stress is detrimental to the homeostasis and could potentially benefit the biological system through preparation and thus may be classified as eustress (Moberg and Mench, 2000). Cattle are exposed to multiple stressors throughout the production phases. In feedlot cattle, this stress can be experienced on the first day of arrival at the feedlot. Although aspects of production are managed in an attempt to prevent stress, no production system can be maintained stress free. Initial stressors faced by cattle entering a feedlot system will more than likely be immunological and metabolic stress due to exposure to a new environment and diets. However, as cattle progress through the feeding period, stress can continue due to events such as extreme weather fluctuations and/or transportation to the abattoir. Evolution of animals has allowed for the development of mechanisms to cope with stressful stimuli, but if the stimuli becomes too overwhelming, the animal must elicit a stress response to combat the stressor (Salak-Johnson and McGlone, 2007).

Hypothalamic–pituitary–adrenal axis

During times of stress, the hypothalamic–pituitary–adrenal (HPA) axis is activated in an attempt to return the body to homeostasis which is a concept first introduced in 1929 (Cannon, 1929). In an attempt to combat the stressful stimuli, one of the body's main defense systems will be to activate the hypothalamus (Aron et al., 2007).

The hypothalamus allows for the connection of the neurological and endocrine systems of the body especially relating to stressful situations (Reece, 2004). Composed of twelve different nuclei, these nuclei areas allow neuron control of endocrine factors that affect endocrine body function (Reece, 2004). During stress, the main function of the hypothalamus is the secretion of corticotropin-releasing hormone (CRH) after stimulation from the higher brain center. During stress, a catecholamine release acts on axons connected to the paraventricular nucleus by binding adrenergic receptors which causes a secretion of CRH into the portal circulation from the hypothalamus (Hadley and Levine, 2007). Corticotropin-releasing hormone acts on the pituitary gland, specifically the anterior pituitary which accounts for two-thirds of the pituitary gland, to secrete hormones including adrenocorticotrophic hormone (ACTH; (Ehlert et al., 2001). Adrenocorticotrophic hormone is secreted from basophilic corticotropic cells of the anterior pituitary to act on the adrenal glands (Reece, 2004).

The adrenal glands are located on the cephalic poles of the kidneys and are divided into two layers which include the adrenal cortex and medulla (Reece, 2004). The adrenal cortex is further divided into three zones that include the zona glomerulosa, zona fasciculate, and zona reticularis. The zona glomerulosa secretes mineralocorticoids and the zona fasciculate secretes glucocorticoids. Lastly, the zona reticularis produces sex hormones that originate from the adrenal cortex (Hadley and Levine, 2007). During stress, the zona fasciculate is activated by the binding of ACTH to produce glucocorticoids which include cortisol and corticosterone. Upon binding, ACTH activates the receptor which works through G protein stimulation. This activation stimulates adenyl cyclase which forms cAMP that activates protein kinase A. This kinase

phosphorylates cholesterol ester hydrolase forming free cholesterol (Reece, 2004). In the mitochondria, free cholesterol is converted to pregnenolone; however, the mitochondria does not obtain the enzyme to convert pregnenolone to cortisol or corticosterone. For this conversation, pregnenolone is transported to the smooth endoplasmic reticulum where cortisol and corticosterone is hydroxylated to form 11-deoxycortisol and 11-deoxycorticosterone. After formation, 11-deoxycortisol and 11-deoxycorticosterone are transported back to the mitochondria where 11-hydroxylation forms cortisol and corticosterone respectively (Reece, 2004). In cattle, the adrenal cortex secretes cortisol at greater concentrations than corticosterone; furthermore, cortisol is released in a diurnal pattern with highest concentrations being released during early morning (Reece, 2004). Control of the HPA axis resulting in the secretion of cortisol is under the negative feedback of cortisol. As cortisol concentrations increase, cortisol down regulates CRH and ACTH release as an inhibitory blocker (Reece, 2004).

Sympathetic nervous system

The sympathetic nervous system (SNS) is associated with active conditions and prepares the body for a “fight or flight” response that will be activated during times of stress (Hadley and Levine, 2007). The SNS is composed of two types of neurons which include preganglionic neurons and postganglionic neurons. Preganglionic neurons are located within the thoracic and lumbar regions in the spinal cord whereas the postganglionic neurons are composed of long axons that transmit signals to different tissue areas of the body (Reece, 2004). Furthermore, chromaffin cells are sympathetic ganglia located in the adrenal medulla which secrete catecholamines and are part of the

SNS (Reece, 2004). Catecholamines are neurotransmitters that are synthesized from tyrosine absorbed from the diet or cleaved from phenylalanine. Once transported into cells, tyrosine hydroxylase forms 3,4-dihydroxyphenylalanine from tyrosine. Next, aromatic L-amino acid decarboxylase transforms 3,4-dihydroxyphenylalanine into dopamine. Dopamine is the precursor for norepinephrine in the presence of dopamine β -hydroxylase, and norepinephrine is converted to epinephrine if cortisol and phenylethanolamine *N*-methyltransferase are available during synthesis (Reece, 2004).

Exogenous hypothalamic–pituitary–adrenal activation

The evaluation of a stress response can be one of the more challenging biological responses to measure due to the fact that in some cases the actual collection of the samples can result in a stress response. Due to this, activation of the HPA axis can be accomplished by administration of exogenous hormones to elicit an acute stress response. To test the sensitivity of the HPA axis, corticotropin-releasing hormone, arginine vasopressin, and ACTH have been utilized as repeatable methods to induce similar responses observed during acute stressors. With utilization of exogenous hormones, constant concentrations can be administered to multiple animals allowing for true evaluation of applied treatment effects on the HPA activation. It was observed that ACTH administration would elicit a cortisol response with increasing doses of ACTH resulting in a greater response (Lay et al., 1996). However, overall plasma peak cortisol concentrations did not differ regardless of initial ACTH dose. Corticotropin-releasing hormone and arginine vasopressin are capable of eliciting similar cortisol responses when administered individually. However, a tandem dose of CRH and arginine vasopressin

results in a more sustained cortisol response (Carroll et al., 2007). These exogenously administered hormones allow for activation of the HPA axis by eliciting similar stress responses observed during normal production phases. Therefore, the utilization of this method is an appropriate and reliable option to test the sensitivity of the HPA axis in regards to a specific treatment.

β -adrenergic agonist and stress

β -adrenergic agonist upon binding β -AR increase the production of cAMP (Mersmann, 1998). This increase in cAMP can result in the availability of nutrients to be utilized by multiple systems. Furthermore, increased circulating concentrations of catecholamines in stress-susceptible pigs produced pale, soft, exudative (PSE) pork due to increased lactate in muscles (Warriss et al., 1990). Furthermore, pigs supplemented with RH were reported to have increased circulating concentrations of catecholamines (Marchant-Forde et al., 2003). Similar findings were observed for cortisol concentrations in lambs and cattle supplemented with β -AA (Bryant et al., 2010; Li et al., 2000). An increase in cortisol concentrations would be expected if β -AA increased stress. In pigs supplemented with RH, no increase in cortisol concentrations were observed (Athayde et al., 2013). In horses, supplementation of ZH for 2 d resulted in increased lactate dehydrogenase, creatine kinase, and aspartate transferase, which was interpreted as an indicator of muscle damage (Wagner et al., 2008). Overall, limited studies have evaluated the stress response of livestock species supplemented with β -AA; however, in the available control studies, β -AA do not appear to significantly alter the ability of

animals to elicit an appropriate stress response in species that are approved to be supplemented with β -AA.

Conclusion

Due to animal wellbeing concerns related to the supplemented with ZH to cattle, the ZH was voluntarily removed from the market in August, 2013. However, at the time of removal, no scientific data were available to support or refute animal welfare concerns regarding ZH supplementation. It was suggested that cattle supplemented with ZH could potentially have an altered stress response when faced with stressful stimuli during production practices such as transportation to the abattoir. At the time, no studies had evaluated the stress response of any livestock species supplemented a β -AA in a controlled setting which would provide crucial baseline concentrations of metabolites affected only by β -AA supplementation. Therefore, the objective of the following study was to evaluate the stress response of beef feedlot heifers supplemented with ZH for a 20 d period followed by a 3 d withdrawal period. To scientifically evaluate the stress response, a CRH and arginine vasopressin endocrine challenge was utilized allowing for a controlled stressful stimulus to be administered across all treatments equally. This allowed for the evaluation of the capability of cattle to elicit a stress response after β -AA supplementation. Furthermore, this approach allowed for the opportunity to measure the overall metabolic status and blood chemistry analysis of cattle supplemented a β -AA during a controlled stress challenge.

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CHAPTER III
THE METABOLIC, STRESS AXIS AND HEMATOLOGY RESPONSE OF
ZILPATEROL HYDROCHLORIDE SUPPLEMENTED BEEF HEIFERS WHEN
EXPOSED TO A DUAL CORTICOTROPIN-RELEASING HORMONE AND
VASOPRESSIN CHALLENGE

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Abstract

The objective of this study was to determine the metabolic, stress, and hematology response of beef heifers supplemented with zilpaterol hydrochloride (ZH) when exposed to an endocrine stress challenge. Heifers ($n = 20$; 556 ± 7 kg BW) were randomized into two treatment groups: 1) Control (CON): no ZH supplementation, and 2) Zilpaterol (ZIL): supplemented with ZH at 8.33 mg / kg (DM basis). The ZIL group were supplemented ZH for 20 d, with a 3-d withdrawal period. On d 24, heifers received an i.v. bolus of corticotropin-releasing hormone (CRH; 0.3 μ g / kg BW) and arginine vasopressin (VP; 1.0 μ g / kg BW) to activate the stress axis. Blood samples were collected at 30-min intervals for serum, and 60-min intervals for plasma and whole blood, from -2 to 8 h relative to the challenge at 0 h (1000 h). Samples were analyzed for glucose, insulin, NEFA, blood urea nitrogen (BUN), cortisol, epinephrine, norepinephrine, and complete blood cell counts. Following the challenge, cattle were harvested over a 3-d period. Liver, *longissimus* muscle (LM), and *Biceps femoris* (BF) samples were collected and analyzed for glucose, lactate, and glycolytic potential (GP). There was a treatment ($P \leq 0.001$) effect for vaginal temperature (VT), with ZIL having a

0.1°C decrease in VT when compared with CON. A treatment x time effect ($P = 0.002$) was observed for NEFA. A treatment effect was observed for BUN; ZIL had decreased BUN concentrations compared with CON ($P < 0.001$) prior to the challenge; however, no treatment x time effect was observed. There was also a treatment effect for cortisol ($P \leq 0.01$) and epinephrine ($P = 0.003$); ZIL had decreased cortisol and epinephrine during the CRH/VP challenge, when compared with CON. There was a time effect for total white blood cells, lymphocytes, and monocytes; each variable increased ($P \leq 0.01$) 2 h post-challenge. Additionally, neutrophil counts decreased ($P \leq 0.01$) in response to CRH/VP challenge in both treatment groups. Glucose concentrations within the LM were greater ($P = 0.03$) in CON when compared with ZIL. Lactate concentrations and GP within the BF were greater in CON ($P = 0.05$) when compared with ZIL. These data suggest there are some variations observed between treatments in terms of response to the CRH/VP challenge; however, in the environmental conditions of this trial, none of the variations observed suggests that the supplementation of ZH detrimentally alters the ability of cattle to effectively respond to stressful stimuli.

Introduction

Zilpaterol hydrochloride (ZH, Zilmax, Merck Animal Health, NJ,) is a β – adrenergic receptor agonist (β -AA) that is approved for the use in feedlot cattle in the United States (Schmidt and Olson, 2007). In 2013, sporadic reports of of lame and slow moving cattle arriving at the at abattoirs was attributed to ZH supplementation (Thomson et al., 2015). Zilpaterol hydrochloride is a β_2 AA that functions as a ligand of the β_2 G protein coupled receptors (seven transmembrane domain receptor; Mersmann, 1998).

Ligand binding to the β_2 – adrenergic receptor activates adenylate cyclase, thus initiating the synthesis of cyclic adenosine monophosphate (cAMP) resulting in regulation of the activity of protein kinase A (Mersmann, 1998).

It is speculated that the binding of ZH to the β_2 – adrenergic receptor causes a shift in cell signaling that up-regulates genes associated with protein accretion, and down-regulates genes associated with protein degradation and lipogenesis (Mersmann, 1998). Feeding of β -AA down-regulates these receptors, resulting in an uncoupling and a reduction in β -adrenergic receptors available in the plasma membrane of cells (Mills, 2002). Chronic feeding of ractopamine to swine resulted in up to a fifty percent decrease in β -adrenergic receptors in adipose tissue (Smith et al., 1994), thus suggesting a potential interaction between the feeding of β -AA and regulation of the stress axis.

Glucocorticoids are regulators of metabolism, including glucose metabolism, and have been documented for many years (Long et al., 1940). Changes in blood metabolites would be expected during the stress response due to energy mobilization that can lead to depletion of energy stores such as liver glycogen (Gupta et al., 2005; Moberg and Mench, 2000). This study was designed to determine the effect of β -AA supplementation on the metabolic, stress, and hematology response to a dual corticotropin-releasing hormone and arginine vasopressin challenge in finished heifers.

MATERIALS AND METHODS

Experimental design

All experimental procedures were in compliance with the *Guide for the Care and Use of Agricultural Animals in Research and Teaching* and approved by the Institutional Animal Care and Use Committee at the University of Nebraska (IACUC #902).

Twenty ovariectomized English-influenced crossbred heifers (initial BW = 556 ± 7 kg BW) from the University of Nebraska Agricultural Research and Development Center feedlot were utilized for this study. Prior to initiation of the study, heifers were acclimated for a 3-wk period. During acclimation, heifers were haltered and acclimated to being restrained in a tie stall environment and to human contact. At the end of the 3-wk acclimation period, heifers were assigned randomly to one of two treatments: 1) Control (CON; n = 10): fed a finishing diet without ZH (Table 1.); and 2) zilpaterol hydrochloride (ZIL; n = 10): fed the same diet supplemented with ZH at 8.33 mg / kg BW on a DM basis. For delivery of ZH, five percent of the high-moisture corn in the diet was replaced with 4.9853% fine ground corn and ZH at 0.0147% calculated to supply ZH at 8.33 mg / kg on a DM basis. Five percent of the CON diet was replaced with fine ground corn to ensure similarity between the two diets. All supplements were individually mixed into individual heifer's daily allotment prior to feeding. Heifers were fed once daily at 0800 for 20 d followed by a 3-d withdrawal period of ZH. During the 3-d period, the five percent high-moisture corn was returned to both the CON and ZIL diets to replace the five percent ground corn supplement. The trial was designed to follow common industry feeding practices of feeding ZIL for a 20-d period followed by a 3-d

mandatory withdrawal before heifers were eligible to be shipped to an abattoir. The transportation of cattle results in activation of the HPA axis (Falkenberg et al., 2013); furthermore, the use of the CRH/VP stress model has resulted in a similar endocrine response (Carroll et al., 2007) when compared with an actual relocation event. Therefore the CRH/VP challenge on d 24 was utilized as a controlled endocrine stressor to produce a similar endocrine response that would be similar to shipping heifers to the abattoir.

Eight days prior to the start of ZH supplementation, heifers were relocated to the University of Nebraska - Lincoln Agricultural Research and Development Center Nutrition Dairy Barn during the month of December 2013. The Nutrition Barn is a 40-stall barn equipped with individual bunks, automatic waters, and dairy mattresses. Prior to heifers being placed into the barn, tie stalls (first ten stalls in a row on opposite sides of an alleyway in the barn) were assigned randomly to treatment, but blocked by treatment group (two heifers per block) so that no CON shared water with the ZIL. Heifers were maintained in individual tie stalls (1.34 m wide by 1.84 m long) for the duration of the trial. Pine shavings were added on top of the dairy mattress and replaced when needed. On d -3 (prior to ZH supplementation, which occurred on d 1), heifers were fitted with indwelling vaginal temperature (VT) monitoring devices (Burdick et al., 2012) that recorded VT at 5-min intervals throughout the duration of the study. On d 20 (last d of ZH supplementation), heifers were removed from tie stalls to obtain a BW (for CRH and VP dose calculations) and fitted with indwelling jugular catheters and then returned to their respective tie stalls. For the jugular cannulation procedure, heifers were restrained in a working chute. A small (2 to 3 cm) incision was made in the skin to more easily access the jugular vein. Indwelling jugular catheters, consisting of 30.48 cm of sterile

Tygon tubing (AAQ04133; US Plastics, Lima, OH; 1.27 mm i.d. and 2.286 mm o.d.), were inserted into the jugular vein using a 14-gauge by 5.08-cm thin-walled stainless steel biomedical needle (o.d. = 3 mm). The catheter was stabilized using tag cement and a 2.08-cm wide porous surgical tape around the incision site. Heifer's entire neck region was wrapped with vet wrap (Vetrap; 3 M Animal Care Products, St. Paul, MN) to ensure stability of the catheterization site. The remaining tubing not inserted into the heifer served as the extension portion of the cannula for collection of blood samples (Burdick Sanchez et al., 2013). Environmental temperature and relative humidity data were collected inside the Nutrition Barn utilizing four HOBO U23 Pro v2 temperature/relative humidity data logger (U23-001, Onset, Bourne, MA). Data from all four probes was averaged and compiled to provide an overall environmental temperature and relative humidity. On d 24, the average environmental temperature was $13^{\circ}\text{C} \pm 1.2^{\circ}\text{C}$ and an average relative humidity of $52\% \pm 3.1\%$. On d 24, the average temperature-humidity index was 24.8 ± 1.6 within the barn. Prior to d 24, two heifers were removed from the trial. One heifer was removed from the trial due to development of a sore underneath the halter and the other heifer was removed due to failure of the jugular catheter on d 13 and 21 respectively; therefore, there were ten heifers in the CON group and eight heifers in the ZIL group.

On d 24 at 0600 h, all residual feed was removed from bunks and daily allotment of feed was not provided until 1900 h (completion of the stress challenge). Starting at 0800 (-2 h) and continuing until 1800 h (8 h), blood samples were collected from each heifer. At 1000 h (immediately following collection of blood sample), each heifer received an i.v. bolus of bovine CRH ($0.3 \mu\text{g} / \text{kg BW}$) and arginine VP ($1.0 \mu\text{g} / \text{kg}$

BW); (Carroll et al., 2007). Following collection of the last blood sample (1800 h), catheters were removed and daily allotment of feed was delivered. A total of three blood samples were collected relative to administration of CRH and VP challenge (1000h). In 30-min intervals during the trial (0800 – 1600 h), one 9 mL blood sample was collected into Sarstedt tubes containing no additive (Sarstedt, Inc., Newton, NC) for isolation of serum for a total of 17 samples. Serum blood samples were allowed to clot for 30 min at room temperature and were then centrifuged at 1,500 x g for 20 min at 4°C. Isolated serum was stored at – 80°C until analyzed for glucose, insulin, NEFA, blood urea nitrogen (BUN), and cortisol concentrations. In 60-min intervals during the trial (0800 – 1800 h), one 9 mL blood sample was collected in Vacutainers containing EDTA (Fisher Scientific, Pittsburg, PA) for isolation of plasma. Plasma blood samples were centrifuged immediately after collection at 1,500 x g for 20 min at 4°C. Isolated plasma was flash frozen in liquid nitrogen and stored at -80°C until analyzed for epinephrine and norepinephrine concentrations (Burdick et al., 2009). Also in 60-min intervals during the trial (0800 – 1800 h), a 3 mL blood sample was collected into Vacutainers containing EDTA (Fisher Scientific, Pittsburg, PA) to determine complete blood cell (CBC) counts using a ProCyte Dx Hematology Analyzer (IDEXX Laboratories, Westbrook, ME).

On d 25, 26, and 27, heifers were harvested at the Loeffel Meat Laboratory located at the University of Nebraska – Lincoln. Heifers were assigned randomly to one of the three harvest days, and within days, harvest order was alternated based on treatment resulting in extended withdrawal times for heifers harvested on different days (4, 5, and 6 d post ZIL supplementation). After evisceration, samples were obtained from liver, *Longissimus* muscle (LM) directly above the 13th rib, and *Biceps femoris* (BF) from

the outer center of the muscle. Within 45 mins of stunning, tissue samples were collected and flash frozen in liquid nitrogen, and stored at -80°C until analyzed for glycolytic potential, glucose, and lactate concentrations.

Serum analysis

Serum glucose concentrations were determined in duplicate samples by modification of the enzymatic Autokit Glucose (Wako Diagnostics, Richmond, VA) to fit a 96-well format. Briefly, 300 μL of prepared working solution was added to 2 μL of serum or prepared standards in a 96-well plate. Plates were incubated at 37°C for 5 min and then read using a plate reader at 505 nm. Concentration of glucose was determined by comparing unknown samples to a standard curve of known glucose concentrations. The minimum detectable concentration was 3.8 mg / dL and the intra- and inter-assay coefficients of variation were 7.0% and 9.5%, respectively. Data are presented as the concentration in mg / dL.

Serum insulin concentrations were determined in duplicate samples by a bovine-specific insulin ELISA according to the manufacturer's instructions (Cat # 80-INSBO-E01; Alpco Diagnostics, Salem, NH). The minimum detectable concentration was 0.1 ng/mL and the intra- and inter-assay coefficients of variation were 11.8% and 17.9%, respectively. Data are presented as the concentration in ng / mL.

Serum concentrations of NEFA were determined in duplicate samples by modification of the enzymatic HR Series NEFA-HR (2) assay (Wako Diagnostics, Richmond, VA) to fit a 96-well format. Briefly, 200 μL of the prepared Color Reagent A were added to 5 μL of serum or prepared standards in a 96-well plate. Plates were incubated at 37°C for 5 min and absorbance was read using a spectrophotometer at 505

nm. Next, 100 μ L of prepared Color Reagent B was added to all wells on the 96-well plate. Plates were incubated for an additional 5 min and read for a second time using a plate reader at 505 nm. Concentrations of NEFA were determined by comparing unknown samples to a standard curve of known NEFA concentrations. The minimum detectable concentration was 0.0014 mmol / L and the intra- and inter-assay coefficients of variation were 7.1% and 6.3%, respectively. Data are presented as the concentration in mmol / L.

Serum concentrations of BUN were determined in duplicate samples by a colorimetric assay according to the manufacturer's directions (K024-H1; Arbor Assays, Ann Arbor, MI) by comparison of unknowns to standard curves generated with known concentrations of urea nitrogen. The minimum detectable BUN concentration was 0.065 mg / dL and the intra- and inter-coefficients of variation were 3.5% and 8.3%, respectively. Data are presented as the concentration in mg / dL.

Serum cortisol concentrations were determined in duplicate samples by enzyme immunoassay according to the manufacturer's instructions (Arbor Assays, Ann Arbor, MI). Serum concentrations of cortisol were determined by comparison to a standard curve of known cortisol concentrations. The minimum detectable concentration was 45.4 pg / mL and intra- and inter-assay coefficients of variation were 18.6% and 16.0%, respectively. Data are presented as the concentration in ng / mL.

Epinephrine and Norepinephrine Analysis

Plasma concentrations of epinephrine and norepinephrine were determined in duplicate samples by enzyme immunoassay according to the manufacturer's directions (17-BCTHU-E02.1; Alpco Diagnostics, Salem, NH) by comparison of unknowns to

standard curves generated with known concentrations of epinephrine or norepinephrine (Burdick et al., 2009). The minimum detectable concentrations were 10 pg / mL and 50 pg / mL for epinephrine and norepinephrine, respectively. The intra- and interassay coefficients of variation were $\leq 17.3\%$ and $\leq 22.3\%$. Data are presented as the concentration in pg / mL.

Tissue analysis

Glycolytic potential values were calculated by the formula: [Glycolytic potential = 2 ([glycogen] + [glucose] + [glucose-6-phosphate]) + (lactate)] (Monin and Sellier, 1985). Duplicate samples of LM, BF, and liver samples were homogenized in 0.6 N perchloric acid, and glycogen was digested with amyloglucosidase and 20 μ L of 5.4 N KOH. Glucose assays were performed with a coupled enzymatic assay kit (hexokinase and glucose-6-phosphate dehydrogenase, Sigma-Aldrich, St. Louis, MO). Lactate content was measured using an enzyme assay including lactate dehydrogenase (Souza et al., 2011).

Statistical analysis

Data were analyzed using the MIXED procedure of SAS specific for repeated measures (SAS Inst. Inc., Cary, NC). Treatment, time, and the treatment by time interaction were included as fixed effects, with heifer within treatment included as the experimental unit. If treatment or the treatment by time interaction were significant, data were further analyzed separately in two different time periods: Baseline, time period prior to administration of CRH and VP (-2 to 0 h); and Challenge, time period following administration of CRH and VP (0 to 8 h). Due to differences in baseline, VT data were analyzed as the change in VT relative to baseline (-2 to -0 h) values, with average

baseline VT values included as a covariate. All heifers were challenged at the same time, following collection of the 0 h sample at 1000 h. The authors measured vaginal temperature for several days prior to the challenge; however, for baseline we focused on the 2 hours prior to coincide with the baseline sample collection which began 2 hours prior to the challenge. Additionally, average baseline values were included as a covariate in the analysis of the change in vaginal temperature. Tissue samples were analyzed with treatment included as a fixed effect and heifer within treatment included as the experimental unit. When main effects were significant, specific treatment comparisons were made using the PDIFF option in SAS, with $P \leq 0.05$ considered significant and $0.05 < P \leq 0.10$ considered a tendency. All data are presented as the $LSM \pm SEM$.

RESULTS

Vaginal Temperature

There were treatment ($P < 0.001$) and time ($P < 0.01$) effects, but not a treatment x time interaction ($P = 0.96$), for VT. Specifically, VT was decreased ($P < 0.001$) in ZIL compared with CON heifers, and varied over time. Due to the treatment effect, with ZIL heifers entering the challenge with decreased VT, the VT data were further analyzed as the change from baseline values (average of values from -2 to 0 h; 0800 to 1000 h), with average baseline values included as a covariate in the analysis. The change in VT relative to baseline values was affected by treatment ($P \leq 0.001$) and time ($P = 0.02$), but there was no treatment x time interaction ($P = 0.99$; Fig. 1). Heifers in the CON group had an overall positive change (increase; $P \leq 0.001$) in VT ($0.068 \pm 0.005^\circ\text{C}$) while ZIL

heifers had an overall negative (decrease) change in VT in response to CRH/VP challenge ($-0.050 \pm 0.005^{\circ}\text{C}$).

Serum Metabolites

There was no treatment effect ($P = 0.62$) or treatment x time interaction ($P = 0.97$) for serum glucose concentrations. There was a time effect ($P < 0.001$) for glucose concentrations. Regardless of the treatment, glucose concentrations increased ($P < 0.001$) within 0.5 h of administering the CRH/VP challenge and remained increased until 2 h post-challenge (Figure 2). There was also no treatment effect ($P = 0.82$) or treatment x time interaction ($P = 0.70$) for serum concentrations of insulin. A time effect ($P < 0.001$) was also observed for serum insulin concentrations. Regardless of treatment, insulin concentrations increased ($P < 0.001$) within 0.5 h of administering CRH/VP challenge and remained increased until 2 h post-challenge (Figure 3).

There was a treatment x time interaction ($P < 0.001$) for serum NEFA concentrations. Prior to challenge, there were no difference ($P = 0.27$) in serum NEFA concentrations between CON and ZIL heifers. Concentrations of NEFA were greater in CON heifers, compared with ZIL heifers at 4 h ($P = 0.04$), at 6 h ($P = 0.02$) and from 7 to 8 h ($P < 0.01$) following the CRH/VP challenge (Figure 4).

Serum BUN concentrations were affected by treatment ($P < 0.001$) and time ($P < 0.001$; Figure 5) but there was no treatment x time interaction ($P = 1.00$). In terms of treatment effect, concentrations of BUN were greater ($P \leq 0.001$) in CON heifers when compared with ZIL heifers (14.0 ± 0.2 mg / dL vs. 11.9 ± 0.2 mg / dL, respectively). This difference in BUN was observed prior to the CRH/VP challenge; baseline

concentrations (-2 to 0 h) were greater ($P \leq 0.001$) in CON heifers compared with ZIL heifers (13.5 ± 0.3 mg / dL vs. 11.5 ± 0.4 mg / dL, respectively).

Serum cortisol concentrations were affected by treatment ($P = 0.002$) and time ($P < 0.001$), but there was no treatment x time interaction ($P = 0.09$). Overall, cortisol concentrations were greater ($P = 0.002$) in CON (26.0 ± 0.7 ng / mL) compared with ZIL heifers (23.2 ± 0.07 ng / mL) during the CRH/VP challenge. Specifically, prior to the CRH/VP challenge, baseline (-2 to 0 h) concentrations of cortisol were similar between the two treatment groups; ($P = 0.17$; Figure 6). Following administration of the CRH/VP challenge cortisol concentrations increased ($P < 0.001$; 0.5 h compared with 0 h) within 0.5 h of the challenge (regardless of treatment) and did not return to near baseline concentrations until 5.5 h ($P = 0.21$; 5.5 h compared with 0 h) after the challenge. While the treatment x time interaction was not significant, there was a tendency ($P = 0.09$) for cortisol concentrations for ZIL heifers to be decreased from 0.5 h to 2 h, when compared with CON heifers.

Epinephrine and Norepinephrine Concentrations

Plasma epinephrine concentrations were affected by treatment ($P = 0.003$) and time ($P < 0.001$), but there was no treatment x time interaction ($P = 0.29$; Figure 7). Due to the treatment effect, data were further analyzed within baseline and challenge periods. Prior to the CRH/VP challenge, CON heifers had greater ($P = 0.005$; 33.5 ± 3.0 pg / mL) baseline epinephrine concentrations (-2 to 0 h), when compared with ZIL heifers (20.4 ± 3.0 pg / mL; Fig 7). Following the challenge, there was a treatment ($P = 0.03$) and time ($P = 0.002$) effect for epinephrine concentrations but no treatment x time interaction ($P =$

0.18). Post CRH/VP challenge, CON heifers had greater (16.7 ± 1.0 pg / mL) epinephrine concentrations, when compared with ZIL heifers (13.4 ± 1.0 pg / mL; Figure 3 B).

There was only a time effect ($P \leq 0.001$) for plasma norepinephrine concentrations (treatment: $P = 0.94$; treatment x time: $P = 0.44$; Figure 8).

Norepinephrine concentrations decreased ($P \leq 0.001$) 1 h following administration of CRH/VP. Concentrations remained decreased for the duration of the study ($P \leq 0.001$, 8 h compared with 0 h; Figure 8) for CON and ZIL heifers.

Complete Blood Cell Counts

There were no main effect differences in total red blood cells ($P = 0.82$), hemoglobin ($P = 0.14$), or platelets ($P = 0.70$; Table 2) between CON and ZIL heifers. There was a tendency ($P \geq 0.06$) for both total red blood cells and hemoglobin to change over time regardless of treatment. There was a time difference ($P \leq 0.001$) for platelets with platelet concentrations increasing relative to the CRH/VP challenge in both CON and ZIL heifers. There was a treatment x time interaction ($P = 0.03$) for hematocrit. Therefore, hematocrit concentrations were further analyzed as baseline and post-challenge. There was a tendency ($P = 0.06$) for a treatment effect and a difference ($P = 0.05$) over time during the baseline period between treatments for hematocrit. Therefore, change from baseline was calculated and analyzed as a percent change between treatments. There was no difference for treatment ($P = 0.85$; Table 2) or treatment x time ($P = 0.80$) for percent change in hematocrit. However, there was a time difference ($P = 0.04$) for percent change in hematocrit such that hematocrit concentrations decreased following administration of CRH/VP.

Total white blood cells (Figure 9), lymphocytes (Figure 10), and neutrophils (Figure 11) were not affected by treatment ($P \geq 0.41$) or a treatment x time interaction ($P \geq 0.60$); however, these variables were affected by time ($P < 0.001$). White blood cells and lymphocytes increased ($P < 0.001$) 2 h following administration of CRH/VP, and remained increased for the duration of the study ($P < 0.001$, 8 h compared with 0 h). In contrast, neutrophils decreased ($P < 0.001$) 3 h following administration of CRH/VP, and remained decreased for the duration of the study ($P < 0.001$, 8 h compared with 0 h). There was a treatment x time ($P = 0.04$) interaction for monocytes (Figure 12). However, no time points were different between treatment groups during the trial. The treatment x time interaction can primarily be contributed to a greater fluctuation of monocyte concentrations within the CON heifers. There was a treatment effect ($P = 0.04$) for eosinophils; eosinophils were greater for CON ($0.13 \times 10^3 / \mu\text{L}$; Table 2), when compared with ZIL heifers ($0.007 \times 10^3 / \mu\text{L}$). There was a treatment x time ($P = 0.04$) interaction for basophil concentrations. At 8 h post CRH/VP challenge, basophil concentrations were greater for ZIL heifers when compared with CON heifers (0.002 vs. 0 , $10^3 / \mu\text{L}$; respectively; Figure 13) during the CRH/VP challenge.

Tissue samples

There were no differences ($P = 0.49$) in concentrations of glucose or lactate, and no difference in glycolytic potential for the liver samples between the CON and ZIL heifers (Table 3). Glucose concentrations within the LM were greater ($P = 0.03$) in CON heifers ($47.95 \pm 12.01 \mu\text{g} / \text{mol}$) compared with ZIL heifers ($35.84 \pm 10.74 \mu\text{g} / \text{mol}$; Figure 5). Supplementation of ZH did not affect lactate concentrations ($P = 0.39$) or total

glycolytic potential ($P = 0.11$) for LM samples (Table 3). Glucose concentrations in the BF tissue samples were not affected ($P = 0.22$) by ZH supplementation (Table 3).

Lactate concentrations in BF tissue samples were greater ($P = 0.05$) in CON ($71.75 \pm 6.90 \mu\text{g} / \text{mol}$) heifers when compared with ZIL heifers ($64.47 \pm 7.79 \mu\text{g} / \text{mol}$; Table 3).

Furthermore, there was a treatment effect for glycolytic potential in BF samples ($P = 0.05$; Table 3) with CON heifers having greater concentrations ($140.47 \pm 23.60 \mu\text{g} / \text{mol}$) compared with ZH-supplemented heifers ($120.05 \pm 17.55 \mu\text{g} / \text{mol}$).

DISCUSSION

Data from the current study would indicate that that activation of the hypothalamic-pituitary-adrenal (HPA) axis will increase both stress and metabolic variables. Activation of the HPA axis is a cascading response, regulated by the magnitude of the stressor (Tsigos and Chrousos, 2002). An acute response may result in brief down-regulation of systems not necessary for survival, such as digestion and reproduction, and stimulate catabolism of energy stores and body tissues to be used for energy (Sapolsky et al., 2000). While there are data to suggest the initiation of a stress response during relocation events (Falkenberg et al., 2013), there are few or no data available to describe how this event is altered due to supplementation of β -AAs.

Studies in cattle using other β -AAs (i.e., clenbuterol) have observed differences in glucose, lipid, and protein metabolism (Eisemann et al., 1988b); yet these metabolites were measured early in the supplementation process rather than following withdrawal as in the current study. When glucose and insulin were measured on d 13 of cimaterol supplementation in steers, there were no differences in glucose or insulin concentrations

compared with control steers (Chikhou et al., 1991b). Additionally, results from a study in finishing steers revealed decreased glucose concentrations after 21 d of supplementation with ZH (Van Bibber et al., 2010). The observed difference in glucose concentrations reported by Van Bibber et al. (2010) differs from the current study where no treatment differences in circulating concentrations of glucose were observed in response to ZH supplementation in cattle. The differences observed between the study by Van Bibber et al. (2010) and the current study could be due to sample frequency and the collection of samples following a 3 d withdrawal in the current study, which the previous studies did not include. In the current study, samples were obtained through indwelling jugular catheters in 30-min increments whereas in the Van Bibber et al. (2010) study, blood samples were only collected once daily by processing cattle through a processing facility. Furthermore, the current study was conducted within an indoor environment where less maintenance energy requirements would be expected (Hoffman and Self, 1970). Comparison of different compounds and species should be conducted with great caution do to the diversity of β -adrenergic receptors and receptor tissue distribution differences in livestock species (Mersmann, 2002).

There is limited reported data regarding the effect of β -AA on body temperature in any species including cattle. The feeding of ZH has been reported to decrease ruminal temperature when compared with control steers not fed ZH (Boyd et al., 2015). As reported by Boyd et al (2015) the decreased body temperature may be attributed to increased respiration rates, allowing ZH supplemented steers the ability to dissipate excess body heat. In this study, authors attributed decreased body temperature to increased respiration rates body heat, which has been published by others (Boyd et al.,

2015). Another biological response typically observed following stimulation of the β -adrenergic receptors is vasodilation of interior tissues (e.g. skeletal muscle, heart, lungs, liver, and gastrointestinal tract; (Dawes et al., 1997). An increase in vasodilation would support the observed decrease in body temperature, as this would allow dissipation of heat from the body surface at a faster rate compared with CON heifers. However, it would be expected to see a decline in vasodilation with continued treatment of β -AA due to a possible down-regulation of the β -adrenergic receptors (Smith et al., 1994). Understanding blood flow changes as a result of β -agonists supplementation, and particularly ZH, would help justify differences observed, in body temperature and also nutrient delivery and blood cell population changes. In the current study, VT supports decreased body temperature with ZH feeding that could potentially benefit the animal during stress allowing the animal to dissipate excess body heat.

Following administration of the CRH/VP challenge, an increase in VT was observed in both treatments within 25 min. While VT remained increased in CON heifers, a return to baseline and sub-baseline values was observed in ZIL heifers. The temporal VT response to the CRH/VP challenge in the current study has similarities to the response observed in weaned Brahman calves challenged with CRH, in that study the authors reported fluctuations in rectal temperature in the 8-h period following CRH administration (Hulbert et al., 2013).

The adrenergic receptors are the receptors that catecholamines (i.e., epinephrine and norepinephrine) bind to initiate a response and are distributed throughout the body. There are two major types of adrenergic receptors, the α - and β -adrenergic receptors, both

of which have several subtypes and are all membrane-bound receptors (Mersmann, 1998). While β -adrenergic receptors are located on most mammalian cells, the β_2 -adrenergic receptors are the primary receptors located in both adipose and skeletal muscle (Mersmann, 1998). Specifically, ZH is considered a β_2 -adrenergic receptor agonist (Hilton et al., 2009). Initial feeding of β -AAs has been reported to increase NEFA concentrations (Mersmann, 1998). During the initial stages of feeding, β -AAs exhibit more lipolytic effects, a biological effect that may be eventually down-regulated during extended feedings and as fat content decreases. In a prior study, concentrations of NEFA measured weekly during ZH supplementation in finishing steers reported no difference in NEFA concentrations (Van Bibber et al., 2010), thus supporting data from the current study as baseline NEFA concentrations did not differ between treatments groups.

The decrease in NEFA concentrations in response to the CRH/VP challenge in the ZH-supplemented heifers is not surprising considering the increase in lean tissue growth, and the decrease in lipogenesis that has been observed in β -AA fed cattle (Sillence, 2004). Feeding of β -AAs leads to decreased lipid deposition, and thus decreased NEFA concentrations when stimulated by a catabolic agent such as cortisol. Studies reported in the literature have demonstrated that β -AAs have anti-lipogenic effects (Dunshea, 1993; Sillence, 2004), data that also supports the results in the current study.

The major growth promoting effects of β -AAs are primarily observed in skeletal muscle (Sillence, 2004), which results in the catabolism of protein from other tissues to be used to build muscle, although only critical when provided inadequate nutrition (Sillence, 2004). Furthermore, β -AA activate β -receptors in muscle and adipose tissue

resulting in decreased lipogenesis, and increased lipolysis and protein accretion (Mersmann, 1998). Therefore, the decreased BUN concentrations in ZH-supplemented heifers is likely due to increased anabolism of protein in skeletal muscle and decreased catabolism of protein in other tissues as these heifers were provided adequate nutrition to support additional lean tissue accretion during the study (Bell et al., 1998a). The decrease in BUN observed in the current study is supported by work with other β -AAs in which the authors observed similar decreases in BUN concentrations in cattle supplemented with cimaterol (Chikhou et al., 1991b). The increase in BUN concentrations post-CRH/VP in both treatment groups is in concert with the increased concentrations of NEFA observed post-challenge, and suggests a greater energy demand during this period than was supplied by glucose alone. Decreased BUN concentrations in ZH-supplemented cattle observed in the current study is supported by work in finishing steers in which the authors reported decreased BUN concentrations after 20 d and 21 d of ZH supplementation, (Parr et al., 2010; Van Bibber et al., 2010).

Baseline cortisol concentrations were not different between treatments in the current study. This is in agreement with a study utilizing a cimaterol treatment in steers, where no difference in cortisol concentrations were observed 1 and 13 d following initiation of oral dosing (Chikhou et al., 1991b). The temporal pattern associated with an acute increase in circulating concentrations of cortisol followed by a subsequent decline in the current study is similar to that observed in Brahman calves in response to a CRH challenge (Hulbert et al., 2013), albeit the magnitude of the cortisol response was greater in the current study. Control heifers produced a greater cortisol response to the CRH/VP challenge compared with ZIL heifers, thus suggesting a possible down-regulation of

aspects associated with HPA axis regulation. As mentioned above, there is cross-talk between the sympathomedullary system and the HPA axis. Thus, the observed cortisol response suggests that supplementation of ZH not only influenced the sympathomedullary system, but may have down-regulated portions of the HPA axis either directly or through actions on the sympathomedullary system.

Differences observed in epinephrine concentrations but not norepinephrine concentrations would theoretically be expected when feeding ZH. Naturally-occurring physiological β -adrenergic agonists consist of norepinephrine, a neurotransmitter, and epinephrine, an adrenal medullary hormone (Mersmann, 1998). Both epinephrine and norepinephrine are capable of binding to the α_1 -, α_2 -, β_1 and β_2 -adrenergic receptors (Robinson et al., 1989); however, β_2 -adrenergic receptors have a greater affinity for epinephrine compared with norepinephrine. Therefore, the lack of down-regulation of the norepinephrine response may be due to the difference in receptor affinity. With ZH being classified as a β_2 -adrenergic receptor agonist it should not affect the regulation of norepinephrine via actions on the α_1 -, α_2 -, and β_1 -adrenergic receptors. Adenylate cyclase in the erythrocytes of frogs has been used to demonstrate a loss of β -adrenergic receptor binding sites in response to catecholamine induced sensitivity. When injected with norepinephrine or isoproterenol (β_1 -adrenergic receptor agonist), cyclic adenosine monophosphate generated from frog erythrocyte membranes decreased compared with controls (Mukherjee et al., 1975). The authors concluded that the decrease in catecholamine responsiveness was due to a reduction in β -adrenergic receptors, or a conformational change resulting in inactive receptors (Mukherjee et al., 1975). In the current study, decreased concentrations of epinephrine in ZIL heifers would further

support a down-regulation of the sympathomedullary axis via action on the β_2 -adrenergic receptors as discussed above.

Few studies have determined the effect of CRH or a combined CRH/VP challenge on immune cell populations, particularly in cattle; however, increased glucocorticoids results in an increase of neutrophils (Burton et al., 2005). Immune cell populations are sensitive to CRH, as well as the intermediate stress hormone, adrenocorticotrophic hormone (ACTH), and cortisol. Hulbert et al. (2013) observed a decrease in total leukocytes at 1 h following a CRH challenge in Brahman calves. This is in contrast to the current study, in which an increase in total WBCs was observed 2 h following CRH/VP administration regardless of treatment. Many factors may contribute to these opposing observations, including age of the cattle, breed of the cattle, and/or the combination of a CRH challenge with VP given simultaneously versus a solitary CRH challenge. While there are limited studies utilizing CRH, the CRH/VP challenge in this study was utilized to mimic an acute stressor. In mice exposed to acute restraint stress, an increase in natural killer (NK) cells, a subset of lymphocytes, was observed (Bauer et al., 2001). Furthermore, the use of cyanoketone, an androstanol, in restraint-stressed rats resulted in less cortisol production during the time of stress (Dhabhar et al., 1996). The blocking of cortisol production with cyanoketone also significantly reduced the stress-induced decrease in leukocytes that has been reported in rats under acute stress (Dhabhar et al., 1996).

The decrease in circulating neutrophils following CRH/VP challenge is indicative of movement of neutrophils out of the blood and into the tissues in preparation for

immune activation (e.g. wounding). In contrast, the increase in circulating total WBC and lymphocytes suggests a mobilization of these cells (Dhabhar et al., 2012). This redistribution of immune cells suggests an activation of the immune response, as several factors, including chemoattractants, cell adhesion molecules, and cytokines are needed for this process to occur (Bauer et al., 2001). Changes in expression of cell adhesion molecules, stimulated by stress hormones, may be responsible for the changes observed in immune cell populations not only in response to CRH/VP challenge, but in response to ZH supplementation.

Glycolytic potential (GP) is a measurement of glucose, lactate, glycogen, and glucose-6-phosphatase that is present in muscle that ultimately can be converted to lactic acid. This index can be used as a measurement of the capacity for postmortem glycolysis in muscle resulting in a decrease of muscle pH in the conversion of muscle to meat (Monin and Sellier, 1985). Glycolytic potential has been evaluated as a potential measurement of meat quality in beef and pork (Hamilton et al., 2003; Wulf et al., 2002). In cattle, GP has been reported to have a curvilinear response with ultimate LM pH after harvest. Muscle samples with a GP less than 100 $\mu\text{mol} / \text{g}$ are associated with a greater postmortem pH that results in decreased lactate production and can cause dark, firm, and dry beef (Wulf et al., 2002). In the current study, GP in the LM was not different in CON and ZH-supplemented heifers. In the BF, ZH-supplemented heifers did have a decreased GP; however, this was associated with less available lactate resulting in a decreased calculated GP and was greater than the 100 $\mu\text{mol} / \text{g}$ threshold that has been suggested for optimal pH postmortem. In previous ZH studies, Hilton et al. (2009) reported ZH supplementation increased beef color resulting in a brighter cherry red favorable beef

color during a 5 d shelf-life study. Furthermore, (Montgomery et al., 2009a) reported ZH supplementation increased LM cherry red color and resulted in a decrease of dark colored LM when compared with control carcasses. The increase in bright cherry red color was attributed to an increase in myoglobin content that resulted in a favorable improvement in muscle color (Montgomery et al., 2009a). In liver samples, there was no difference in glucose, lactate, or GP observed in CON and ZH-supplemented heifers. In the rat model, it has been reported protein kinases activity on glycogen synthase in skeletal muscle predominantly take place in the liver (Imazu et al., 1984). Furthermore, the lack of differences in glucose and GP between treatment groups of liver samples would further support the fact that no differences in serum glucose were observed in the current study. If blood glucose concentrations decreased, glucagon would be released from the α -cells of the pancreas to raise blood glucose concentrations (Navarro et al., 1999). These data suggest that ZH-supplemented heifers do not have decreased available energy stores in muscle or liver samples that could cause muscle fatigue from lack of an energy source.

Also, these data demonstrate that a simulated stress induced by administration of CRH/VP in beef cattle elicits shifts in immune cell populations and metabolic variables; and stress hormones, metabolic variables, and immune cell responses are altered in beef heifers supplemented with ZH when cattle are exposed to a simulated stress challenge. However, within the environment of this study, supplementation of ZH did not appear to hinder finishing heifer's ability to mount a stress, immune, or metabolic response during a neurohormone-induced stimulated stress challenge.

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Table 1. Composition of finishing diets fed to control (CON) and zilpaterol (ZIL) heifers as a percent of DM basis during a corticotropin-releasing hormone (CRH; 0.3 µg / kg BW) and vasopressin (VP; 1.0 µg / kg BW) challenge in finishing heifers.

Item	Treatment	
	Control	ZIL ¹
Ingredient (%)		
High Moisture Corn	51.00	51.00
Sweet Bran®	40.00	40.00
Wheat Straw	5.00	5.00
Fine Ground Corn	1.8710	1.8710
Limestone	1.6400	1.6400
Salt	0.3000	0.3000
Tallow	0.1000	0.1000
Beef Trace Mineral	0.0500	0.0500
Rumensin-90	0.0150	0.0150
Vitamin A-D-E	0.0165	0.0165
Tylan-40	0.0075	0.0075
Supplement ¹		
Fine Ground Corn	5.0	4.9853
Zilpaterol Hydrochloride	-	0.0147

¹ Heifers receiving zilpaterol hydrochloride for 20-d period with 3-d withdrawal

²The control supplement contained fine ground corn only. Zilpaterol hydrochloride (ZH) supplement contained (DM basis) 0.0147% Zilmax® (Merck Animal Health) Type A medicated article and 4.9853% fine ground corn and supplied zilpaterol hydrochloride (ZH) supplementation (8.33 mg / kg on a DM basis). Supplement was fed for 20 d.

Table 2. Changes in blood variables in response to CRH/VP challenge in heifers supplemented with zilpaterol hydrochloride (ZH, 8.33 mg / kg on a DM basis).

Variable	Treatment		SEM	<i>P</i> -value
	Control	ZIL		
Red blood cells, $10^5 / \mu\text{L}$	6.24	6.31	0.20	0.82
Hemoglobin, g / dL	10.7	11.2	0.25	0.14
Hematocrit change, %	-0.87	-0.79	0.30	0.85
Platelets, $10^3 / \mu\text{L}$	313.66	300.92	23.9	0.70
Eosinophils, $10^3 / \mu\text{L}$	0.13	0.07	0.02	0.04

Table 3. Effect of zilpaterol hydrochloride (ZH) supplementation (8.33 mg / kg on a DM basis) on tissue samples following 20 d ZH supplementation, 3 d withdrawal period, and a corticotropin-releasing hormone and vasopressin challenge administered on d 24.

Item	CON	ZIL ¹	SEM	<i>P</i> -value
<i>Liver</i>				
Glucose	152.75	146.46	6.70	0.51
Lactate	11.54	11.11	0.85	0.71
GP ²	317.03	304.02	13.38	0.49
<i>LM³</i>				
Glucose	47.95	35.84	3.81	0.03
Lactate	63.83	68.91	4.18	0.39
GP ²	159.73	140.60	8.17	0.11
<i>BF⁴</i>				
Glucose	34.36	27.79	3.77	0.22
Lactate	71.75	64.47	2.44	0.05
GP ²	140.47	120.05	6.99	0.05

¹ Heifers receiving zilpaterol hydrochloride for 20-d period with 3-d withdrawal

² Glycolytic Potential

³ *Longissimus* muscle

⁴ *Biceps Femoris*

Fig. 1 Vaginal temperature of heifers fed zilpaterol hydrochloride (ZH; 8.33 mg / kg on a DM basis) for 20 d followed by a 3 d withdrawal, or fed the control diet for 23 d and then subjected to a corticotropin-releasing hormone (CRH; 0.3 μ g / kg BW) and vasopressin (VP; 1.0 μ g / kg BW) challenge administered on d 24 at time 0.

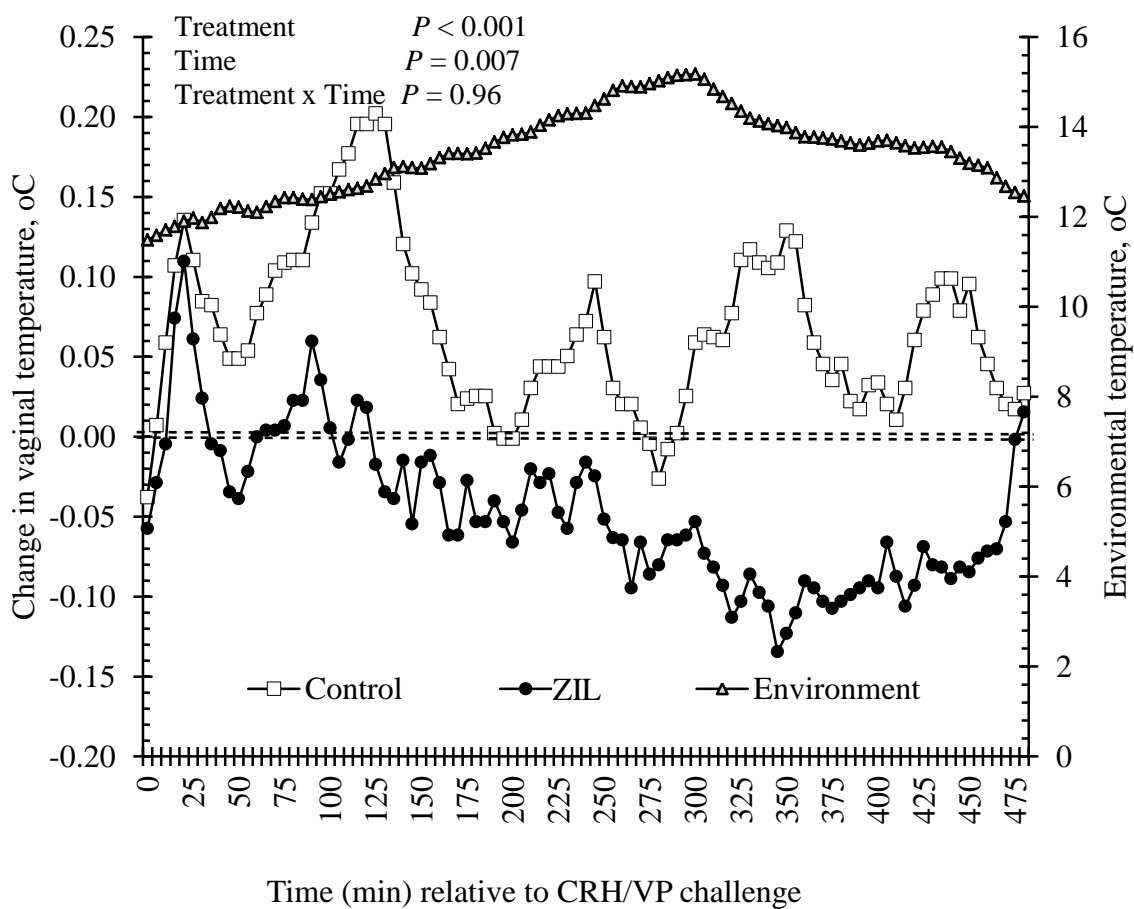


Fig. 2 Serum glucose response of heifers fed zilpaterol hydrochloride (ZH; 8.33 mg / kg on a DM basis) for 20 d followed by a 3 d withdrawal, or fed the control diet for 23 d and then subjected to a corticotropin-releasing hormone (CRH; 0.3 μ g / kg BW) and vasopressin (VP; 1.0 μ g / kg BW) challenge administered on d 24 at time 0 h. Control represents heifers that were not fed ZH while ZIL represents heifers fed ZH for 20 d followed by a 3 d withdrawal.

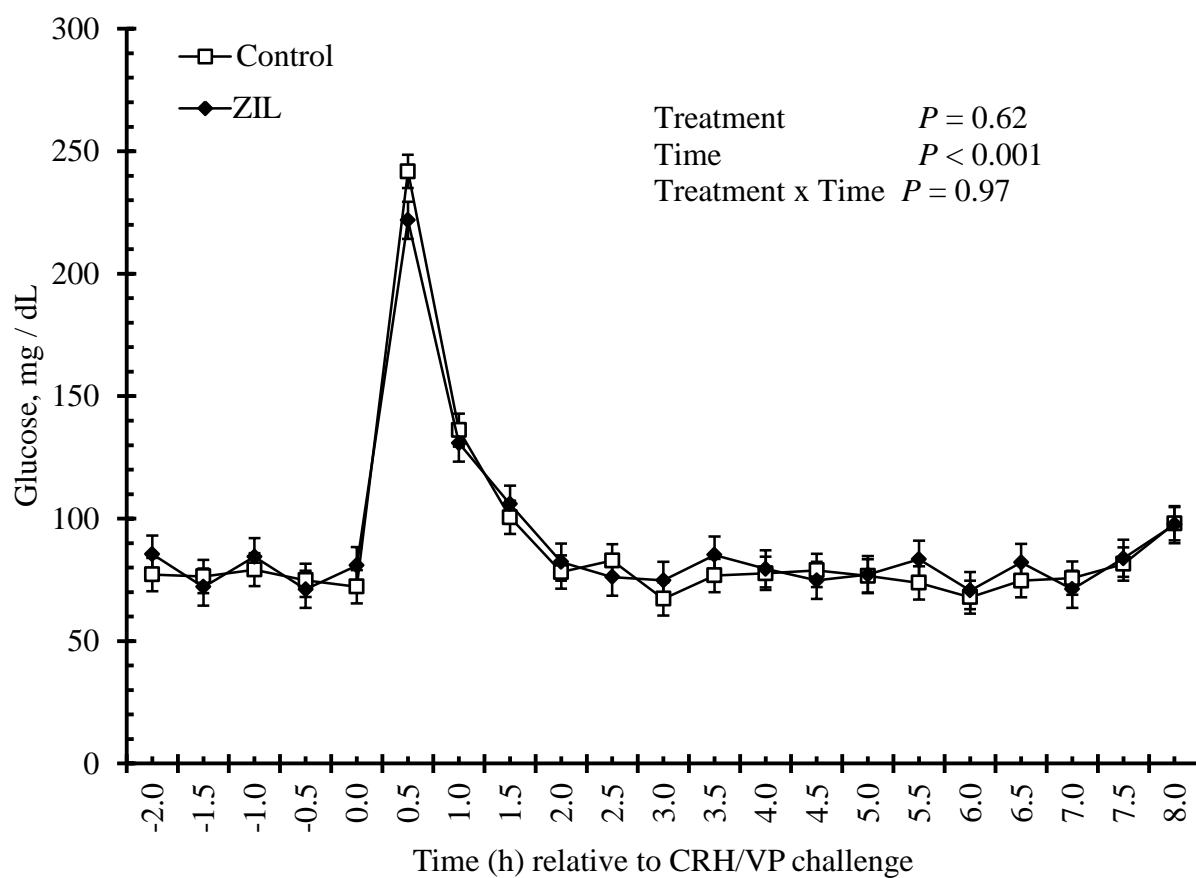


Fig. 3 Serum insulin response of heifers fed zilpaterol hydrochloride (ZH; 8.33 mg / kg on a DM basis) for 20 d followed by a 3 d withdrawal, or fed the control diet for 23 d and then subjected to a corticotropin-releasing hormone (CRH; 0.3 μ g / kg BW) and vasopressin (VP; 1.0 μ g / kg BW) challenge administered on d 24 at time 0 h. Control represents heifers that were not fed ZH while ZIL represents heifers fed ZH for 20 d followed by a 3 d withdrawal.

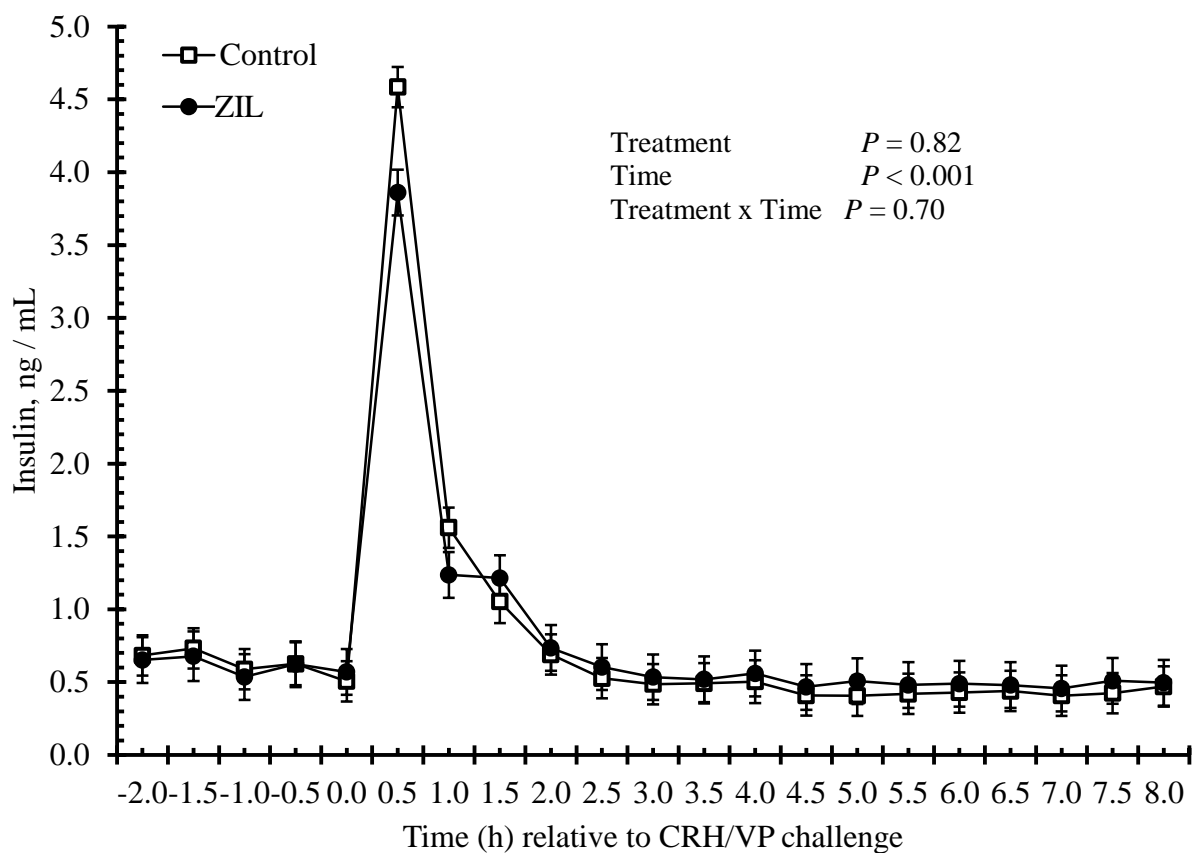


Fig. 4 Serum non-esterified fatty acid (NEFA) response of heifers fed zilpaterol hydrochloride (ZH; 8.33 mg / kg on a DM basis) for 20 d followed by a 3 d withdrawal, or fed the control diet for 23 d and then subjected to a corticotropin-releasing hormone (CRH; 0.3 μ g / kg BW) and vasopressin (VP; 1.0 μ g / kg BW) challenge administered on d 24 at time 0 h. Control represents heifers that were not fed ZH while ZIL represents heifers fed ZH for 20 d followed by a 3 d withdrawal. * represents individual time points that are different ($P \leq 0.05$)

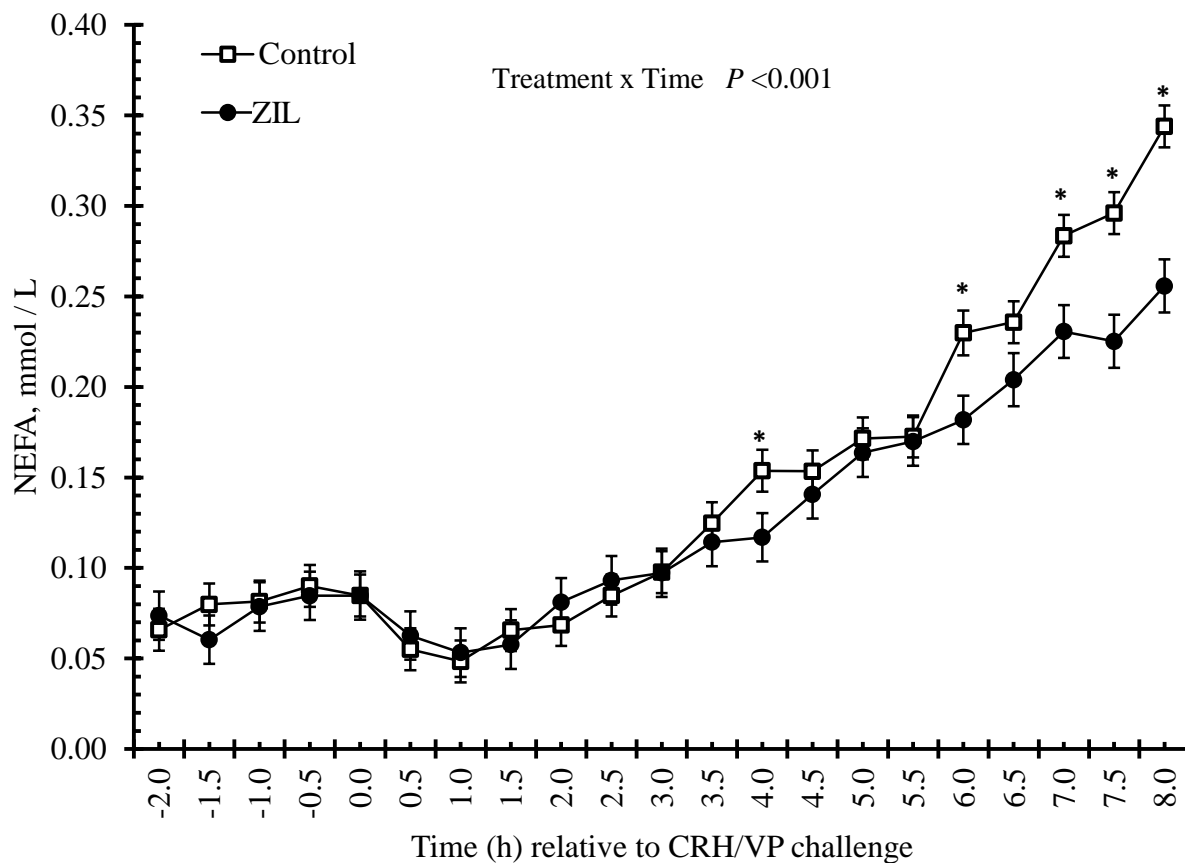


Fig. 5 Serum blood urea nitrogen (BUN) response of heifers fed zilpaterol hydrochloride (ZH; 8.33 mg / kg on a DM basis) for 20 d followed by a 3 d withdrawal, or fed the control diet for 23 d and then subjected to a corticotropin-releasing hormone (CRH; 0.3 μ g / kg BW) and vasopressin (VP; 1.0 μ g / kg BW) challenge administered on d 24 at time 0 h. Control represents heifers that were not fed ZH while ZIL represents heifers fed ZH for 20 d followed by a 3 d withdrawal.

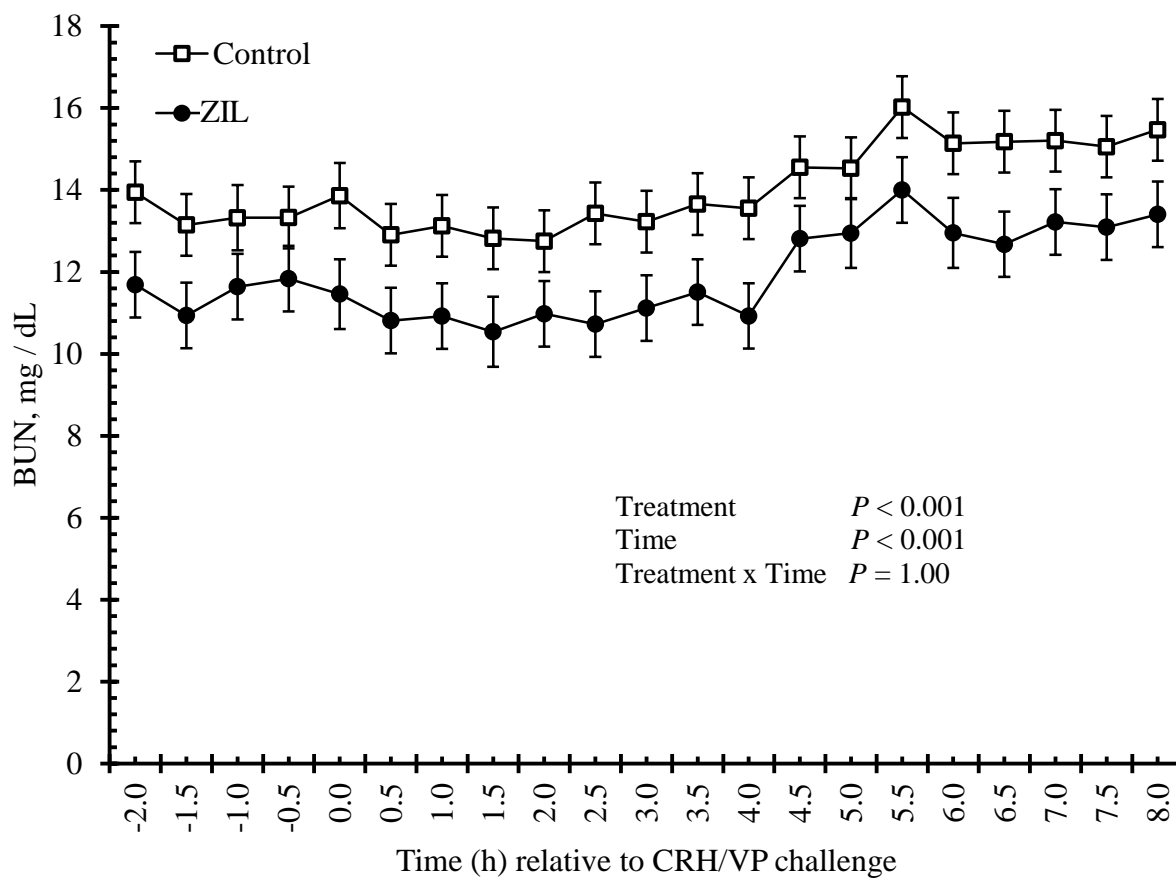


Fig. 6 Serum cortisol response of heifers fed zilpaterol hydrochloride (ZH; 8.33 mg / kg on a DM basis) for 20 d followed by a 3 d withdrawal, or fed the control diet for 23 d and then subjected to a corticotropin-releasing hormone (CRH; 0.3 μ g / kg BW) and vasopressin (VP; 1.0 μ g / kg BW) challenge administered on d 24 at time 0.

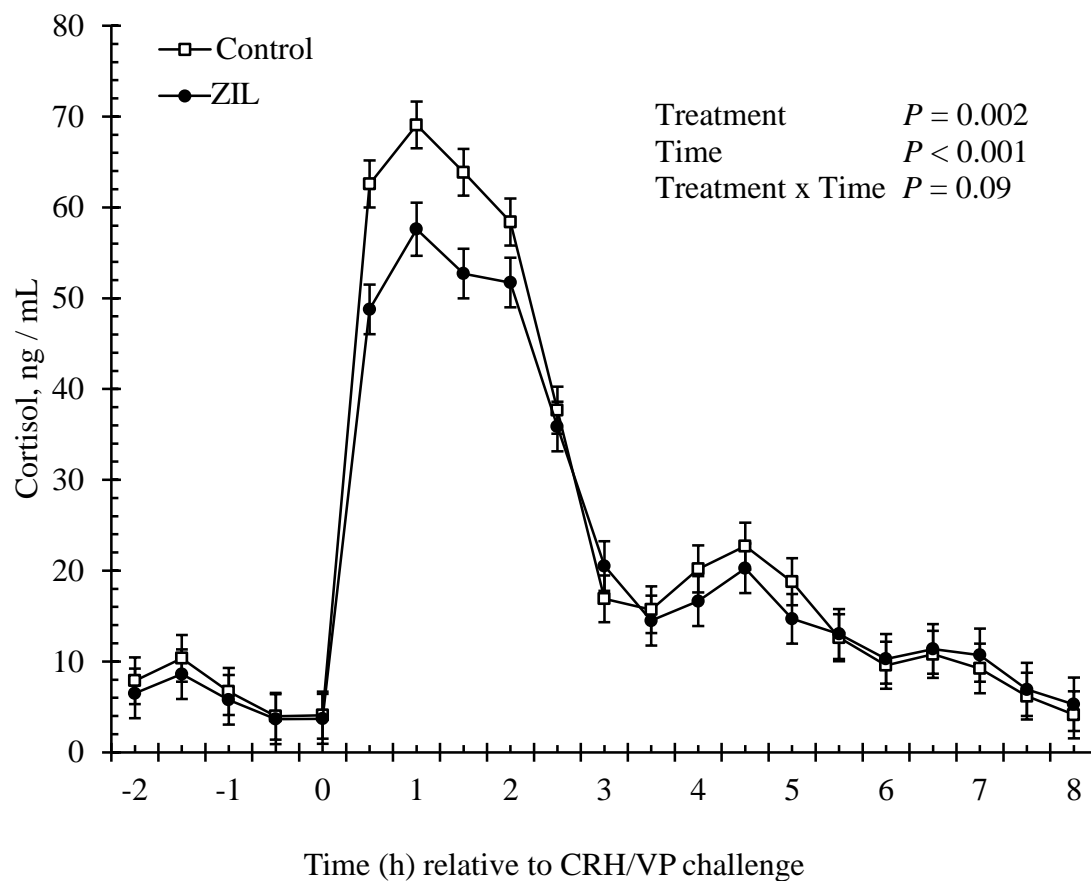


Fig. 7 Plasma epinephrine response of heifers fed zilpaterol hydrochloride (ZH; 8.33 mg / kg on a DM basis) for 20 d followed by a 3 d withdrawal, or fed the control diet for 23 d and then subjected to a corticotropin-releasing hormone (CRH; 0.3 μ g / kg BW) and vasopressin (VP; 1.0 μ g / kg BW) challenge administered on d 24 at time 0.

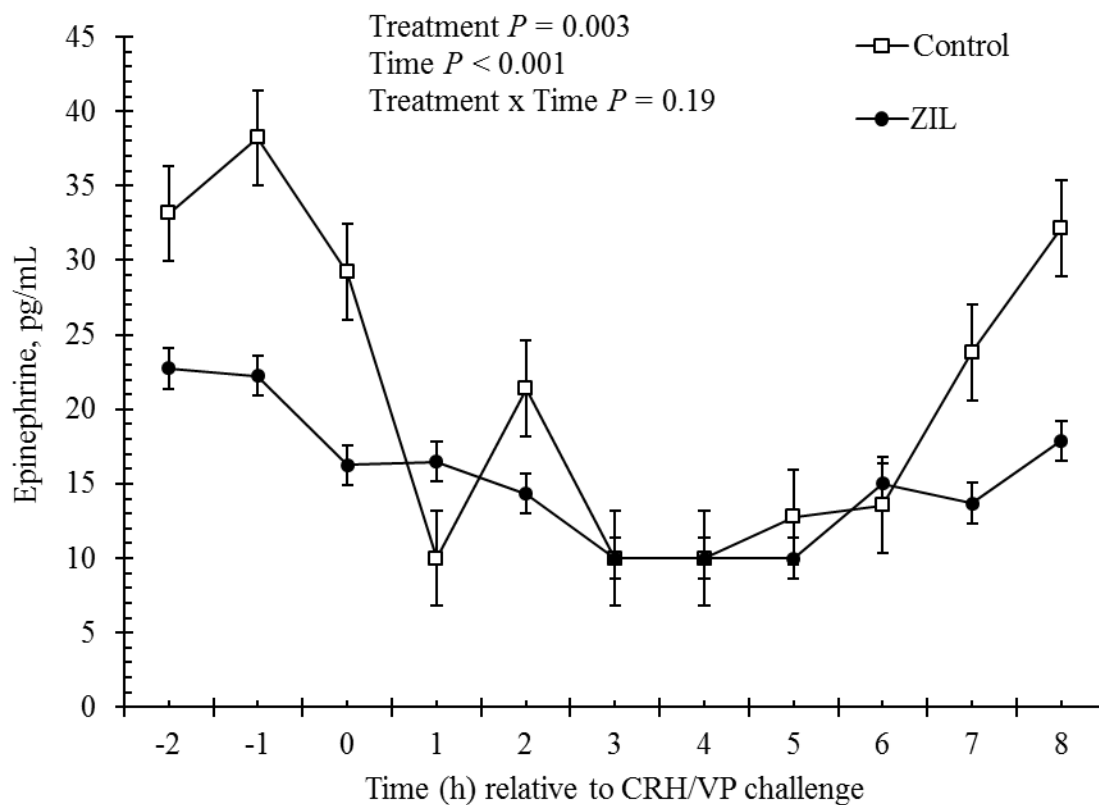


Fig. 8 Plasma norepinephrine response of heifers fed zilpaterol hydrochloride (ZH; 8.33 mg / kg on a DM basis) for 20 d followed by a 3 d withdrawal, or fed the control diet for 23 d and then subjected to a corticotropin-releasing hormone (CRH; 0.3 μg / kg BW) and vasopressin (VP; 1.0 μg / kg BW) challenge administered on d 24 at time 0.

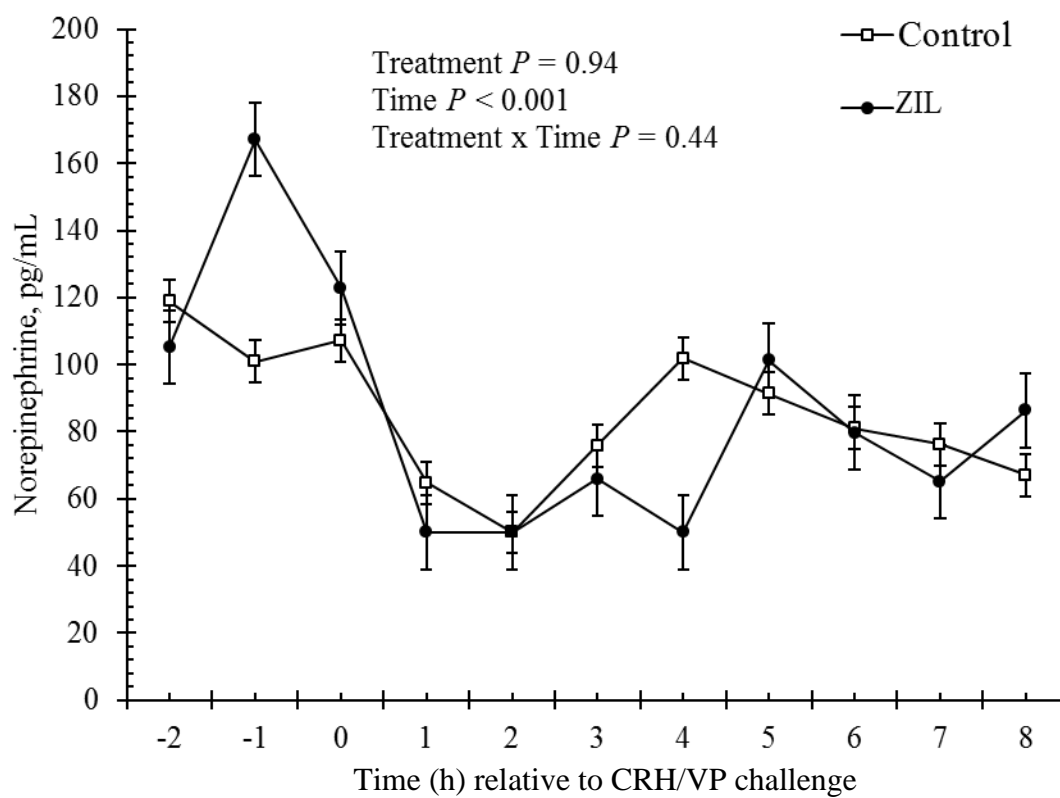


Fig. 9 Total white blood cell (WBC) count response of heifers fed zilpaterol hydrochloride (ZH; 8.33 mg / kg on a DM basis) for 20 d followed by a 3 d withdrawal, or fed the control diet for 23 d and then subjected to a corticotropin-releasing hormone (CRH; 0.3 μ g / kg BW) and vasopressin (VP; 1.0 μ g / kg BW) challenge administered on d 24 at time 0.

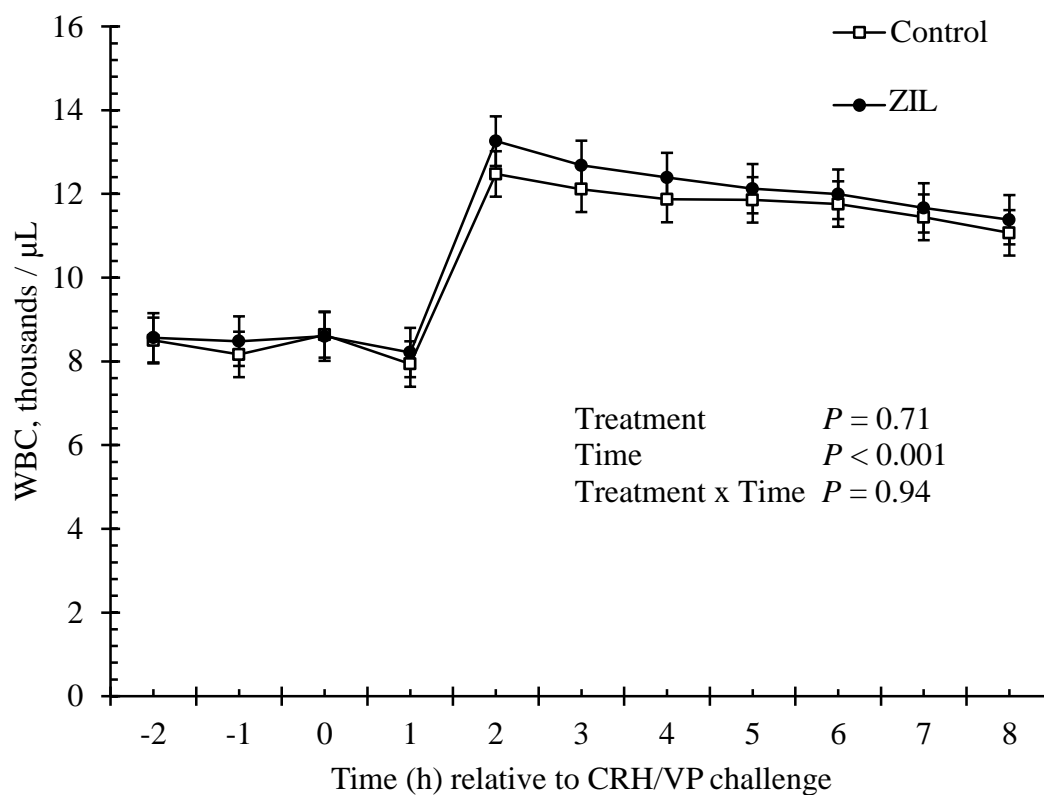


Fig. 10 Lymphocyte count response of heifers fed zilpaterol hydrochloride (ZH; 8.33 mg / kg on a DM basis) for 20 d followed by a 3 d withdrawal, or fed the control diet for 23 d and then subjected to a corticotropin-releasing hormone (CRH; 0.3 μ g / kg BW) and vasopressin (VP; 1.0 μ g / kg BW) challenge administered on d 24 at time 0.

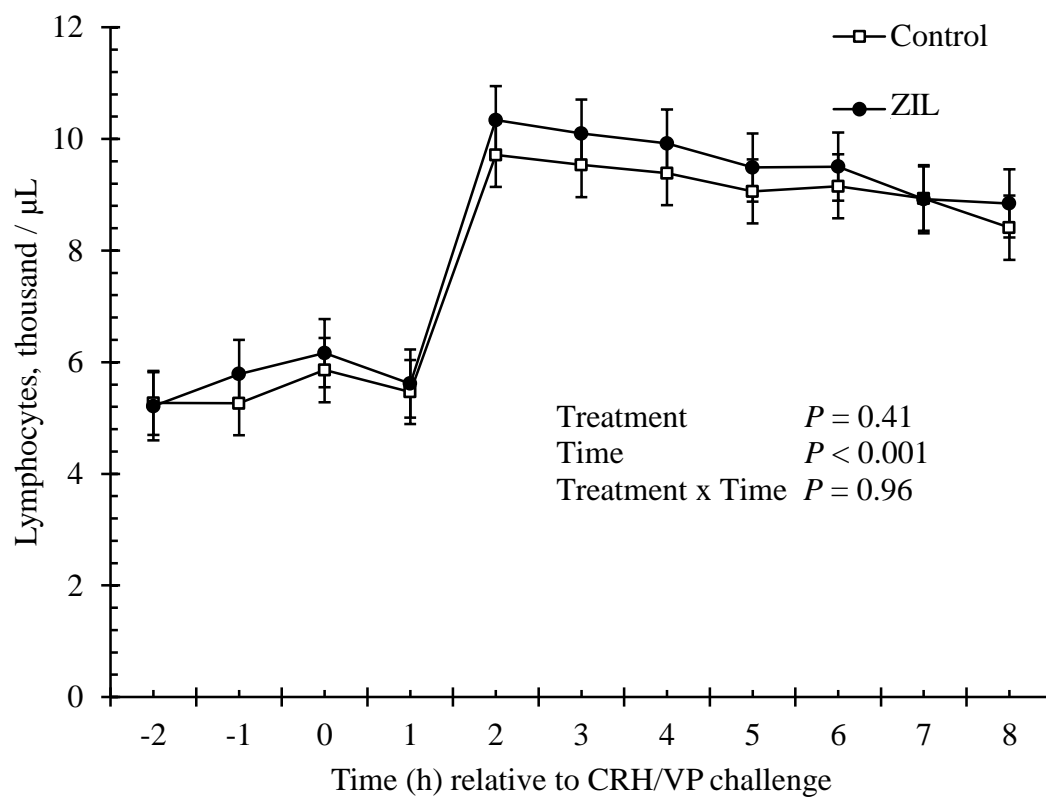


Fig. 11 Neutrophil count response of heifers fed zilpaterol hydrochloride (ZH; 8.33 mg / kg on a DM basis) for 20 d followed by a 3 d withdrawal, or fed the control diet for 23 d and then subjected to a corticotropin-releasing hormone (CRH; 0.3 μ g / kg BW) and vasopressin (VP; 1.0 μ g / kg BW) challenge administered on d 24 at time 0.

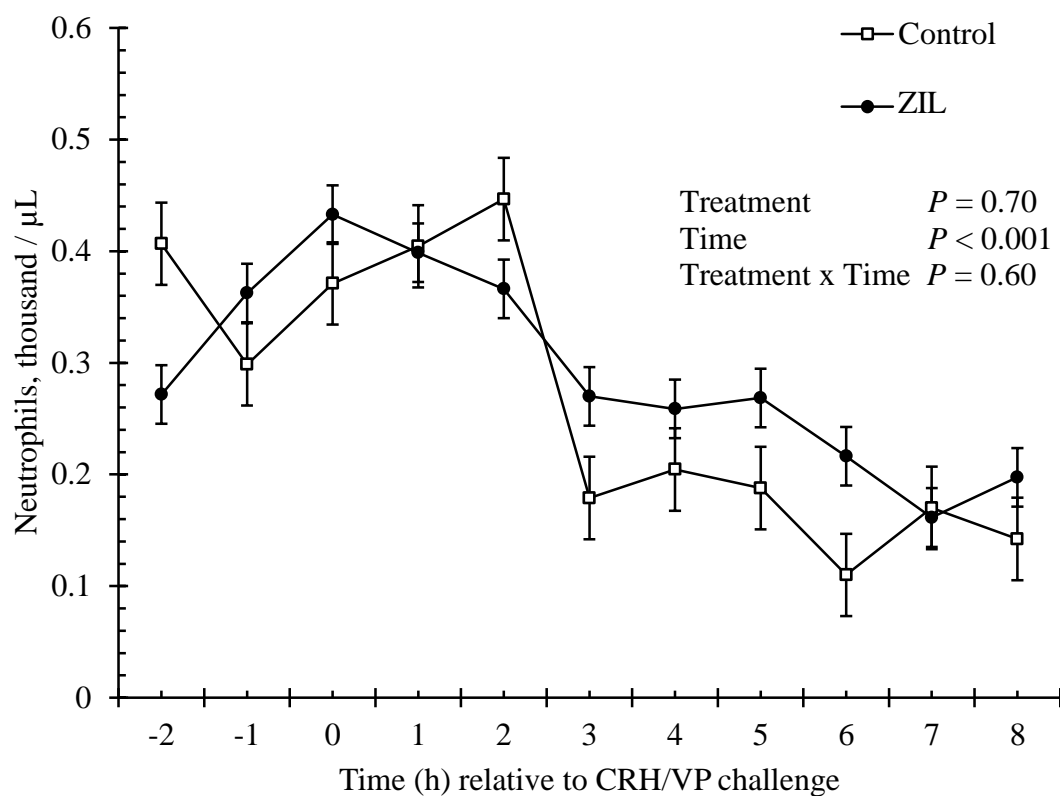


Fig. 12 Monocyte count response of heifers fed zilpaterol hydrochloride (ZH; 8.33 mg / kg on a DM basis) for 20 d followed by a 3 d withdrawal, or fed the control diet for 23 d and then subjected to a corticotropin-releasing hormone (CRH; 0.3 μ g / kg BW) and vasopressin (VP; 1.0 μ g / kg BW) challenge administered on d 24 at time 0.

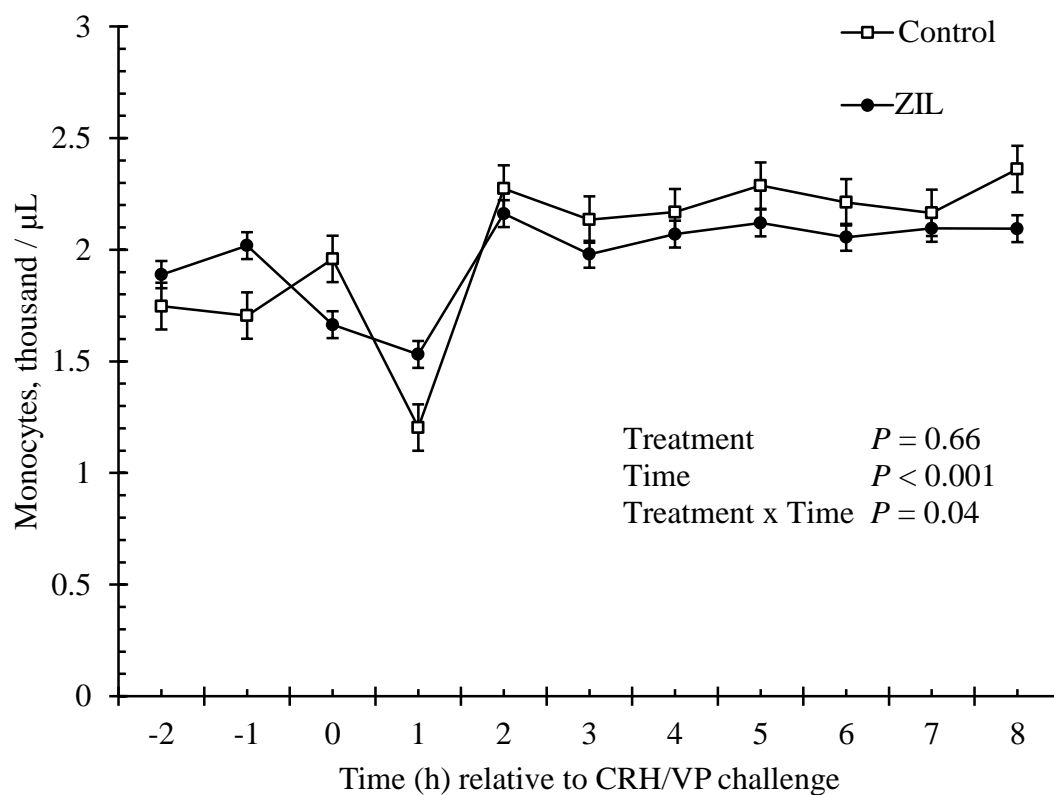
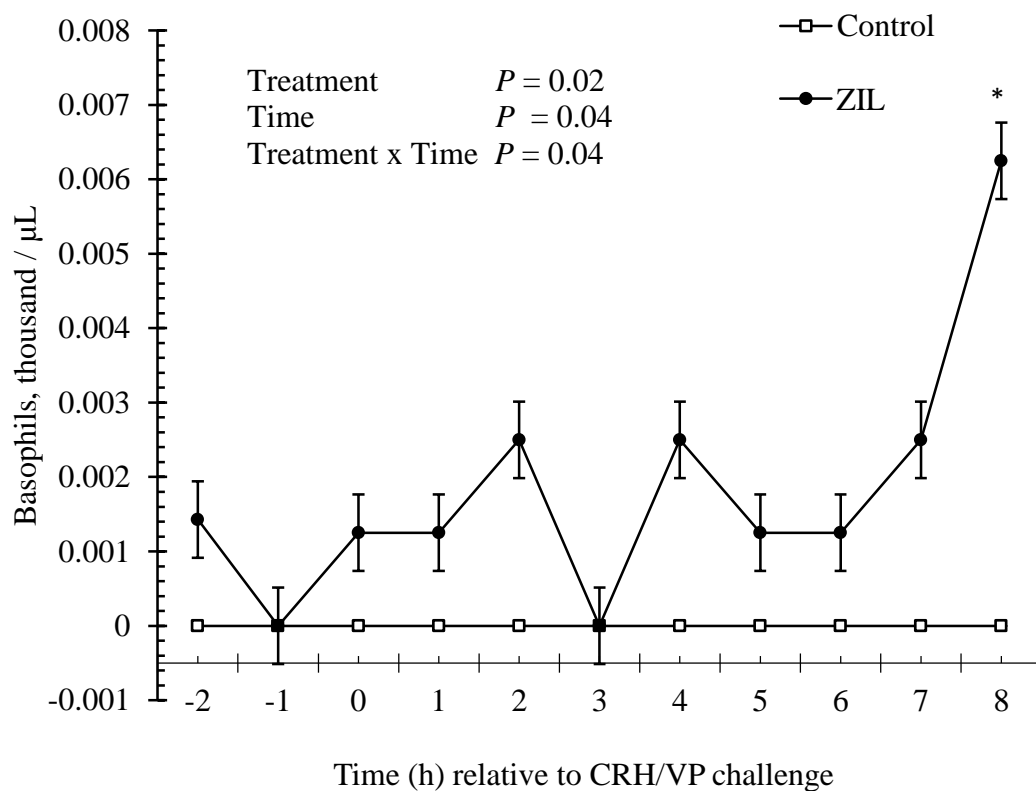


Fig. 13 Basophil count response of heifers fed zilpaterol hydrochloride (ZH; 8.33 mg / kg on a DM basis) for 20 d followed by a 3 d withdrawal, or fed the control diet for 23 d and then subjected to a corticotropin-releasing hormone (CRH; 0.3 μ g / kg BW) and vasopressin (VP; 1.0 μ g / kg BW) challenge administered on d 24 at time 0. * represents individual time points that are different ($P \leq 0.05$).



CHAPTER IV
BLOOD CHEMISTRY AND SELECT ORGAN WEIGHT, HISTOLOGY, AND
CARDIAC MORPHOLOGY OF BEEF HEIFERS SUPPLEMENTED WITH
ZILPATEROL HYDROCHLORIDE AND EXPOSED TO A DUAL
CORTICOTROPIN-RELEASING HORMONE AND VASOPRESSIN STRESS
CHALLENGE

Abstract

The objective of this study was to: 1) determine if administration of a corticotropin-releasing hormone (CRH) and vasopressin (VP) challenge following 20 d ZH supplementation altered the blood chemistry profile in cattle and 2) determine if supplementation of Zilpaterol Hydrochloride (ZH) altered select organ weights, histology and cardiac anatomical features at harvest. Crossbred heifers ($n = 20$; 556 ± 7 kg BW) were randomized into two treatment groups: 1) Control (CON): no ZH, and 2) Zilpaterol (ZIL): ZH at 8.33 mg / kg (DM basis) for 20 d. On d 20 of supplementation, heifers were fitted with indwelling jugular catheters. On d 24, starting at 0800h and continuing until 1600h, blood samples were collected at 60-min intervals. At 1000h, heifers received an i.v. bolus of CRH ($0.3 \mu\text{g} / \text{kg BW}$) and VP ($1.0 \mu\text{g} / \text{kg BW}$) to activate the stress axis. Serum from blood samples was separated and stored at -80°C until analyzed for a large animal chemistry panel. Following the CRH/VP challenge, heifers were harvested on d 25, 26, and 27 (5, 6, and 7 d post ZH supplementation); BW, HCW, select organ weights, histology, and total heart necropsy were performed. A treatment effect ($P \leq 0.02$) was observed for Ca and K, ZIL heifers had decreased concentrations during the CRH/VP challenge and increased concentrations of creatinine ($P = 0.02$) during the CRH/VP

challenge, when compared to control heifers. A treatment x time interaction ($P = 0.02$) was observed for P; concentrations were similar between treatments from -2h to 6h post-challenge, while CON heifers had decreased P at 7 h post-challenge. Control heifers had greater ($P \leq 0.05$) concentrations of alkaline phosphatase and sorbitol dehydrogenase, when compared with ZIL heifers. Liver ($P = 0.06$) and kidney ($P = 0.08$) weights as a percent of BW tended ($P = < 0.08$) to be reduced in ZIL heifers. Gross liver weights tended ($P = 0.08$) to be less in ZIL heifers. Other organ (heart, lung, adrenals) to BW ratios remained similar ($P \geq 0.41$). These data suggest that there are some variations observed between treatments in terms of response to ZH supplementation and the CRH/VP challenge; however, in the environmental conditions of this study, limited variation in blood metabolic responses and organ weights suggests that the supplementation of ZH did not detrimentally alter the physiology of cattle.

Keywords: β -agonist, cattle, heart, pathology, serum profile

INTRODUCTION

β -adrenergic agonist can be defined as a phenylethanolamine similar to the natural adrenergic neurotransmitters epinephrine and norepinephrine (Smith, 1998). The binding of β -adrenergic agonists (β -AA) to β -adrenergic receptors (β -AR) forms a complex which activates the $G_{\text{stimulatory}}$ (G_s) protein. The activation of the G_s protein's α -subunit activates the enzyme adenylyl cyclase which produces cyclic adenosine monophosphate (cAMP), an intracellular signaling molecule that binds the regulatory subunit of protein kinase A. This binding releases the catalytic subunit allowing for the phosphorylation of intracellular proteins (Mersmann, 1998).

Zilpaterol hydrochloride (ZH) is a β_2 – adrenergic receptor agonist (β_2 -AA) that is approved for the use in feedlot cattle in Mexico, South Africa, and the United States (Schmidt and Olson, 2007). Increases in ADG have been observed (Montgomery et al., 2009a) with slight decreases in DMI with ZH supplementation resulting in increased feed efficiency (Mersmann, 2002) and an increase in gain to feed ratio (Avendaño-Reyes et al., 2006). Zilpaterol hydrochloride is associated with an increase in lean tissue deposition in carcass components (addition of 13 and 11 kg in HCW for steers and heifers, respectively; (Montgomery et al., 2009a) and a decrease in adipose tissue (Mersmann, 2002; Mersmann, 1998).

Recently, the supplementation of ZH was implicated as a possible cause of reported lameness at harvest (Thomson et al., 2015). A recent report demonstrated an epidemiologic risk of mortality associated with ZH supplementation during the late feeding period, but did not investigate the cause of the mortality (Loneragan et al., 2014).

Furthermore, limited controlled studies have investigated the impact of ZH supplementation on organ morphology (May et al., 2014). Therefore, the objective of this trial was to evaluate the impact of ZH supplementation on blood chemistry concentrations and internal organ weight and morphology of feedlot heifers at harvest.

MATERIALS AND METHODS

Experimental design

All experimental procedures were in compliance with the *Guide for the Care and Use of Agricultural Animals in Research and Teaching* and approved by the Institutional Animal Care and Use Committee at the University of Nebraska (IACUC #902).

Twenty ovariectomized English-influenced crossbred heifers ($n = 20$; 556 ± 7 kg BW) from the University of Nebraska Agricultural Research and Development Center feedlot were selected for this study. Prior to initiation of the study, heifers were acclimated for a 3 wk period. During acclimation, heifers were haltered and acclimated to being restrained in a tie stall environment and to human contact. At the end of the 3 wk acclimation period, heifers were randomly assigned to one of two treatments: 1) Control (CON; $n = 10$), fed a finishing diet without ZH (Table 1.); and 2) zilpaterol (ZIL; $n = 10$), fed the same finishing diet supplemented with ZH (Zilmax[®]; Merck Animal Health) at a rate of 8.33 mg / kg BW on a DM basis. For delivery of ZH, five percent of the high-moisture corn in the diet was replaced with 4.9853% fine ground corn and ZH at 0.0147% calculated to supply ZH at 8.33 mg / kg on a DM basis. Five percent of the CON diet was replaced with fine ground corn to ensure nutritional similarity between the two diets. All supplements were individually mixed into individual heifer's daily

allotment prior to feeding. Heifers were fed once daily at 0800 for 20 d followed by a 3 d withdrawal period of ZH. During the 3 d period, the five percent high-moisture corn was returned to both the CON and ZIL diets to replace the five percent ground corn supplement.

Eight d prior to the start of ZH supplementation, heifers were relocated to the University of Nebraska - Lincoln Agricultural Research and Development Center Nutrition Dairy Barn during the month of Dec 2013. The Nutrition Barn is a 40-stall barn equipped with individual bunks, automatic waters, and dairy mattresses. Prior to heifers being placed into tie stalls, stalls were randomly assigned to treatment, but blocked by treatment group (two heifers per block) so that no CON heifers shared water with a ZIL heifer. Heifers were maintained in individual tie stalls (1.34 m wide by 1.84 m long) for the duration of the trial. Pine shavings were added on top of the dairy mattress and replaced when needed. On d 20 (last d of ZH supplementation), heifers were removed from tie stalls to obtain a BW (for corticotropin-releasing hormone (CRH) and vasopressin (VP) dose calculations) and fitted with indwelling jugular catheters and then returned to tie stalls. For the jugular cannulation procedure, heifers were restrained in a working chute and the neck area was prepped utilizing betadine scrub and ethanol wipes. A small (2–3 cm) incision was made in the skin to more easily access the jugular vein. Indwelling jugular catheters, consisting of 30.48 cm of sterile Tygon[®] tubing (AAQ04133; US Plastics, Lima, OH, USA; 1.27 mm i.d. and 2.286 mm o.d.), were inserted into the jugular vein using a 11-gauge by 8.3-cm thin-walled stainless steel biomedical needle (o.d. = 3 mm). The catheter was stabilized using tag cement and a 2.08-cm wide porous surgical tape around the incision site. Heifer's entire neck region

was wrapped with vet wrap (Vetrap™; 3 M Animal Care Products, St. Paul, MN, USA) to ensure stability of the catheterization site. The remaining tubing served as the extension portion of the cannula for collection of blood samples (Sanchez et al., 2013). Environmental temperature and relative humidity data were collected inside the Nutrition Barn utilizing four HOBO U23 Pro v2 temperature/relative humidity data logger (U23-001, Onset, Bourne, MA, USA). Data from all four probes was averaged and compiled to provide an overall environmental temperature and relative humidity. On d 24, the average environmental temperature was $13\text{ }^{\circ}\text{C}\pm 1.2\text{ }^{\circ}\text{C}$ and an average relative humidity of $52\%\pm 3.1\%$. On d 24, the average temperature-humidity index was 24.8 ± 1.6 within the barn. Prior to d 24, two heifers were removed from the trial. One heifer was removed from the trial due to development of a sore underneath the halter and the other heifer was removed due to failure of the jugular catheter on d 21 respectively; therefore, there were ten heifers in the CON group and eight heifers in the ZIL group.

The transportation of cattle results in activation of the hypothalamic-pituitary-adrenal (HPA) axis (Falkenberg et al., 2013); furthermore, the use of the CRH/VP stress model has resulted in a similar endocrine response (Carroll et al., 2007) when compared with an actual relocation event. Therefore a CRH/VP challenge on d 24 was utilized as a controlled endocrine stressor to produce a similar endocrine response that would be similar to shipping heifers to the abattoir. On d 24 at 0600 h, all feed residual was removed from bunks and heifers were not provided daily allotment of feed until 1700 h (completion of the stress challenge). Starting at 0800 h and continuing until 1600 h, 18 mL of blood were collected in Sarstedt tubes containing no additive (Sarstedt, Inc., Newton, NC USA) from each heifer in 60-min intervals. Blood samples were allowed to

clot for 30 min at room temperature and then centrifuged at 1500 g for 20 min at 4°C. Isolated serum was stored at -80°C until analyzed for a large animal chemistry profile, β -hydroxybutyrate, lactate, and lactate dehydrogenase. At 1000 h (immediately following collection of blood sample), each heifer received an i.v. bolus of bovine CRH (0.3 μ g / kg BW) and arginine VP (1.0 μ g / kg BW; (Carroll et al., 2007). Following collection of the last blood sample at 1600 h, catheters were removed and the daily allotment of feed was delivered.

On d 25, 26, and 27, heifers were transported to the University of Nebraska – Lincoln Loeffel Meat Laboratory and harvested under USDA Inspection. Heifers were randomly assigned to one of the three harvest d with an equal number of CON and ZIL harvested on each day. Individuals collecting organ weights/measurements and conducting histologic examinations were blinded to treatment. At time of evisceration, organ weights were collected. Following weighing of organs, tissue samples of each organ, the pulmonary artery, and the *longissimus* were collected, placed into 10% neutral buffered formalin and stored until processed routinely for hematoxylin and eosin staining. All tissue slides were evaluated by a certified American College of Veterinary Pathologist that was blinded to treatment.

Serum analysis

Serum samples were shipped overnight to Kansas State Veterinary Diagnostic Laboratory (Manhattan, KS 66506) the laboratory on dry ice. At the diagnostic laboratory, a large animal chemistry profile was performed on serum samples utilizing a Roche Cobas C501 chemistry analyzer for serum samples. Serum concentrations of total protein (mg/dL), albumin (g/dL), globulin (g/dL), total calcium (mg/dL), phosphorus

(mg/dL), potassium (mmol/L), sodium (mmol/L), sodium potassium ratio, chloride (mmol/L), bicarbonate (mmol/L), anion gap (mmol/L), creatinine (mg/dL), creatine kinase (CK, U/L), aspartate transaminase (AST, U/L), alkaline phosphatase (ALP, U/L), γ – glutamyltransferase (γ GT, U/L), and sorbitol dehydrogenase (SDH, U/L) were measured.

Serum β -hydroxybutyrate (β HB) concentrations were determined in duplicate samples by utilizing a β HB assay kit (MAK041; Sigma-Aldrich, St. Louis, MO, USA) in a 96-well format. Plates were incubated at 37°C for 30 min and then read using a plate reader at 450 nm. Concentration of β HB was determined by comparing unknown samples to a standard curve of known β HB concentrations. Data are presented as the concentration in ng / μ L.

Serum lactate concentrations were determined in duplicate samples by utilizing a lactate assay kit (MAK064; Sigma-Aldrich, St. Louis, MO, USA) in a 96-well format. Plates were incubated at 37°C for 30 min and then read using a plate reader at 570 nm. Concentration of lactate was determined by comparing unknown samples to a standard curve of known lactate concentrations. Data are presented as the concentration in ng/ μ L.

Serum lactate dehydrogenase (LDH) concentrations were determined in duplicate samples by utilizing a LDH activity assay kit (MAK066; Sigma-Aldrich, St. Louis, MO, USA) in a 96-well format. Plates were incubated at 37°C for 2 min and then an initial absorbance was measured using a plate reader at 450 nm. After the initial reading, subsequent absorbance was measured every 5 min at 450 nm until the most active unknown sample value was greater than the highest standard. The final measurement utilized in calculating the enzyme activity was the penultimate reading or the value

before the most active sample is near or exceeds the end of the linear range of the standard curve. Concentration of LDH was determined by comparing unknown samples to a standard curve of known LDH concentrations. Data are presented as the concentration in milliunits/mL.

Statistical analysis

A completely randomized design was utilized in the current study. Data were analyzed using the MIXED procedure of SAS specific for repeated measures (SAS Inst. Inc., Cary, NC USA). For serum blood metabolites, treatment, time, and the treatment by time interaction were included as fixed effects, with heifer within treatment included as the experimental unit. Organ weights were analyzed with treatment included as a fixed effect and heifer within treatment included as the experimental unit. When main effects were significant, specific treatment comparisons were made using the PDIF option in SAS, with $P \leq 0.05$ considered significant and $0.05 < P \leq 0.10$ considered a tendency. All data are presented as the $LSM \pm SEM$.

RESULTS

A time ($P < 0.001$) affect and treatment x time interaction ($P = 0.001$) was observed between CON and ZIL heifers (not treatment affect; $P < 0.58$). When individual time points between treatments were evaluated, no time points were different; serum total protein concentrations were 7.60 ± 0.11 g/dL and 7.51 ± 0.11 g/dL for CON and ZIL heifers, respectively (Table 2). There was a treatment ($P = 0.02$) and time ($P < 0.001$) effect for serum albumin but no treatment x time interaction ($P = 0.60$). Serum albumin concentrations were 3.64 ± 0.05 g/dL and 3.47 ± 0.05 g dL for CON and ZIL

heifers, respectively (Table 2). A time ($P < 0.001$) effect and a treatment x time ($P = 0.001$) interaction was observed for serum globulin concentrations between CON and ZIL heifers; but no treatment effect ($P < 0.61$). When individual time points were evaluated, no time points were different between treatment groups, and serum globulin concentrations were 3.95 ± 0.14 g/dL and 4.04 ± 0.14 g/dL for CON and ZIL heifers, respectively (Table 2).

Serum calcium concentrations were affected by treatment ($P = 0.001$) and time ($P < 0.001$); there was no treatment x time interaction ($P = 0.34$; Table 2). Overall, serum calcium concentrations were greater in CON heifers (9.75 ± 0.07 mg/dL) compared with ZIL heifers (9.49 ± 0.07 mg/dL). For serum phosphorus concentrations, there was no treatment ($P < 0.60$) effect, but a time ($P < 0.001$) effect and treatment x time ($P = 0.02$) interaction was observed. When individual time points were evaluated, no time points were different between treatment groups. Serum phosphorus concentrations were 5.67 ± 0.19 mg/dL compared with 5.80 ± 0.19 mg/dL for CON and ZIL heifers, respectively. A treatment ($P = 0.02$) and time ($P < 0.001$) effect were observed for serum potassium; but no treatment x time interaction ($P < 0.13$). Serum potassium concentrations were 3.90 ± 0.02 mmol/L compared with 3.82 ± 0.02 mmol/L for CON and ZIL heifers, respectively. Serum sodium concentrations were not affected by treatment ($P = 0.95$) or treatment x time ($P = 0.53$) interaction. However, there was a time ($P < 0.001$) effect for serum sodium concentrations. Serum sodium/potassium ratio was affected by treatment ($P = 0.03$) and time ($P < 0.001$). Furthermore, there was a tendency ($P = 0.06$) for a treatment x time interaction for serum sodium-potassium ratio. Serum sodium-potassium ratio were 35.6 ± 0.25 compared with 36.4 ± 0.25 for CON vs.

ZIL heifers, respectively. There was no treatment ($P = 0.19$), time ($P = 0.37$), or treatment x time ($P = 0.26$) interaction for serum chloride concentrations. Serum chloride concentrations were 98.8 ± 0.77 and 97.4 ± 0.77 for CON and ZIL heifers respectively. There was a time ($P < 0.001$) effect and a tendency ($P = 0.06$) for a treatment difference for serum bicarbonate, but no treatment x time ($P = 0.88$) interaction. Serum bicarbonate concentrations were 25.7 ± 0.43 mmol/L and 26.9 ± 0.43 mmol/L for CON and ZIL heifers, respectively. There was a treatment ($P = 0.03$) and time effect ($P < 0.001$), but no treatment x time ($P = 0.66$) interaction for serum anion gap. Serum anion gap was 18.7 ± 0.30 and 17.8 ± 0.30 for CON and ZIL heifers respectively.

Serum creatinine concentrations were affected by treatment ($P = 0.003$) and time ($P = 0.02$), but there was only a tendency ($P = 0.06$) for a treatment x time interaction. Overall, creatinine concentrations were less ($P = 0.003$) in CON heifers (1.13 ± 0.06 mg/dL), when compared with ZIL heifers (1.40 ± 0.06 mg/dL). Creatine kinase concentrations were not affected by treatment ($P = 0.17$), time ($P = 0.55$), nor was there a treatment x time ($P = 0.40$). Serum creatine kinase concentrations were 131.8 ± 49 U/L compared with 227.1 ± 49 U / L for CON and ZIL heifers, respectively.

Serum β HB concentrations were not affected by treatment ($P = 0.11$) or treatment x time ($P = 0.83$), but there was a time ($P < 0.001$) effect. There was a time effect ($P = 0.001$) and a tendency ($P = 0.09$) for a treatment effect, but no treatment x time ($P = 0.72$) interaction for serum lactate concentrations. Overall, serum lactate concentrations were 22.95 ± 1.02 ng/ μ L and 20.52 ± 1.02 ng/ μ L for CON and ZIL heifers, respectively.

There was no treatment ($P = 0.39$) or treatment x time ($P = 0.41$) interaction for serum LDH but there was a time ($P < 0.001$) effect.

Liver Enzymes

There was a treatment ($P = 0.05$) and time ($P < 0.001$) effect, but no treatment x time interaction ($P = 0.88$) for serum ALP concentrations. Overall, serum concentrations of ALP were 111.6 ± 8.02 U/L for CON heifers and 88.5 ± 8.02 U/L for ZIL heifers. There was no treatment ($P = 0.22$) or treatment x time ($P = 1.0$) interaction, but there was a time ($P < 0.001$) effect for serum AST. There was no treatment ($P = 0.28$) effect for serum γ GT. However, there was a tendency ($P = 0.08$) for a treatment x time interaction. Overall, serum γ GT concentrations were 48.66 ± 6.41 U/L and 39.00 ± 6.41 U/L for CON vs. ZIL heifers, respectively. There was a treatment ($P = 0.05$) and time ($P < 0.001$) effect, but no treatment x time ($P = 0.64$) interaction for serum SDH. Overall, SDH concentrations were 64.34 ± 6.86 U/L for ZIL heifers, when compared with 45.04 ± 6.86 U/L for CON heifers.

Initial BW, final live weight, HCW, and dressing percentage are reported in Table 3. There was no difference in initial live BW ($P = 0.79$), final live BW ($P = 0.90$), or HCW ($P = 0.17$) between CON and ZIL heifers (Table 3). There was a difference ($P < 0.001$) in dressing percent with ZIL heifers having a greater dressing percent, when compared to CON heifers (65.8 ± 0.5 vs. $62.3 \pm 0.5\%$; Table 3). In terms of select organ weights, there was no effect of treatment; weights for the heart ($P = 0.36$), lungs ($P = 0.30$), total kidneys ($P = 0.11$), left kidney ($P = 0.31$), and left adrenal gland ($P = 0.79$) were similar (Table 4). There was a tendency ($P = 0.08$) for liver weights to be greater in

CON heifers (7.7 ± 0.10 kg) when compared with ZIL heifers (6.9 ± 0.10 kg; Table 4). Also, there was a tendency ($P = 0.10$) for right adrenal gland weights to be greater in CON heifers (14.3 ± 0.69 g) than in ZIL heifers (12.7 ± 0.69 g; Table 4). There was a treatment difference ($P = 0.03$) for right kidney weights; right kidney weights were greater in CON heifers (595.3 ± 28.9 g) compared with ZIL heifers (504.8 ± 28.9 g; Table 4). As a percentage of BW, there was no treatment effect on heart ($P = 0.45$), lung ($P = 0.41$), and total adrenal gland ($P = 0.42$) weights (Table 5). However, as a percentage of BW, there was a tendency ($P = 0.06$) for CON heifers to have greater liver and total kidney ($P = 0.08$) weights than ZIL heifers. Liver and kidney weights as a percentage of final BW were 0.65 ± 0.05 vs 0.61 ± 0.05 for liver and 0.0021 ± 0.001 vs 0.0018 ± 0.001 for kidney comparing CON vs ZIL, respectively (Table 5).

Between CON and ZIL heifers, no treatment differences were observed between CON and ZIL heifers for aortic valve to left atrial ventricular valve diameter ratio ($P = 0.64$), aortic valve to pulmonary valve diameter ratio ($P = 0.16$), aortic valve circumference ($P = 0.98$), or the aortic valve / right ventricular valve diameter ratio ($P = 0.96$; Table 6). Also, there were no differences for inter-ventricular septum thickness ($P = 0.30$), left atrial ventricular valve circumference ($P = 0.64$), left atrial ventricular valve / right atrial ventricular valve diameter ratio ($P = 0.64$), left ventricle free wall thickness ($P = 0.97$), or for the left ventricle + interventricular septum / right ventricle weight ratio ($P = 0.14$) between treatment groups (Table 6). There was a tendency ($P = 0.07$) for left ventricle + interventricular septum / total heart weight ratio to be less in CON (0.58 ± 0.05), when compared with ZIL heifers (0.61 ± 0.05 ; Table 6). There was no difference in left ventricle free wall thickness / right ventricle free wall thickness ratio ($P = 0.13$), left

ventricle thickness / septal thickness ratio ($P = 0.36$), or left ventricle + septum weight ($P = 0.97$; Table 6). In addition, there was no difference in pulmonary valve / left atrial ventricular valve ratio ($P = 0.47$), pulmonary valve / right atrial ventricular valve ratio ($P = 0.30$), or pulmonary valve circumference ($P = 0.11$; Table 6). There was no difference ($P = 0.94$) for right atrial ventricular valve circumference. There was a tendency ($P = 0.09$) for right ventricle free wall thickness to be thicker in CON (2.11 ± 0.05 cm), when compared to ZIL heifers (1.99 ± 0.05 cm; Table 6). There was no difference ($P = 0.33$) in right ventricle free wall weight between treatment groups. There was a difference ($P = 0.04$) in right ventricle free wall / inter-ventricular septal thickness ratio with CON having a greater ratio when compared with ZIL (0.51 ± 0.02 vs 0.47 ± 0.02 , respectively; Table 6). However, there was no difference ($P = 0.72$) for right ventricle / total heart weight ratio between treatments (Table 6). Furthermore, there was no difference ($P = 0.85$) in left ventricle + inter-ventricular septum as a percent of BW or right ventricle ($P = 0.30$) as a percent of BW (Table 7). Gross lesions of hepatitis were seen in two CON heifers and gross nephritis was seen in one ZIL heifer; catheter infection observed during the trial). Histologic evaluation of lungs demonstrated a very mild regional eosinophilic interstitial pneumonia in one CON heifer. Pulmonary artery changes were adventitial and consisted of periarterial fibrosis and subacute inflammation and were noted in two CON and one ZIL heifer. Livers had mild periportal hepatitis in 6/10 CON and 7/9 ZIL heifers. The two CON heifers with gross hepatitis had histologic fibrosis in addition to mild lymphocytic infiltrates. The hepatic lesions were typical mild periportal infiltrates and rare isolated small lymphocytic foci. Renal lesions were mild focal chronic interstitial nephritis consisting of very small, sparse, scattered, interstitial aggregates of lymphocytes

and plasma cells (6 CON and 6 ZIL heifers). A single foci of lymphocytes was seen in the adrenal medulla of one CON heifer. Skeletal muscle and cardiac muscle was histologically normal in all heifers.

DISCUSSION

To our knowledge, the evaluation of complete blood chemistry profiles of cattle supplemented with ZH for a 20 d period in a controlled environment has not been reported. However, similar measurements have been reported in feedlot lambs supplemented with ZH (correct?). Lopez-Carlos et al. (2010) reported that ZH supplementation at either 0.10, 0.20, or 0.30 mg/kg of BW/d for the last 42 d of feeding had no impact on serum total protein (don't need this citation at the end of the sentence since you start with it) (López-Carlos et al., 2010). Serum total protein concentrations account for albumin and other globulin fractions and are indicators of overall serum protein status (Evans and Duncan, 2003). Neither supplementation of ZH or ractopamine hydrochloride had an impact on overall serum protein status in feedlot lambs (López-Carlos et al., 2010). Furthermore, the feeding of clenbuterol to Hereford steers did not impact serum total protein (Ricks et al., 1984). In the current study, ZH supplementation decreased serum total protein in heifers; however, the decrease was minimal (based upon reported normal references for cattle). Furthermore, a decrease in total protein would be anticipated due to a decrease of albumin in ZIL supplemented heifers.

Blood pH affects blood calcium concentrations, with an increase in pH resulting in an increase in calcium concentrations (Russell and Roussel, 2007). Furthermore,

decreases in albumin can result in decreased calcium concentrations (Russell and Roussel, 2007). In the current study, serum calcium concentrations were decreased in ZIL heifers which also had decreased albumin concentrations. Ricks et al., (1984), reported no difference in blood calcium or phosphorus concentrations of Hereford steers supplemented clenbuterol in samples collected immediately prior to harvest. Due to approximately 99% of mineral storage in bone, serum circulating concentrations of minerals may be an unreliable measurement on total body mineral status of an animal (Russell and Roussel, 2007). Potassium is the major intracellular cation which is extensively regulated due to organ interactions (Russell and Roussel, 2007). In the current study, potassium concentrations were decreased in ZIL heifers when compared with CON. The decrease in serum potassium concentrations in ZIL heifers could possibly be explained due to an increase in lean muscle deposition observed during the utilization of ZH (Vasconcelos et al., 2008). Muscle has been reported to contain 2.43 ± 0.9 mg/g of potassium (Mariam et al., 2004). Serum sodium concentrations correlated to total body sodium due to the fact that sodium is a major extracellular cation which is the main contributor to osmotic force and is confined to extracellular fluid (Russell and Roussel, 2007). No differences for serum sodium would be expected in the current study as all heifers had *ab libitum* access to water. The treatment difference observed for Na:K ratio with ZIL heifers having greater concentrations when compared with CON heifers would be expected due to the observed differences between treatment groups for potassium.

Chloride is the major anion in extracellular fluid and usually resembles sodium concentrations due to the reabsorption of sodium and chloride together in the kidneys

(Russell and Roussel, 2007). In the current study, serum chloride concentrations were not affected by treatment which would be anticipated when compared with serum sodium concentrations. The reference range for bicarbonate in cattle is 17 – 29 mmol/L (Kaneko et al., 2008). In the current study, both CON and ZIL heifers had bicarbonate concentrations within the reported reference range. However, bicarbonate tended to be decreased in CON heifers. Ramirez et al. (1992) reported a decrease in bicarbonate concentrations of Holstein cows reared at sea level when compared with Holstein cows reared at 3,000 m. Cows reared in high altitude are exposed to ambient hypoxia resulting in the development of decreased bicarbonate concentrations to compensate abnormal acid-base concentrations (Ramirez et al., 1992). Clenbuterol a β_2 -AA has been reported to increase blood flow and oxygen uptake in the hind-quarters of cattle during early supplementation (Eisemann and Huntington, 1987). Due to an increase in oxygen uptake observed in β_2 -AA supplemented cattle, greater bicarbonate concentrations observed in ZIL heifers may suggest an increase in oxygen availability with ZH supplementation.

The reported reference range for anion gap for cattle is 13.9 – 20.2 mmol/L (Kaneko et al., 2008). Alterations in the anion gap are commonly utilized to determine acid/base disorders; an increase in lactate or β HB concentrations (Gabow et al., 1980). In the current study, β HB concentrations were within the normal reference range for anion gap for CON and ZIL heifers. However, CON heifers had a greater anion gap which could possibly be explained due to a tendency for CON heifers to have greater serum lactate. Creatinine concentrations were greater in ZIL heifers but were within the normal reported reference range; 1.2 – 1.9 mg/dL (Kaneko et al., 2008); however, CON heifers had decreased creatinine concentrations when compared with normal reported reference

ranges. The increase in creatinine would be expected due to a 19 kg increase in HCW for ZIL heifers, when compared with CON heifers. During normal muscle metabolism, creatine is broken down into creatinine which can be utilized as an indicator of muscle mass in the serum (Russell and Roussel, 2007). Furthermore, serum creatinine is positively correlated with HCW, dressing percentage, and proportion of lean meat in a carcass (Istasse et al., 1990). Cimaterol, a β_2 -AA (Signorile et al., 1995), increased plasma creatinine concentrations in Friesian steers during long term supplementation (Chikhou et al., 1993). Creatine kinase is a key enzyme for cellular energetics (Wallimann et al., 1992). In the current study, there was no difference in creatine kinase between CON and ZIL heifers. Thomson et al., 2015 reported no increase in creatine kinase activity in steers under normal feedlot conditions supplemented ZH or ractopamine hydrochloride when compared with a control group. Creatine kinase has been reported to increase when steers are transported (Warriss et al., 1995).

β -hydroxybutyrate is a ketone body produced during the metabolism of NEFA in the liver and can indicate a negative energy balance (Ospina et al., 2010). No difference was observed between treatment groups for β HB in the current study. This lack of a difference is similar to those reported in steers supplemented clenbuterol (Eisemann et al., 1988a). Furthermore, there was also no difference observed for β HB concentrations for steers supplemented ZH for a 23 d duration (Van Bibber-Krueger et al., 2015). Van Bibber-Krueger et al. (2015) suggested that ZH supplementation in cattle did not alter the metabolization rate of β HB. During anaerobic conditions, lactate is formed by the oxidation of NADH by pyruvate, thus allowing glycolysis to continue (Reece et al., 2015). In the current study, there was a tendency for lactate to be decreased in ZIL

supplemented heifers. Thomson et al. (2015) and Van Bibber-Krueger et al. (2015) reported no difference in lactate concentrations of steers supplemented ZH. Differences observed between these studies could be explained by frequency of sampling, as the current study evaluated lactate concentrations during a 10 h CRH/VP challenge following a 3 d ZH withdrawal. Van Bibber-Krueger et al. (2015) observed a suggested association for decreased plasma lactate concentrations as d on ZH increased; however, no samples were obtained during the 3 d ZH withdrawal period. These results are in conflict with those reported from previous β -AA supplementation. The inclusion of P-5369 and Q-2636 in milk replacer increased lactate concentrations in calves (Blum and Flueckiger, 1988). Eisemann et al. (1988) observed increased lactate concentrations when clenbuterol was supplemented to steers. Furthermore, arterial cimaterol infusion increased lactate concentrations as cimaterol infusion rates increased (Byrem et al., 1996). Observed differences between the current study and previous studies could be again, related to the time at which serum samples were collected. In the current study heifers had been withdrawn from ZH supplementation for 3 d, while in the abovementioned studies, serums samples were collected during the supplementation period. The glycolytic enzyme LDH catalyzes the conversion of lactate to pyruvic acid (Doornenbal et al., 1988). The reference range for LDH in the cow is 692 – 1445 U/L (Kaneko et al., 2008). In the current study, there was no difference in LDH concentrations between CON and ZIL heifers. When ZH was administered to horses, an increase in LDH was observed (Wagner et al., 2008). Differences observed in these studies can possibly be explained by species differences as ZH is not approved for utilization in the horse (and can be fatal).

In the current study, serum concentrations for alkaline phosphatase (ALP), aspartate transaminase, γ -glutamyltransferase, and sorbitol dehydrogenase were determined. Alkaline phosphatase catalyzes the cleavage of P_i from phosphate esters with production of aspartate transaminase originating in the liver of mature animals (Doornenbal et al., 1988). The reference values for aspartate transaminase in the cow are 78 – 132 U/L (Kaneko et al., 2008). Ricks et al. (1984) reported no difference in aspartate transaminase in Hereford steers supplemented clenbuterol. In the current study, aspartate transaminase concentrations were decreased in ZIL heifers when compared with CON. A possible hypothesis for the decrease in aspartate transaminase concentrations could be related to the decrease in liver weight observed in the current study. It has been reported that in dogs with increased corticosteroid concentrations, aspartate transaminase concentrations are increased due to the formation of a more heat-stable isoenzyme of aspartate transaminase (Teske et al., 1986). Previous work from our lab observed a decrease in cortisol in ZIL supplemented heifers which could explain the increase in aspartate transaminase observed in CON heifers. Aspartate transaminase is an enzyme that catalyzes the transfer of an α -amino group from an amino acid to an α -keto acid (Doornenbal et al., 1988). In the current study, no difference was observed in aspartate transaminase concentrations. An increase in aspartate transaminase concentrations was observed in Friesian calves administered cimaterol (Chikhou et al., 1993). However, Chikhou et al. (1993) did not observe a difference in liver weights in cimaterol treated calves, possibly explaining the differences between these studies. γ -glutamyltransferase is a membrane bound enzyme that catalyzes the transfer of γ -glutamyl groups from γ -glutamylpeptides to other peptides or amino acids with a

normal reference range of 6.1 – 17.4 U/L (Kaneko et al., 2008). In the current study, there was no difference in γ -glutamyltransferase between treatment groups; however, both treatment groups were above normal reference ranges. A potential cause of increased γ -glutamyltransferase in the current study could be associated with the CRH/VP challenge administered. In canines, glucocorticoids have been associated with an increase in γ -glutamyltransferase concentrations (Shull and Hornbuckle, 1979). Lastly, sorbitol dehydrogenase concentrations were increased in CON heifers in the current study; however, both treatment groups had elevated sorbitol dehydrogenase concentrations compared with reference ranges for the cow which are 4.3 – 15.3 U/L (Kaneko et al., 2008). Feed restriction in llamas resulted in an increase in hepatic lipidosis which resulted in elevated sorbitol dehydrogenase concentrations (Tornquist et al., 2001). Therefore, the increase in sorbitol dehydrogenase in the current study could potentially be a result of increased lipolysis during the CRH/VP challenge allowing heifers to mount a stress challenge.

Experiments that have evaluated and reported the effects of β -AA on major organs of feedlot cattle are limited. In the current study, there was no difference in final BW or HCW potentially due to the number of animals on study; however, there was a numerical difference in HCW with ZIL supplemented heifers having a 19 kg increase when compared with CON heifers. Montgomery et al. (2009a) reported an 11 kg increase in HCW with a 20 d supplementation of ZH in heifers. In the current study, there was a positive increase in dressing percentage observed when ZH was supplemented. Montgomery et al. (2009b) reported a dressing percentage increase of 1.2 percentage units with the feeding of ZH. The current study was not designed to evaluate

the carcass effects of ZH but a more in-depth analysis of organ parameters with the supplementation of ZH. The increase in dressing percentage of ZIL heifers would indicate a ZH response was initiated.

In the current study, no differences were observed in heart and lung weights between treatment groups. No difference was reported in combined heart and lung weights of beef steers fed ZH for 20 d (Holland et al., 2010). However, the feeding of clenbuterol (β_2 -AA) in normal mice decreased heart weights by 8% when compared with control mice (Sharma et al., 1997). Furthermore, May et al. (2014) reported a tendency for a decrease in heart weights of Holstein steers supplemented ZH for 20 d but no difference in lung weights between zilpaterol and control steers. A decrease in kidney and liver mass of zilpaterol supplemented steers has been reported, when compared with control steers was observed (May et al., 2014). In the current study, there was only a difference observed for right kidney weights, with ZIL supplemented heifers having lighter right kidney weights; however, overall kidney weights did not differ between treatment groups. Furthermore, there was a tendency for liver weights to be greater in the CON heifers when compared with ZIL heifers in the current study. The feeding of salbutamol (β_2 -AA) to pigs has been reported to decrease kidney and liver weights when compared with control pigs (Hansen et al., 1994). The decreased in renal and liver mass could be a result of decreased gut fill. The repartitioning of energy to skeletal muscle growth and a decrease in feed intake resulting in less gut fill could explain the decrease in renal and liver mass of ZIL supplemented cattle (Montgomery et al., 2009b).

To our knowledge, data on the effect of β -AA's on adrenal weights has not been reported in the literature. The comparing of organ weight ratios to BW can result in

misleading data due to differences in animal adipose and muscle tissues (Joseph, 1908), as well as the previously reported effect of ZH on HCW (Montgomery et al., 2009a; Montgomery et al., 2009b). Reported percent's for bovine heart weight ratios to BW are 0.26, 0.52, and 0.38% for steers, bulls, and female, respectively (Joseph, 1908). In the current study, heifer heart weight ratios to BW were within this reported range for cattle.

Minimum published anatomic data is available for measurements of the dissection of the bovine heart. The total cardiac to BW ratios and the left ventricle and septum to right ventricle weight ratios were within the normal range (Leifsson, 2011). The expanded data and reference of a broader range of normal cardiac dimensional anatomy published here will be useful in evaluating finishing cattle affected by or at risk of pulmonary hypertension and chronic right heart failure which appears to be a growing problem (Neary et al., 2013). The gross lesions of liver fibrosis were only observed in CON heifers and the kidney lesion was in a heifer with a documented catheter infection and transient sepsis during the study. The mild chronic periportal hepatitis and chronic interstitial nephritis seen equally in both groups are common background lesions seen in cattle in standard production systems. These likely reflect residual inflammation of neonatal infections. The mild adventitial inflammation seen around the pulmonary artery in two ZIL and one CON heifers are of undetermined etiology but suspected to be residual scarring and inflammation due to past resolved pulmonary infection. None of the histologic lesions were more prevalent in ZIL heifers and were typical of incidental changes seen in standard commercial cattle. No pathologic changes were observed in these cattle associated with ZH treatment that might suggest a direct link between treatment and reported mortality risk in some production systems. Changes in feed

delivery and other production practices associated with implementing of supplement
delivery and documentation of causes of mortality during the last 25 d of feeding need
further prospective study.

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Table 1. Composition of finishing diets fed to control (CON) and zilpaterol (ZIL) heifers as a percent of DM basis during a corticotropin-releasing hormone (CRH; 0.3 µg / kg BW) and vasopressin (VP; 1.0 µg / kg BW) challenge in finishing heifers.

Item	Treatment	
	Control	ZIL ¹
Ingredient (%)		
High Moisture	51.00	51.00
Corn		
Sweet Bran ²	40.00	40.00
Wheat Straw	5.00	5.00
Fine Ground Corn	1.8710	1.8710
Limestone	1.6400	1.6400
Salt	0.3000	0.3000
Tallow	0.1000	0.1000
Beef Trace Mineral	0.0500	0.0500
Rumensin-90 ³	0.0150	0.0150
Vitamin A-D-E	0.0165	0.0165
Tylan-40 ³	0.0075	0.0075
Supplement ⁴		
Fine Ground Corn	5.0	4.9853
Zilpaterol Hydrochloride	-	0.0147

¹Heifers receiving zilpaterol hydrochloride for 20-d period with 3-d withdrawal

²Sweet Bran, (Cargill Corn Milling; Blair, NE)

³Elanco Animal Health; Greenfield, IN

⁴The control supplement contained fine ground corn only. Zilpaterol hydrochloride (ZH) supplement contained (DM basis) 0.0147% Zilmax® (Merck Animal Health) Type A medicated article and 4.9853% fine ground corn and supplied zilpaterol hydrochloride (ZH) supplementation (8.33 mg / kg on a DM basis). Supplement was fed for 20 d.

Table 2. Effect of zilpaterol hydrochloride (ZH) supplementation (8.33 mg / kg on a DM basis) on serum metabolites following 20 d ZH supplementation, 3 d withdrawal period, and a corticotropin-releasing hormone and vasopressin challenge administered on d 24.

Item	CON	ZIL ¹	SEM	Treatment	Time	Trt x Time
<i>Blood metabolite</i>						
Total Protein, g / dL	7.60	7.51	0.11	0.58	<0.001	0.001
Albumin, g / dL	3.64	3.45	0.05	0.02	<0.001	0.60
Globulin, g / dL	3.95	4.04	0.14	0.61	<0.001	0.001
Calcium, mmol / L ²	9.75	9.49	0.07	0.01	<0.001	0.34
Phosphorus, mg / dL	5.67	5.80	0.19	0.60	<0.001	0.02
Potassium, mmol / L	3.90	3.82	0.02	0.02	<0.001	0.13
Sodium, mmol / L	138.3	138.3	0.24	0.95	<0.001	0.53
Na-K ratio ³	35.64	36.42	0.25	0.03	<0.001	0.06
Chloride, mmol / L	98.84	97.44	0.19	0.19	0.37	0.26
Bicarbonate, mmol / L	25.73	26.92	0.43	0.06	<0.001	0.88
Anion Gap ⁴	18.71	17.78	0.30	0.03	<0.001	0.66
Creatinine, mg / dL	1.13	1.40	0.06	0.003	0.02	0.06
CK, U / L ⁵	131.8	227.1	49.0	0.17	0.55	0.40
BHB, ng / μ L ⁶	89.13	83.73	2.35	0.11	<0.001	0.83
Lactate, ng / μ L	22.94	20.52	1.02	0.09	<0.001	0.73
LDH, milliunits / mL ⁷	919.93	973.81	45.36	0.39	<0.001	0.41
<i>Liver enzymes</i>						
ALP, U / L ⁸	111.6	88.5	8.02	0.05	<0.001	0.88
AST, U / L ⁹	156.8	131.1	15.1	0.22	<0.001	1.0
GGT, U / L ¹⁰	48.66	39.00	6.41	0.28	<0.001	0.08
SDH, U / L ¹¹	64.34	45.04	6.86	0.05	<0.001	0.64

¹Heifers receiving zilpaterol hydrochloride for 20-d period with 3-d withdrawal

²Total serum calcium

³Serum sodium-potassium ratio

⁴Calculated serum anion gap

⁵Serum creatine kinase

⁶Serum beta-hydroxybutyrate

⁷Serum lactate dehydrogenase

⁸Serum alkaline phosphatase

⁹Serum aspartate transaminase

¹⁰Serum gamma glutamyltransferase

¹¹Serum sorbitol dehydrogenase

Table 3. Effect of zilpaterol hydrochloride (ZH) supplementation (8.33 mg / kg on a DM basis) on live weights (kg), HCW (kg), and dressing percentage following 20 d ZH supplementation, 3 d withdrawal period, and a corticotropin-releasing hormone and vasopressin challenge administered on d 24.

Item	CON	ZIL ¹	SEM	<i>P</i> -value
<i>Weights</i>				
Initial weight, kg	550	546	11.2	0.79
Final weight, kg	578	575	13.7	0.90
HCW, kg	359	378	9.2	0.17
Dressing percent, %	62.3	65.8	0.5	<0.001

¹ Heifers receiving zilpaterol hydrochloride for 20-d period with 3-d withdrawal

Table 4. Effect of zilpaterol hydrochloride (ZH) supplementation (8.33 mg / kg on a DM basis) on major organ weights following 20 d ZH supplementation, 3 d withdrawal period, and a corticotropin-releasing hormone and vasopressin challenge administered on d 24.

Item	CON	ZIL ¹	SEM	<i>P-value</i>
<i>Organ</i>				
Heart, kg	2.64	2.51	0.10	0.36
Liver, kg	7.70	6.90	0.31	0.08
Lung, kg	3.76	3.52	0.17	0.30
Total Kidney, g	1197.24	1057.5	60.4	0.11
Right Kidney, g	595.3	504.8	28.9	0.03
Left Kidney, g	602.0	552.7	34.0	0.31
Total Adrenal, g	28.8	26.8	1.39	0.32
Right Adrenal, g	14.3	12.7	0.69	0.10
Left Adrenal, g	14.5	14.2	0.84	0.79

¹ Heifers receiving zilpaterol hydrochloride for 20-d period with 3-d withdrawal

Table 5. Effect of zilpaterol hydrochloride (ZH) supplementation (8.33 mg / kg on a DM basis) on major organ weights as a percent of total BW following 20 d ZH supplementation, 3 d withdrawal period, and a corticotropin-releasing hormone and vasopressin challenge administered on d 24.

Item	CON	ZIL ¹	SEM	<i>P-value</i>
<i>Organ</i>				
Heart	0.46	0.44	0.02	0.45
Liver	1.33	1.19	0.05	0.06
Lung	0.65	0.61	0.03	0.41
Total Kidney	0.0021	0.0018	0.00009	0.08
Total Adrenal	0.005	0.005	0.0004	0.42

¹ Heifers receiving zilpaterol hydrochloride for 20-d period with 3-d withdrawal

Table 6. Effect of zilpaterol hydrochloride (ZH) supplementation (8.33 mg / kg on a DM basis) on heart measurements following 20 d ZH supplementation, 3 d withdrawal period, and a corticotropin-releasing hormone and vasopressin challenge administered on d 24.

Item	CON	ZIL ¹	SEM	<i>P</i> -value
Heart parameter				
A/LAV ²	0.71	0.73	0.03	0.64
A/P ³	0.98	1.04	0.03	0.16
Aorta, cm ⁴	11.61	11.60	0.38	0.98
AR/RAV ⁵	0.69	0.69	0.04	0.96
IVS, TH ⁶	4.12	4.29	0.12	0.30
LAV, cm ⁷	16.53	16.13	0.61	0.64
LAV/RAV ⁸	0.97	0.95	0.03	0.64
LVFW, TH ⁹	3.54	3.54	0.08	0.97
LV+S/RV ¹⁰	3.28	3.57	0.13	0.14
LV+S/THW ¹¹	0.58	0.61	0.05	0.07
LVFWT/RVFWT ¹²	1.68	1.79	0.05	0.13
LVT/ST ¹³	0.86	0.82	0.03	0.36
LVW+S ¹⁴	1.53	1.53	0.06	0.97
P/LAV ¹⁵	0.72	0.70	0.02	0.47
P/RAV ¹⁶	0.70	0.66	0.03	0.30
PUL, cm ¹⁷	11.89	11.20	0.30	0.11
RAV, cm ¹⁸	17.14	17.08	0.60	0.94
RVFW, TH ¹⁹	2.11	1.99	0.05	0.09
RVFW ²⁰	467.60	436.60	22.40	0.33
RVFWT /IVST ²¹	0.51	0.47	0.02	0.04
RV/THW ²²	0.18	0.17	0.06	0.72

¹ Heifers receiving zilpaterol hydrochloride for 20-d period with 3-d withdrawal

² Aortic valve / left atrial ventricular valve diameter ratio

³ Aortic valve / pulmonary valve diameter ratio

⁴ Aortic valve circumference in cm

⁵ Aortic valve / right atrial ventricular valve diameter ratio

⁶ Interventricular septum thickness in cm

⁷ Left atrial ventricular valve circumference in cm

⁸ Left atrial ventricular valve / right atrial ventricular valve diameter ratio

⁹ Left ventricle free wall thickness in cm

¹⁰ Left ventricle + interventricular septum / right ventricle weight ratio

¹¹ Left ventricle + interventricular septum / total heart weight

¹² Left ventricle free wall thickness / right ventricle free wall thickness

¹³ Left ventricle thickness / septal thickness

¹⁴ Left ventricle + septum weight kg.

¹⁵ Pulmonary valve / left atrial ventricular valve ratio

¹⁶ Pulmonary valve / right atrial ventricular valve ratio

¹⁷ Pulmonary valve circumference in cm

¹⁸ Right atrial ventricular valve circumference in cm

¹⁹ Right ventricle free wall thickness in cm

²⁰ Right ventricle free wall weight in g

²¹ Right ventricle free wall / interventricular septal thickness ratio

²² Right ventricle / total heart weight ratio

Table 7. Effect of zilpaterol hydrochloride (ZH) supplementation (8.33 mg / kg on a DM basis) on heart parameter weights as a percent of total BW following 20 d ZH supplementation

Item	CON	ZIL ¹	SEM	<i>P-value</i>
<i>Heart parameters</i>				
LV+S BW ²	0.26	0.27	0.01	0.85
RV BW ³	0.08	0.08	0.003	0.30

¹ Heifers receiving zilpaterol hydrochloride for 20-d period with 3-d withdrawal ¹

² Left ventricle + interventricular septum as percent of BW

³ Right ventricle as percent of BW

CHAPTER V

OVERALL SUMMARY, CONCLUSIONS, AND IMPLICATIONS FOR BEEF HEIFERS SUPPLEMENTED WITH ZILPATEROL HYDROCHLORIDE AND EXPOSED TO A DUAL CORTICOTROPIN-RELEASING HORMONE AND VASOPRESSIN STRESS CHALLENGE

Performance

Initial BW, final live weight, HCW, and dressing percentage are reported in. There was no difference in initial live BW ($P = 0.79$), final live BW ($P = 0.90$), or HCW ($P = 0.17$) between treatment groups. There was a difference ($P < 0.001$) in dressing percent with CON heifers having a decreased dressing percentage when compared to dressing percentage of ZIL heifers (62.3 ± 0.5 vs 65.8 ± 0.5 percent, respectively).

In the current study, there was no difference in final BW or HCW potentially due to the number of animals on study; however, there was a numerical difference in HCW with ZIL supplemented heifers having a 19 kg increase when compared with CON heifers. Montgomery et al., (2009a) reported an 11 kg increase in HCW with a 20 d supplementation of ZH in heifers. In the current study, there was a positive increase in dressing percentage observed when ZH was supplemented. Montgomery et al., (2009b) reported an increase in dressing percentage of 1.2% units when feeding ZH was utilized. The current study was not designed to evaluate the carcass effects of ZH but a more in-depth analysis of organ parameters with the supplementation of ZH. The increase in dressing percentage of ZIL heifers would indicate a ZH response was initiated.

Vaginal Temperature

Vaginal temperature (VT) was decreased ($P < 0.001$) in ZIL compared with CON heifers, and varied over time. Due to the treatment effect, with ZIL heifers entering the challenge with decreased VT, the VT data were further analyzed as the change from baseline values (average of values from -2 to 0 h; 0800 to 1000 h), with average baseline values included as a covariate in the analysis. The change in VT relative to baseline values was affected by treatment ($P \leq 0.001$) and time ($P = 0.02$), but there was no treatment x time interaction ($P = 0.99$). Heifers in the CON group had an overall positive change (increase; $P \leq 0.001$) in VT ($0.068 \pm 0.005^\circ\text{C}$) while ZIL heifers had an overall negative (decrease) change in VT in response to CRH/VP challenge ($-0.050 \pm 0.005^\circ\text{C}$). There is limited reported data regarding the effect of β -AA on body temperature in any species including cattle. The feeding of ZH has been reported to decrease ruminal temperature when compared with control steers not fed ZH (Boyd et al., 2015). As reported by Boyd et al (2015) the decreased body temperature may be attributed to increased respiration rates, allowing ZH supplemented steers the ability to dissipate excess body heat. In this study, authors attributed decreased body temperature to increased respiration rates body heat, which has been published by others (Boyd et al., 2015). Another biological response typically observed following stimulation of the β -adrenergic receptors is vasodilation of interior tissues (e.g. skeletal muscle, heart, lungs, liver, and gastrointestinal tract; (Dawes et al., 1997). An increase in vasodilation would support the observed decrease in body temperature, as this would allow dissipation of heat from the body surface at a faster rate compared with CON heifers. However, it

would be expected to see a decline in vasodilation with continued treatment of β -AA due to a possible down-regulation of the β -adrenergic receptors (Smith et al., 1994).

Serum Metabolites

There was no treatment effect observed for serum glucose and insulin concentrations. Studies in cattle using other β -AAs (i.e., clenbuterol) have observed differences in glucose, lipid, and protein metabolism (Eisemann et al., 1988b); yet these metabolites were measured early in the supplementation process rather than following withdrawal as in the current study. When glucose and insulin were measured on d 13 of cimaterol supplementation in steers, there were no differences in glucose or insulin concentrations compared with control steers (Chikhou et al., 1991b). Additionally, results from a study in finishing steers revealed decreased glucose concentrations after 21 d of supplementation with ZH (Van Bibber et al., 2010). The observed difference in glucose concentrations reported by Van Bibber et al. (2010) differs from the current study where no treatment differences in circulating concentrations of glucose were observed in response to ZH supplementation in cattle. The differences observed between the study by Van Bibber et al. (2010) and the current study could be due to sample frequency and the collection of samples following a 3 d withdrawal in the current study, which the previous studies did not include. In the current study, samples were obtained through indwelling jugular catheters in 30 min increments whereas in the Van Bibber et al. (2010) study, blood samples were only collected once daily via restraining within working chute. Furthermore, the current study was conducted within a controlled environment, thus a possible reduction in ME requirements (Hoffman and Self, 1970).

There was a treatment x time interaction ($P < 0.001$) for serum NEFA concentrations. Concentrations of NEFA were greater in CON heifers, compared with ZIL heifers at 4 h ($P = 0.04$), at 6 h ($P = 0.02$) and from 7 to 8 h ($P < 0.01$) following the CRH/VP challenge. Initial feeding of β -AAs has been reported to increase NEFA concentrations (Mersmann, 1998). During the initial stages of feeding, β -AAs exhibit more lipolytic effects, a biological effect that may be eventually down-regulated during extended feedings and as fat content decreases. In a prior study, concentrations of NEFA measured weekly during ZH supplementation in finishing steers reported no difference in NEFA concentrations (Van Bibber et al., 2010), thus supporting data from the current study as baseline NEFA concentrations did not differ between treatments groups. The decrease in NEFA concentrations in response to the CRH/VP challenge in the ZH-supplemented heifers is not surprising considering the increase in lean tissue growth, and the decrease in lipogenesis that has been observed in β -AA fed cattle (Sillence, 2004). Feeding of β -AAs leads to decreased lipid deposition, and thus decreased NEFA concentrations when stimulated by a catabolic agent such as cortisol. Studies reported in the literature have demonstrated that β -AAs have anti-lipogenic effects (Dunshea, 1993; Sillence, 2004), data that also supports the results in the current study.

Serum BUN concentrations were affected by treatment ($P < 0.001$) and time ($P < 0.001$). Concentrations of BUN were greater in CON heifers when compared with ZIL heifers. The major growth promoting effects of β -AAs are primarily observed in skeletal muscle (Sillence, 2004), which results in the catabolism of protein from other tissues to be used to build muscle, although only critical when provided inadequate nutrition (Sillence, 2004). Furthermore, β -AA activate β -receptors in muscle and adipose tissue

resulting in decreased lipogenesis, and increased lipolysis and protein accretion (Mersmann, 1998). Therefore, the decreased BUN concentrations in ZH supplemented heifers is likely due to increased anabolism of protein in skeletal muscle and decreased catabolism of protein in other tissues as these heifers were provided adequate nutrition to support additional lean tissue accretion during the study (Bell et al., 1998a). The decrease in BUN observed in the current study is supported by work with other β -AAs in which the authors observed similar decreases in BUN concentrations in cattle supplemented with cimaterol (Chikhou et al., 1991b). The increase in BUN concentrations post-CRH/VP in both treatment groups is in concert with the increased concentrations of NEFA observed post-challenge, and suggests a greater energy demand during this period than was supplied by glucose alone. Decreased BUN concentrations in ZH-supplemented cattle observed in the current study is supported by work in finishing steers in which the authors reported decreased BUN concentrations after 20 d and 21 d of ZH supplementation, (Parr et al., 2010; Van Bibber et al., 2010).

Serum cortisol concentrations were affected by treatment ($P = 0.002$) and time ($P < 0.001$). Cortisol concentrations were greater in CON when compared with ZIL heifers during the CRH/VP challenge. Baseline cortisol concentrations were not different between treatments in the current study. This is in agreement with a study utilizing cimaterol treatment in steers, where no difference in cortisol concentrations were observed 1 and 13 d following initiation of oral dosing (Chikhou et al., 1991b). The temporal pattern associated with an acute increase in circulating concentrations of cortisol followed by a subsequent decline in the current study is similar to that observed in Brahman calves in response to a CRH challenge (Hulbert et al., 2013), albeit the

magnitude of the cortisol response was greater in the current study. Control heifers produced a greater cortisol response to the CRH/VP challenge compared with ZIL heifers, thus suggesting a possible down-regulation of aspects associated with HPA axis regulation.

Epinephrine and Norepinephrine Concentrations

Plasma epinephrine concentrations were affected by treatment ($P = 0.003$) and time ($P < 0.001$). Due to the treatment effect, data were further analyzed within baseline and challenge periods. Prior to the CRH/VP challenge, CON heifers had greater ($P = 0.005$) baseline epinephrine concentrations (-2 to 0 h), when compared with ZIL heifers. Following the challenge, there was a treatment ($P = 0.03$) and time ($P = 0.002$) effect for epinephrine concentrations with CON heifers having greater epinephrine concentrations, when compared with ZIL heifers. For plasma norepinephrine concentrations, there was only a time effect ($P \leq 0.001$) observed. Norepinephrine concentrations decreased ($P \leq 0.001$) 1 h following administration of CRH/VP. Concentrations remained decreased for the duration of the study ($P \leq 0.001$) for CON and ZIL heifers.

Differences observed in epinephrine concentrations but not norepinephrine concentrations would theoretically be expected when feeding ZH. Naturally-occurring physiological β -adrenergic agonists consist of norepinephrine, a neurotransmitter, and epinephrine, an adrenal medullary hormone (Mersmann, 1998). Both epinephrine and norepinephrine are capable of binding to the α_1 -, α_2 -, β_1 and β_2 -adrenergic receptors (Robinson et al., 1989); however, β_2 -adrenergic receptors have a greater affinity for epinephrine compared with norepinephrine. Therefore, the lack of down-regulation of

the norepinephrine response may be due to the difference in receptor affinity. With ZH being classified as a β_2 -adrenergic receptor agonist it should not affect the regulation of norepinephrine via actions on the α_1 -, α_2 -, and β_1 -adrenergic receptors. Adenylate cyclase in the erythrocytes of frogs has been used to demonstrate a loss of β -adrenergic receptor binding sites in response to catecholamine induced sensitivity. When injected with norepinephrine or isoproterenol (β_1 -adrenergic receptor agonist), cyclic adenosine monophosphate generated from frog erythrocyte membranes decreased compared with controls (Mukherjee et al., 1975). I hypothesize that the decrease in catecholamine responsiveness was due to a reduction in β -adrenergic receptors, or a conformational change resulting in inactive receptors (Mukherjee et al., 1975). In the current study, decreased concentrations of epinephrine in ZIL heifers would further support a down-regulation of the sympathomedullary axis via action on the β_2 -adrenergic receptors.

Complete Blood Cell Counts

There were no main effect differences in total red blood cells ($P = 0.82$), hemoglobin ($P = 0.14$), or platelets ($P = 0.70$) between CON and ZIL heifers. There was a tendency ($P \geq 0.06$) for both total red blood cells and hemoglobin to change over time regardless of treatment. There was a time difference ($P \leq 0.001$) for platelets with platelet concentrations increasing relative to the CRH/VP challenge in both CON and ZIL heifers. There was a treatment x time interaction ($P = 0.03$) for hematocrit. Therefore, hematocrit concentrations were further analyzed as baseline and post-challenge. There was a tendency ($P = 0.06$) for a treatment effect and a difference ($P = 0.05$) over time during the baseline period between treatments for hematocrit. Therefore, change from baseline was calculated and analyzed as a percent change between treatments. There was

no difference for treatment ($P = 0.85$) or treatment x time ($P = 0.80$) for percent change in hematocrit. However, there was a time difference ($P = 0.04$) for percent change in hematocrit such that hematocrit concentrations decreased following administration of CRH/VP. Total white blood cells, lymphocytes, and neutrophils were not affected by treatment ($P \geq 0.41$); however, these variables were affected by time ($P < 0.001$). White blood cells and lymphocytes increased ($P < 0.001$) 2 h following administration of CRH/VP, and remained increased for the duration of the study ($P < 0.001$, 8 h compared with 0 h). In contrast, neutrophils decreased ($P < 0.001$) 3 h following administration of CRH/VP, and remained decreased for the duration of the study ($P < 0.001$, 8 h compared with 0 h). There was a treatment x time ($P = 0.04$) interaction for monocytes. However, no time points were different between treatment groups during the trial. The treatment x time interaction can primarily be contributed to a greater fluctuation of monocyte concentrations within the CON heifers. There was a treatment effect ($P = 0.04$) for eosinophils with greater concentrations for CON when compared with ZIL heifers. There was a treatment x time ($P = 0.04$) interaction for basophil concentrations. At 8 h post CRH/VP challenge, basophil concentrations were greater for ZIL heifers when compared with CON heifers during the CRH/VP challenge.

Few studies have determined the effect of CRH or a combined CRH/VP challenge on immune cell populations, particularly in cattle; however, increased glucocorticoids results in an increase of neutrophils (Burton et al., 2005). Immune cell populations are sensitive to CRH, as well as the intermediate stress hormone, adrenocorticotrophic hormone (ACTH), and cortisol. Hulbert et al. (2013) observed a decrease in total leukocytes at 1 h following a CRH challenge in Brahman calves. This is in contrast to

the current study, in which an increase in total WBCs was observed 2 h following CRH/VP administration regardless of treatment. Many factors may contribute to these opposing observations, including age of the cattle, breed of the cattle, and/or the combination of a CRH challenge with VP given simultaneously versus a solitary CRH challenge. While there are limited studies utilizing CRH, the CRH/VP challenge in this study was utilized to mimic an acute stressor. In mice exposed to acute restraint stress, an increase in natural killer (NK) cells, a subset of lymphocytes, was observed (Bauer et al., 2001). Furthermore, the use of cyanoketone, an androstanol, in restraint-stressed rats resulted in less cortisol production during the time of stress (Dhabhar et al., 1996). The blocking of cortisol production with cyanoketone also significantly reduced the stress-induced decrease in leukocytes that has been reported in rats under acute stress (Dhabhar et al., 1996).

The decrease in circulating neutrophils following CRH/VP challenge is indicative of movement of neutrophils out of the blood and into the tissues in preparation for immune activation (e.g. wounding). In contrast, the increase in circulating total WBC and lymphocytes suggests a mobilization of these cells (Dhabhar et al., 2012). This redistribution of immune cells suggests an activation of the immune response, as several factors, including chemoattractants, cell adhesion molecules, and cytokines are needed for this process to occur (Bauer et al., 2001). Changes in expression of cell adhesion molecules, stimulated by stress hormones, may be responsible for the changes observed in immune cell populations not only in response to CRH/VP challenge, but in response to ZH supplementation.

Blood Chemistry Profile

Serum calcium concentrations were affected by treatment ($P = 0.001$) and time ($P < 0.001$). Overall, serum calcium concentrations were greater in CON heifers when compared with ZIL heifers. For serum phosphorus concentrations, there was no treatment ($P < 0.60$) effect, but a time ($P < 0.001$) effect and treatment x time ($P = 0.02$) interaction was observed. However, when individual time points were evaluated, no time points were different between treatment groups. A treatment ($P = 0.02$) and a time ($P < 0.001$) effect were observed for serum potassium. Serum sodium concentrations were not affected by treatment ($P = 0.95$) or treatment x time ($P = 0.53$) interaction; however, there was a time ($P < 0.001$) effect for serum sodium concentrations. Serum sodium-potassium ratio was affected by treatment ($P = 0.03$) and time ($P < 0.001$). Furthermore, there was a tendency ($P = 0.06$) for a treatment x time interaction for serum sodium-potassium ratio. There was no treatment ($P = 0.19$), time ($P = 0.37$), or treatment x time ($P = 0.26$) interaction for serum chloride concentrations. There was a time ($P < 0.001$) effect and a tendency ($P = 0.06$) for a treatment difference for serum bicarbonate, but no treatment x time ($P = 0.88$) interaction. There was a treatment ($P = 0.03$) and time ($P < 0.001$) effect, but no treatment x time ($P = 0.66$) interaction for serum anion gap.

There was no treatment effect ($P < 0.58$) for serum total protein, but a time ($P < 0.001$) effect and treatment x time interaction ($P = 0.001$) was observed between CON and ZIL heifers. However, when individual time points were evaluated, no time points were different between treatment groups. There was a treatment ($P = 0.02$) and time ($P < 0.001$) effect for serum albumin but no treatment x time interaction ($P = 0.60$). Serum albumin concentrations were increased in CON heifers when compared with ZIL heifers. There was no treatment effect ($P < 0.61$) for serum globulin concentrations, but a time (P

< 0.001) effect and a treatment x time ($P = 0.001$) interaction was observed between CON and ZIL heifers. However, when individual time points were evaluated, no time points were different between treatment groups.

To our knowledge, the evaluation of complete blood chemistry profiles of cattle supplemented with ZH for a 20 d period in a controlled environment has not been reported. However, similar measurements have been reported in feedlot lambs supplemented with. Lopez-Carlos et al. (2010) reported that ZH supplementation at either 0.10, 0.20, or 0.30 mg / kg of BW / d for the last 42 d of feeding had no impact on serum total protein (López-Carlos et al., 2010). Serum total protein concentrations account for albumin and other globulin fractions and are indicators of overall serum protein status (Evans and Duncan, 2003). Neither supplementation of ZH or ractopamine hydrochloride had an impact on overall serum protein status in feedlot lambs (López-Carlos et al., 2010). Furthermore, the feeding of clenbuterol to Hereford steers did not impact serum total protein (Ricks et al., 1984). In the current study, ZH supplementation decreased serum total protein in heifers; however, the decrease was minimal. Furthermore, a decrease in total protein would be expected due to a decrease of albumin in ZIL supplemented heifers.

Blood pH affects blood calcium concentrations, with an increase in pH resulting in an increase in calcium concentrations (Russell and Roussel, 2007). Furthermore, decreases in albumin can result in decreased calcium concentrations (Russell and Roussel, 2007). In the current study, serum calcium concentrations were decreased in ZIL heifers which also had decreased albumin concentrations. Ricks et al., (1984), reported no difference in blood calcium or phosphorus concentrations of Hereford steers

supplemented clenbuterol in samples collected immediately prior to harvest. Due to approximately 99 percent of mineral storage in bone, serum circulating concentrations of minerals may be an unreliable measurement on total body mineral status of an animal (Russell and Roussel, 2007). Potassium is the major intracellular cation which is extensively regulated due to organ interactions (Russell and Roussel, 2007). In the current study, potassium concentrations were decreased in ZIL heifers when compared with CON. The decrease in serum potassium concentrations in ZIL heifers could possibly be explained due to an increase in lean muscle deposition observed during the utilization of ZH (Vasconcelos et al., 2008). Muscle has been reported to contain 2.43 ± 0.92 mg/g of potassium (Mariam et al., 2004). Serum sodium concentrations reflect total body sodium due to the fact that sodium is a major extracellular cation which is the main contributor to osmotic force and is confined to extracellular fluid (Russell and Roussel, 2007). No differences for serum sodium would be expected in the current study since all heifers had *ab libitum* access to water. The treatment difference observed for Na:K ratio with ZIL heifers having greater concentrations when compared with CON heifers would be expected due to the observed differences between treatment groups for potassium.

Chloride is the major anion in extracellular fluid and usually resembles sodium concentrations due to the reabsorption of sodium and chloride together in the kidneys (Russell and Roussel, 2007). In the current study, serum chloride concentrations were not affected by treatment which would be expected when compared with serum sodium concentrations. The reference range for bicarbonate in cattle is 17 – 29 mmol/L (Kaneko et al., 2008). In the current study, both treatment groups had bicarbonate concentrations

within the reference range; however, bicarbonate tended to be decreased in CON heifers. Ramirez et al. (1992) reported a decrease in bicarbonate concentrations of Holstein cows reared at sea level when compared with Holstein cows reared at 3,000 m. Cows reared in high altitude are exposed to ambient hypoxia resulting in the development of decreased bicarbonate concentrations to compensate abnormal acid-base concentrations (Ramirez et al., 1992). Clenbuterol a β_2 -AA has been reported to increase blood flow and oxygen uptake in the hind-quarters of cattle during early supplementation (Eisemann and Huntington, 1987). Due to an increase in oxygen uptake observed in β_2 -AA supplemented cattle, greater bicarbonate concentrations observed in ZIL heifers could suggest an increase in oxygen availability with ZH supplementation. The reference range for anion gap in bovine is 13.9 – 20.2 mmol/L (Kaneko et al., 2008). Alterations in the anion gap are commonly utilized to determine acid/base disorders with an increase in lactate or β HB concentrations (Gabow et al., 1980).

Serum creatinine concentrations were affected by treatment ($P = 0.003$) and time ($P = 0.02$), but there was only a tendency ($P = 0.06$) for a treatment x time interaction. Overall, creatinine concentrations were less in CON heifers when compared with ZIL heifers. Creatine kinase concentrations were not affected by treatment ($P = 0.17$), time ($P = 0.55$), or treatment x time ($P = 0.40$). Serum beta-hydroxybutyrate concentrations were not affected by treatment ($P = 0.11$) or treatment x time ($P = 0.83$), but there was a time ($P < 0.001$) effect. For serum lactate concentrations, there was a time effect ($P = 0.001$) and a tendency ($P = 0.09$) for a treatment effect, but no treatment x time ($P = 0.72$) interaction observed. There was no treatment ($P = 0.39$) or treatment x time ($P = 0.41$)

interaction for serum lactate dehydrogenase (LDH) but there was a time ($P < 0.001$) effect.

In the current study both treatment groups were within the normal reference range for anion gap; however, CON heifers had a greater anion gap which could possibly be explained due to a tendency for CON heifers to have greater serum lactate. Creatinine concentrations were greater in ZIL heifers but were within the normal reference range which is 1.2 -1.9 mg/dL (Kaneko et al., 2008); however, CON heifers had decreased creatinine concentrations when compared with normal reference ranges. The increase in creatinine would be expected due to a numerical increase in HCW which was 19 kg for ZIL when compared with CON heifers. During normal muscle metabolism, creatine is broken down into creatinine which can be utilized as an indicator of muscle mass in the serum (Russell and Roussel, 2007). Furthermore, serum creatinine is positively correlated with carcass weight, dressing percentage, and proportion of lean meat in a carcass (Istasse et al., 1990). Cimaterol, a β_2 -AA (Signorile et al., 1995), increased plasma creatinine concentrations in Friesian steers during long term supplementation (Chikhou et al., 1993). Creatine kinase is a key enzyme for cellular energetics (Wallimann et al., 1992). In the current study, there was no difference in creatine kinase between treatment groups. Thomson et al., (2015) reported no increase in creatine kinase activity in steers under normal feedlot conditions supplemented ZH or ractopamine hydrochloride when compared with a control group. Creatine kinase has been reported to increase when steers are transported (Warriss et al., 1995).

β -hydroxybutyrate is a ketone body produced during the metabolism of NEFA in the liver and can indicate a negative energy balance (Ospina et al., 2010). No difference

was observed between treatment groups for β HB in the current study, which is similar to that reported in steers supplemented clenbuterol (Eisemann et al., 1988a). Furthermore, there was also no difference observed for β HB concentrations for steers supplemented ZH for a 23 d duration (Van Bibber-Krueger et al., 2015). Van Bibber-Krueger et al. (2015) suggested that ZH supplementation in cattle did not alter the metabolization rate of BHB. During anaerobic conditions, lactate is formed by the oxidation of NADH by pyruvate allowing glycolysis to continue (Reece et al., 2015). In the current study, there was a tendency for lactate to be decreased in ZIL supplemented heifers. Thomson et al. (2015) and Van Bibber-Krueger et al. (2015) reported no difference in lactate concentrations of steers supplemented ZH. Differences observed between these studies could be explained by frequency of sampling, as the current study evaluated lactate concentrations during a 10 h CRH/VP challenge following a 3 d ZH withdrawal. Van Bibber-Krueger et al. (2015) observed a numerical decrease in plasma lactate concentrations as days on ZH increased; however, no samples were obtained during the withdrawal period of ZH. These findings are different than what have been observed with previous β -AA supplementation. The inclusion of P-5369 and Q-2636 in milk replacer increased lactate concentrations in calves (Blum and Flueckiger, 1988). Eisemann et al. (1988) observed increased lactate concentrations with the inclusion of clenbuterol in steer diets. Furthermore, arterial cimaterol infusion increased lactate concentrations as cimaterol infusion rates increased (Byrem et al., 1996). Observed differences between the current study and these can possibly be explained with time of sampling as in the current study heifers had been withdrawn from ZH supplementation for a 3-d period. The glycolytic enzyme LDH catalyzes the conversion of lactate to

pyruvic acid (Doornenbal et al., 1988). The reference range for LDH in the cow is 692 – 1445 U / liter (Kaneko et al., 2008). In the current study, there was no difference in LDH concentrations between treatment groups. When ZH was administered to horses, an increase in LDH was observed (Wagner et al., 2008). Differences observed in these studies can possibly be explained by species differences as ZH is not approved for utilization in the horse.

Liver Enzymes

There was a treatment ($P = 0.05$) and time ($P < 0.001$) effect for serum alkaline phosphatase (ALP) concentrations. There was no treatment ($P = 0.22$) or treatment x time ($P = 1.0$) interaction, but there was a time ($P < 0.001$) effect for serum aspartate transaminase (AST). There was no treatment ($P = 0.28$) effect for serum gamma glutamyltransferase (γ GT). However, there was a tendency ($P = 0.08$) for a treatment x time interaction. There was a treatment ($P = 0.05$) and time ($P < 0.001$) effect, but no treatment x time ($P = 0.64$) interaction for serum sorbitol dehydrogenase (SDH).

Alkaline phosphatase catalyzes the cleavage of P_i from phosphate esters with production of ALP originating in the liver of mature animals (Doornenbal et al., 1988). Ricks et al. (1984) reported no difference in ALP in Hereford steers supplemented clenbuterol. In the current study, ALP concentrations were decreased in ZIL heifers when compared with CON. One explanation to a decrease in ALP concentrations could be the decrease in liver weight observed in the current study. Furthermore, in dogs with increased corticosteroid concentrations, ALP concentrations are increased due to the formation of a more heat stable isoenzyme of ALP (Teske et al., 1986). The decrease in

cortisol in ZIL supplemented heifers which explain the increase in ALP observed in CON heifers. Aspartate transaminase is an enzyme that catalyzes the transfer of an α -amino group from an amino acid to an α -keto acid (Doornenbal et al., 1988). In the current study, no difference was observed in AST concentrations. An increase in AST concentrations was observed in Friesian calves administered cimaterol (Chikhou et al., 1993). However, Chikhou et al. (1993) did not observe a difference in liver weights in cimaterol treated calves which could explain the differences between these studies. Gamma glutamyltransferase is a membrane bound enzyme that catalyzes the transfer of gamma glutamyl groups from gamma glutamylpeptides to other peptides or amino acids (Kaneko et al., 2008). In the current study, there was no difference in γ GT between treatment groups; however, both treatment groups were above normal reference ranges. A potential cause of increased γ GT in the current study could be the CRH/VP challenge administered. In canines, glucocorticoids have been associated with an increase in γ GT concentrations (Shull and Hornbuckle, 1979). Lastly, SDH concentrations were increased in CON heifers in the current study; however, both treatment groups had elevated SDH concentrations compared with reference ranges for the cow which are 4.3 – 15.3 U/L (Kaneko et al., 2008). Feed restriction in llamas resulted in an increase in hepatic lipidosis which resulted in elevated SDH concentrations (Tornquist et al., 2001). Therefore, the increase in SDH in the current study could potentially be a result of increased lipolysis during the CRH/VP challenge allowing heifers to mount a stress challenge.

Tissue samples

There were no differences ($P = 0.49$) in concentrations of glucose or lactate, and no difference in glycolytic potential for the liver samples between the CON and ZIL heifers. Glucose concentrations within the LM were greater ($P = 0.03$) in CON heifers when compared with ZIL heifers. Supplementation of ZH did not affect lactate concentrations ($P = 0.39$) or total glycolytic potential ($P = 0.11$) for LM samples. Glucose concentrations in the BF tissue samples were not affected ($P = 0.22$) by ZH supplementation. Lactate concentrations in BF tissue samples were greater ($P = 0.05$) in CON heifers when compared with ZIL heifers. Furthermore, there was a treatment effect for glycolytic potential in BF samples ($P = 0.05$) with CON heifers having greater concentrations when compared with ZH-supplemented heifers.

Glycolytic potential (GP) is a measurement of glucose, lactate, glycogen, and glucose-6-phosphatase that is present in muscle that ultimately can be converted to lactic acid. This index can be used as a measurement of the capacity for postmortem glycolysis in muscle resulting in a decrease of muscle pH in the conversion of muscle to meat (Monin and Sellier, 1985). Glycolytic potential has been evaluated as a potential measurement of meat quality in beef and pork (Hamilton et al., 2003; Wulf et al., 2002). In cattle, GP has been reported to have a curvilinear response with ultimate LM pH after harvest. Muscle samples with a GP less than 100 $\mu\text{mol/g}$ are associated with a greater postmortem pH that results in decreased lactate production and can cause dark, firm, and dry beef (Wulf et al., 2002). In the current study, GP in the LM was not different in CON and ZH supplemented heifers. In the BF, ZH-supplemented heifers did have a decreased GP; however, this was associated with less available lactate resulting in a decreased calculated GP and was greater than the 100 $\mu\text{mol/g}$ threshold that has been suggested for

optimal pH postmortem. In previous ZH studies, Hilton et al. (2009) reported ZH supplementation increased beef color resulting in a brighter cherry red favorable beef color during a 5 d shelf-life study. Furthermore, (Montgomery et al., 2009a) reported ZH supplementation increased LM cherry red color and resulted in a decrease of dark colored LM when compared with control carcasses. The increase in bright cherry red color was attributed to an increase in myoglobin content that resulted in a favorable improvement in muscle color (Montgomery et al., 2009a). In liver samples, there was no difference in glucose, lactate, or GP observed in CON and ZH supplemented heifers. In the rat model, it has been reported protein kinases activity on glycogen synthase in skeletal muscle predominantly take place in the liver (Imazu et al., 1984). Furthermore, the lack of differences in glucose and GP between treatment groups of liver samples would further support the fact that no differences in serum glucose were observed in the current study. If blood glucose concentrations decreased, glucagon would be released from the α -cells of the pancreas to raise blood glucose concentrations (Navarro et al., 1999). These data suggest that ZH supplemented heifers do not have decreased available energy stores in muscle or liver samples that could cause muscle fatigue from lack of an energy source.

Organ analysis

In terms of select organ weights, there was no effect of treatment. Weights for the heart ($P = 0.36$), lungs ($P = 0.30$), total kidneys ($P = 0.11$), left kidney ($P = 0.31$), and left adrenal gland ($P = 0.79$) were similar. There was a tendency ($P = 0.08$) for liver weights to be greater in CON heifers when compared with ZIL heifers. Also, there was a tendency ($P = 0.10$) for right adrenal gland weights to be greater in CON heifers when compared with ZIL heifers. There was a treatment difference ($P = 0.03$) for right kidney

weights such that weights were greater in CON heifers when compared with ZIL heifers. As a percentage of BW, there was no treatment effect on heart ($P = 0.45$), lung ($P = 0.41$), and total adrenal gland ($P = 0.42$) weights. However, as a percentage of BW, there was a tendency ($P = 0.06$) for CON heifers to have greater liver and total kidney ($P = 0.08$) weights than ZIL heifers.

Between CON and ZIL heifers, no treatment differences were observed between CON and ZIL heifers for aortic valve to left atrial ventricular valve diameter ratio ($P = 0.64$), aortic valve to pulmonary valve diameter ratio ($P = 0.16$), aortic valve circumference ($P = 0.98$), or the aortic valve / right ventricular valve diameter ratio ($P = 0.96$). Also, there were no differences for interventricular septum thickness ($P = 0.30$), left atrial ventricular valve circumference ($P = 0.64$), left atrial ventricular valve / right atrial ventricular valve diameter ratio ($P = 0.64$), left ventricle free wall thickness ($P = 0.97$), or for the left ventricle + interventricular septum / right ventricle weight ratio ($P = 0.14$) between treatment groups. There was a tendency ($P = 0.07$) for left ventricle + interventricular septum / total heart weight ratio to be less in CON, when compared with ZIL heifers. There was no difference in left ventricle free wall thickness / right ventricle free wall thickness ratio ($P = 0.13$), left ventricle thickness / septal thickness ratio ($P = 0.36$), or left ventricle + septum weight ($P = 0.97$). Furthermore, there was no difference in pulmonary valve / left atrial ventricular valve ratio ($P = 0.47$), pulmonary valve / right atrial ventricular valve ratio ($P = 0.30$), or pulmonary valve circumference ($P = 0.11$). There was no difference ($P = 0.94$) for right atrial ventricular valve circumference. There was a tendency ($P = 0.09$) for right ventricle free wall thickness to be thicker in CON vs ZIL heifers. However, there was no difference ($P = 0.33$) in right ventricle free wall

weight between treatment groups. There was a difference ($P = 0.04$) in right ventricle free wall / interventricular septal thickness ratio with CON having a greater ratio when compared with ZIL heifers. However, there was no difference ($P = 0.72$) for right ventricle / total heart weight ratio between treatments. Furthermore, there was no difference ($P = 0.85$) in left ventricle + interventricular septum as a percent of BW or right ventricle ($P = 0.30$) as a percent of BW. Gross lesions of hepatitis were seen in two CON heifers. Gross nephritis was seen in one ZIL heifer that was noted to have a catheter infection during the study. Histologic evaluation of lungs demonstrated a very mild regional eosinophilic interstitial pneumonia in one CON heifer. Pulmonary artery changes were adventitial and consisted of periarterial fibrosis and subacute inflammation and were noted in two CON and one ZIL heifer. Livers had mild periportal hepatitis in 6 of 10 CON and 7 of 9 ZIL heifers. The two CON heifers with gross hepatitis had histologic fibrosis in addition to mild lymphocytic infiltrates. The hepatic lesions were typical mild periportal infiltrates and rare isolated small lymphocytic foci. Renal lesions were mild focal chronic interstitial nephritis consisting of very small, sparse, scattered, interstitial aggregates of lymphocytes and plasma cells (6 CON and 6 ZIL heifers). A single foci of lymphocytes was seen in the adrenal medulla of one CON heifer. Skeletal muscle and cardiac muscle was histologically normal in all heifers.

Experiments that have evaluated and reported the effects of β -AA on major organs of feedlot cattle are limited. In the current study, no differences were observed in heart and lung weights between treatment groups. No difference was reported in combined heart and lung weights of beef steers fed ZH for 20 d (Holland et al., 2010). However, the feeding of clenbuterol (β_2 -AA) in normal mice decreased heart weights by

8% when compared with control mice (Sharma et al., 1997). Furthermore, May et al. (2014) reported a tendency for a decrease in heart weights of Holstein steers supplemented ZH for 20 d but no difference in lung weights between zilpaterol and control steers. Furthermore, a decrease in kidney and liver mass of zilpaterol supplemented steers when compared with control steers was observed (May et al., 2014). In the current study, there was only a difference observed for right kidney weights, with ZIL supplemented heifers having less right kidney weights; however, overall kidney weights did not differ between treatment groups. Furthermore, there was a tendency for liver weights to be greater in the CON heifers when compared with ZIL heifers in the current study. The feeding of salbutamol (β_2 -AA) to pigs has been reported to decrease kidney and liver weights when compared with control pigs (Hansen et al., 1994). The decreased in renal and liver mass could be a result of decreased gut fill. The repartitioning of energy to skeletal muscle growth and a decrease in feed intake resulting in less gut fill could explain the decrease in renal and liver mass of ZIL supplemented cattle (Montgomery et al., 2009b).

To our knowledge, data on the effect of β -AA's on adrenal weights has not been reported in the literature. The comparing of organ weight ratios to BW can result in misleading data due to differences in animal adipose and muscle tissues (Joseph, 1908), as well as the previously reported effect of ZH on HCW (Montgomery et al., 2009a; Montgomery et al., 2009b). Reported percent's for bovine heart weight ratios to BW are 0.26, 0.52, and 0.38% for steers, bulls, and female respectively (Joseph, 1908). In the current study, heifer heart weight ratios to BW were within this range for cattle.

Minimum published anatomic data is available for measurements of the dissection of the bovine heart. The total cardiac to BW ratios and the left ventricle and septum to right ventricle weight ratios were within the normal range (Leifsson, 2011). The expanded data and reference of a broader range of normal cardiac dimensional anatomy published here will be useful in evaluating finishing cattle affected by or at risk of pulmonary hypertension and chronic right heart failure which appears to be a growing problem (Neary et al., 2013). The gross lesions of liver fibrosis were seen only in CON heifers and the kidney lesion was in a heifer with a documented catheter infection and transient sepsis during the study. The mild chronic periportal hepatitis and chronic interstitial nephritis seen equally in both groups are common background lesions seen in cattle in standard production systems. These likely reflect residual inflammation of neonatal infections. The mild adventitial inflammation seen around the pulmonary artery in two ZIL and one CON heifers are of undetermined etiology but suspected to be residual scarring and inflammation due to past resolved pulmonary infection. None of the histologic lesions were more prevalent in ZIL heifers and were typical of incidental changes seen in standard commercial cattle. No pathologic changes were observed in these cattle associated with ZH treatment that might suggest a direct link between treatment and reported mortality risk in some production systems. Changes in feed delivery and other production practices associated with implementing of supplement delivery and documentation of causes of mortality during the last 25 d of feeding need further prospective study.

Conclusions and Implications

Data from the current study would indicate that that activation of the hypothalamic-pituitary-adrenal (HPA) axis will increase both stress and metabolic variables in both CON and ZIL heifers respectively. Activation of the HPA axis is a cascading response, regulated by the magnitude of the stressor (Tsigos and Chrousos, 2002). An acute response may result in brief down-regulation of systems not necessary for survival, such as digestion and reproduction, and stimulate catabolism of energy stores and body tissues to be used for energy (Sapolsky et al., 2000). While there are data to suggest the initiation of a stress response during relocation events (Falkenberg et al., 2013), there are few or no data available to describe how this event is altered due to supplementation of β -AAs. Therefore, this data indicates the biological response of cattle supplemented β -AAs to a controlled endocrine stress. Furthermore, these data demonstrate that a simulated stress induced by administration of CRH/VP in beef cattle elicits shifts in immune cell populations and metabolic variables; and stress hormones, metabolic variables, and immune cell responses are altered in beef heifers supplemented with ZH when cattle are exposed to a simulated stress challenge. However, within the environment of this study, supplementation of ZH did not appear to hinder finishing heifer's ability to mount a stress, immune, or metabolic response during a neurohormone-induced stimulated stress challenge.

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Appendix I

β -Hydroxybutyrate Assay

Reference: Sigma-Aldrich MAK041 Kit Bulletin, 2012

Preparation of Reagents:

Briefly centrifuge vials of each reagent before opening. Be sure to use ultrapure water for preparation. Avoid repeated freeze/thaw cycles to maintain integrity.

1. β -Hydroxybutyrate Assay Buffer: Allow to come to room temperature before use.
2. β -Hydroxybutyrate Enzyme Mix: Reconstitute with 220 μ l of β -Hydroxybutyrate assay buffer. Mix well via pipette then aliquot and store, protected from light, at -20°C. Use within 2 months of reconstitution and keep cold while in use.
3. β -Hydroxybutyrate Substrate Mix: Reconstitute with 220 μ l of β -Hydroxybutyrate assay buffer. Mix well via pipette then aliquot and store, protected from light, at -20°C. Use within 2 months of reconstitution and keep cold while in use.

Preparation of Standards:

1. β -Hydroxybutyrate Standards: Reconstitute with 100 μ l of water to generate a 10mM solution. Mix well via pipetting, then aliquot and store, protected from light, at -20°C.
2. Dilute 10 μ l of the 10mM β -Hydroxybutyrate Standard Solution with 90 μ l β -Hydroxybutyrate Assay Buffer to prepare a 1mM standard solution.
3. For 2 plates mix 50 μ l of the 10mM β -Hydroxybutyrate Standard Solution with 450 μ l of the β -Hydroxybutyrate Assay Buffer.
4. In 0.6 ml tubes make up the standards according to the following chart:

1mM Standard Solution (μ l)	Beta-Hydroxybutyrate Assay Buffer (μ l)	nmole / well Value
0	400	0
16	384	2
32	368	4
48	352	6
64	336	8
80	320	10
96	304	12

- Once the standards are made up in the tubes, add 50 μ l of the appropriate standard into duplicate wells on the plate.

Preparation of Samples:

Add 8 μ l of each sample into duplicate wells on the plate.

Add 42 μ l of Assay Buffer to each well to get a final volume of 50 μ l

Assay Reaction:

- Set up the Reaction Mixes according to the scheme below. 50 μ l of the appropriate Reaction Mix is required for each well in the plate.

Reagent	Samples and Standards	Blank
Beta-Hydroxybutyrate Assay Buffer	46 μ l	48 μ l
Beta-Hydroxybutyrate Enzyme Mix	2 μ l	-
Beta-Hydroxybutyrate Substrate Mix	2 μ l	2 μ l

For 1-96 well plate, mix:

- 4600 μ l of β -Hydroxybutyrate Assay Buffer
 - 200 μ l of β -Hydroxybutyrate Enzyme Mix
 - 200 μ l of β -Hydroxybutyrate Substrate Mix
- Add 50 μ l of the appropriate Reaction Mix to each of the wells. Mix well using a horizontal shaker and incubate the reaction for 20 minutes at room temperature. Protect the plate from light during the incubation.
 - Measure the absorbance at 450 nm (A_{450}) on the plate reader using the Beta Protocol.

Results:

Calculations:

The background for the assays is the value obtained for the 0 (blank) Standard. Correct for the background by subtracting the 0 (blank) value from all readings.

Use the values obtained from the Standards to plot a Standard Curve (A new standard curve must be set up each time the assay is run). Blank values must also be subtracted from the sample readings to obtain a corrected measurement. Use the corrected value in all calculations.

Concentration of Beta-Hydroxybutyrate:

$$S_a/S_v = C$$

S_a = Amount of Beta-Hydroxybutyrate in unknown sample (nmole) from standard curve.

S_v = Sample volume (μ l) added into the wells

C = Concentration of Beta-Hydroxybutyrate in sample

Appendix II Lactate Assay

Reference: Sigma-Aldrich MAK064 Kit Bulletin, 2012

Reagent Preparation:

Lactate Assay Buffer: Allow buffer to come to room temperature before use.

Lactate Probe: Warm to room temperature before use. Protect from the light. Mix well by pipetting, then aliquot and store at -20°C.

Lactate Enzyme Mix: Reconstitute in 220 μ l of Lactate Assay Buffer. Mix well via pipette, then aliquot and store at -20°C. Use within 2 months of reconstitution.

Preparation of Lactate Standard:

1. Take 10 μ l Lactate Standard (100 nmole / μ l) and dilute with 990 μ l of Assay Buffer to result in 1 ml of Diluted Standard (1 nmole / μ l).
2. In 0.6 ml tubes make up the standards according to the following chart:

Diluted Standard	Lactate Assay Buffer (μ l)	Std. Value (nmole / well)
0	250	0
10	240	0.2
20	230	0.4
30	220	0.6
40	210	0.8
50	200	1.0

3. Once standards are prepared in tubes, add 50 μ l of the appropriate standard into duplicate wells on the plate.

Preparation of Samples:

1. Samples must be deproteinized using a 10 kDaMWCO spin filter.
2. Add 500 μ l of sample to filter.

3. Spin tube for 1.5 hours at 14,000g.
4. Remove filter and store sample at -80°C until ready to use.

Assay Reaction:

1. Set up the Master Reaction Mix according to the following scheme:

Reagent	Master Reaction Mix (cell)	Master Reaction Mix (plate)
Lactate Assay Buffer	46 μ L	4.600 ml
Lactate Enzyme Mix	2 μ L	200 μ L
Lactate Probe	2 μ L	200 μ L

2. Add 50 μ l of the Master Reaction Mix to each of the duplicate wells.
3. Mix well using a horizontal shaker or by pipetting.
4. Incubate the reaction for 30 minutes at room temperature. Protect the plate from light during the incubation
5. Measure the absorbance at 570 nm (A_{570})

Procedure:

1. Allow Buffer to come to room temperature. Allow Lactate probe to come to room temperature (protect from light)
2. Reconstitute Lactate Enzyme Mix in 220 μ l of Lactate Assay Buffer, mix well
3. Take 10 μ l of Lactate Standard and reconstitute with 990 μ l of Assay Buffer to get your 1 ml of Diluted Standard (1nmole / μ l).
4. Prepare standards according to the previous chart. (This should give enough standard for 2 plates)
5. Place 50 μ l of each standard into duplicate wells on the plate. (Do not dilute with assay buffer).
6. Add 3 μ l of sample to each duplicate well.
7. Add 47 μ l of Lactate Assay Buffer to each well that has a sample in it. (Do not add to the standards)

8. Make up the Master Reaction Mix according to assay chart in previous section (add probe last and use immediately.)
9. Add 50 μL of the Master Reaction Mix to each well.
10. Place plate on incubator/shaker and shake for 1 minute, then continue incubating for 29 more minutes. (Protect from light).
11. Measure the absorbance using Lactate Procedure at 570 nm on plate reader.

Results:

The background for the assay is the value obtained for the 0 (blank) lactate standard. Correct for the background by subtracting the blank value from all readings. Background values can be significant and must be subtracted from all readings.

Use the values obtained from the appropriate lactate standards to plot a standard curve. The amount of lactate present in the samples may be determined from the standard curve.

(Note: A new standard curve must be set up each time the assay is run.)

Concentration of Lactate:

$$S_a/S_v = C$$

$$C \times 89.07 \text{ ng / nmole} = \text{ng} / \mu\text{L}$$

S_a = Amount of lactate acid in unknown sample (nmole) from standard curve

S_v = Sample volume (μL) added into the wells.

C = Concentration of lactate acid in sample

Appendix III Lactate Dehydrogenase Assay

Reference: Sigma-Aldrich MAK066 Kit Bulletin, 2013

Preparation of Reagents:

Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

1. LDH Assay Buffer: Allow buffer to come to room temperature before use.
2. LDH Substrate Mix: Reconstitute in 1 mL of water. Mix well via pipette and keep cold while in use. (Substrate Mix is stable for one week at 4 ° C and 1 month at -20°C.

Preparation of NADH Standards:

1. Reconstitute in 400 μ L of water to generate 1.25 mM standard solution
2. Mix well via pipette and keep cold while in use. The NADH Standard Solution is stable for one week at 4 ° C and 1 month at -20°C.
3. In 0.6 mL tubes make up the standards according to the following chart:

1.25mM Standard Solution (μ l)	LDH Assay Buffer (μ l)	nmole / well Value
0	250	0
10	240	2.5
20	230	5
30	220	7.5
40	210	10
50	200	12.5

4. Once standards are made up in the tubes, add 50 μ L of the appropriate standard into duplicate wells on the plate.

Preparation of Samples:

1. Dilute samples in tubes off of the plate :
 - 2 μ L of sample

- 198 μL of LDH Assay Buffer (dilution factor 100)
2. Then add 50 μL of each diluted sample into duplicate wells on the plate (amount of sample going into plate 0.5 μL).

Assay Reaction:

1. Set up Reaction Mixes according to the scheme below, 50 μL of the appropriate Reaction Mix is required for each well.

Reagent	Master Reaction Mix
LDH Assay Buffer	48 μl
LDH Substrate Mix	2 μl

2. Add 50 μL of the Master Reaction Mix to each of the wells. Protect the plate from light while incubating.
3. Shake 1 minute while incubating. Incubate an additional minute. After these 2 minutes, take the initial measurement (T_{initial}). Measure the absorbance at 450 nm at the initial time ($(A_{450})_{\text{initial}}$). Note: It is essential ($(A_{450})_{\text{initial}}$) is in the linear range of the standard curve.
4. Incubate the plate at 37°C taking measurements (A_{450}) every 5 minutes. Protect the plate from light during the incubation.
5. Continue taking measurements until the value of the most active sample is greater than the value of the highest standard (12.5 nmole / well). At this time the most active sample is near or exceeds the end of the linear range of the standard curve.
6. The final measurement [$(A_{450})_{\text{final}}$] for calculating the enzyme activity would be penultimate reading or the value before the most active sample is near or exceeds the end of the linear range of the standard curve. The time of the penultimate reading is T_{final} .

Note: It is essential the final measurement falls within the linear range of the standard curve.