8-2016

EVALUATION OF HEMATOLOGICAL AND SERUM BIOCHEMICAL PROFILES ASSOCIATED WITH THE SUPPLEMENTATION OF ZILPATEROL HYDROCHLORIDE TO FEEDLOT HEIFERS

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By

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A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Animal Science

Under the Supervision of Professor Steven J. Jones

Lincoln, Nebraska

August 2016

EVALUATION OF HEMATOLOGICAL AND SERUM BIOCHEMICAL PROFILES ASSOCIATED WITH THE SUPPLEMENTATION OF ZILPATEROL HYDROCHLORIDE TO FEEDLOT HEIFERS

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University of Nebraska, 2016

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Supplementation of zilpaterol hydrochloride (ZH; Zilmax®) to cattle has been suggested to have a negative impact on well-being of cattle. The objective of this study was to evaluate the hematological and serum biochemical profiles in feedlot heifers supplemented with ZH in a minimal stress environment. Heifers were randomly assigned to one of two treatment groups: Control (CON no ZH), or ZH (supplemented with ZH at 8.33mg/kg of feed on a DM basis). The study was conducted over a period of 25 d (-2 to 23), with three serum collection periods [-2 to 4 d (ZH supplementation began on d 0); 13-16 d; and 21 to 23 d (withdrawal period)]. Serum samples were collected for a large animal chemistry profile analysis and blood samples were collected for hematological profile analysis. Liver, *longissimus dorsi* (LM), and *biceps femoris* (BF) samples were collected for vitamin E concentration analysis. There was a treatment effect for serum calcium ($P = 0.008$) with concentrations being greater in CON. A treatment effect was observed for serum bicarbonate ($P = 0.03$), with ZH having greater concentrations. A treatment x time interaction was observed for glucose ($P = 0.02$), BUN ($P < 0.001$), Cl ($P = 0.04$), creatinine ($P \leq 0.001$), and creatine kinase ($P \leq 0.001$). In heifers fed ZH, creatinine, creatine kinase, and Cl concentrations in the serum were greater than CON heifers whereas glucose and BUN concentrations were decreased in the ZH heifers. There was a treatment x time interaction for

alkaline phosphatase (ALP, $P < 0.001$) and gamma-glutamyltransferase (GGT, $P = 0.004$). Overall, concentrations of ALP and GGT were greater in CON heifers. In the hematological profile, there was a treatment effect ($P = 0.02$) for hematocrit, where the ZH heifers had greater hematocrit. These data suggest that in this controlled environment, there were no negative impacts observed on the homeostasis with supplementation of ZH and most variables were within the normal homeostatic range of feedlot cattle.

Acknowledgements

During my time at the University of Nebraska, I would not have been as successful in my graduate program without the support and help of the many friendships I had gained.

I would like to extend my gratitude to my advisor, Dr. Steve Jones, for providing me with the opportunity to be a part of the meat science graduate program. His understanding of how important my family is to me was greatly appreciated. In addition, he was very cooperative with my schedule while traveling with the meat judging team. I am so grateful that I was given the opportunity to coach while at Nebraska. I appreciate everything that Dr. Jones has done for me and have thoroughly enjoyed my time as his graduate student.

Additionally, I would like to thank Dr. Ty Schmidt for serving as my research project PI, committee member, and meat judging faculty advisor. Without his support and guidance, I would not have been as successful during my graduate student program. If I wasn't ever given the opportunity to be a part of the judging program, I would have never made the connections in the meat industry that were vital to finding my future career.

I would like to extend my gratitude to Dr. Calkins for serving as a member on my committee under such short notice. His guidance and encouragement were beneficial to my graduate program experience. Dr. Calkins has exposed me to several valuable networking opportunities that have immensely helped me grow as a professional.

I am grateful for the many people that helped during my research project, especially the USDA crew from Lubbock, TX and the undergraduate workers Ashley McCoy and Joseph Bennier. I would also like to thank Merck Animal Health for funding my research.

Furthermore, I would like to acknowledge all the hard work and assistance I received from Sherri Pitchie, Tommi Jones, and Calvin Schrock. Sherri has always been there for me whether it is filling out paperwork for judging trips, helping make reservations, or just giving me

advice. I greatly appreciate everything she has done for me during my time at Nebraska. I also appreciate all the advice and contributions that Tommi has made towards my laboratory work. She has exceptional organization skills and with her I was able to successfully complete my lab work for my research. Calvin's cooperation and patience towards the judging team and me were definitely appreciated as well. Without Calvin's support the judging team would not be as successful and Nebraska would not be able to host all the workouts and workshops for other companies and universities to utilize.

I am most grateful for my fellow graduate students and my time at Nebraska would not have been complete without them. Katherine Domenech, Michael Chao, Chad Bower, Regan Stanley, Hope Voegele, Kelly McCarty, and Emery Kunze provided an exceptional team atmosphere and I have thoroughly enjoyed helping with their research endeavors. Words cannot express how grateful I am to have worked alongside Joe Buntyn while in graduate school. I appreciate all of the support, guidance, and honesty I have received from Joe. Also, I cannot thank Brandy Cleveland enough for all of the excellent training, advice, and fun memories with the meat judging team.

Finally, I am indebted to my family for providing me endless support and encouragement over the past several years. They have provided me with the drive to push myself to do the best that I possibly can. They have been very understanding throughout my time in Nebraska and I would not be where I am today without them. I am especially thankful for my parents, who helped keep me focused and my spirits high while completing my master's degree.

Table of Contents

Introduction

The supplementation of β-adrenergic agonists (β-AA) to livestock has been a topic of discussion for the past forty years. The β-AAs ractopamine hydrochloride (RH) and zilpaterol hydrochloride (ZH) are supplemented to swine and beef cattle to improve feed efficiency and promote increased lean muscle growth. The use of β-AAs has resulted in an increase in ADG (Montgomery et al., 2009) with decreases in DMI resulting in an increase in feed efficiency (Mersmann, 1998) and an increase in gain to feed ratio (Avendaño-Reyes et al., 2006). Administration of β-AAs has also been determined to result in increases in lean muscle in conjunction with decreases in fat deposition.

In the past few years, concerns have developed due to personnel at the abattoir and feedlots observing lameness, slow movement, possible muscle fatigue, and even increased mortality. Some have suggested that these mobility issues could be a result of supplementation of ZH. A recent study suggested a possible association between β-AA and an increase in both the cumulative risk and incidence of mortality in feedlot cattle (Loneragan et al., 2014).

The objective of this study was to conduct a very intensive and controlled trial to evaluate the hematological and serum biochemical profiles of feedlot heifers during supplementation and the withdrawal of ZH for the prescribed 23 d prior to harvest. When evaluating the profiles, alterations in serum metabolites and hematological profile were observed to determine if supplementation of ZH had a detrimental effect on the overall physiological health of the heifers.

Review of Literature

I. Introduction of Beta- Adrenergic Agonists

A. Definition of the Beta-Adrenergic Agonist

The term "adrenergic" relates to nerve cells in which epinephrine and norepinephrine act as neurotransmitters. The β-adrenergic agonists (β-AA) bind to β-adrenergic receptors (β-AR) that are located on numerous cell types with in the body. β-adrenergic agonists are potent compounds that have been well documented in acting as growth promotants across many species. Agonists are defined as compounds that either directly or indirectly elicit a response similar to endogenous neurotransmitters by binding to β-receptors (Reece, 2015). A commonly known response of an agonist is the relaxation of muscles, specifically around the airways. Antagonists are the opposite of agonists and are defined as compounds that directly or indirectly prevent or block the effects of the endogenous neurotransmitters (Reece, 2015). When β-agonists bind to a receptor, they elicit a physiological response similar to the naturally occurring endogenous agonists circulating in the body. The endogenous β-agonists commonly found are epinephrine, norepinephrine, and dopamine (Mersmann, 1998).

The physiological β-AAs, epinephrine and norepinephrine are classified as phenethylamines which are amines that are naturally occurring in the body and contain one amino group connected to a phenyl group via a two carbon chain (Pleadin et al 2012). Endogenous epinephrine and norepinephrine are classified as compounds called catecholamines. Catecholamines are compounds derived from the amino acids tyrosine and phenylalanine (Hadley and Levine, 2007). The catecholamine norepinephrine is produced by the sympathetic nervous system and is synthesized from tyrosine. Norepinephrine circulates in relativity high concentrations in the blood whereas; epinephrine is circulating in much lower concentrations

when compared to norepinephrine. Norepinephrine is synthesized inside the nerve axon and is stored within the vesicles. It is released when an action potential travels down the nerve which depolarizes the membrane causing calcium to enter the axon (Hadley and Levine, 2007). Epinephrine is secreted from the medulla of the adrenal glands and is synthesized from norepinephrine.

The β-AAs ractopamine hydrochloride (RH), cimaterol, salbutamol, clenbuterol, and zilpaterol hydrochloride (ZH) are all examples of phenethylamines that have been studied in livestock.

Beta-agonists chemical structures all possess the same phenyl group with the ethanolamine group. The ethanolamine group is composed of a two carbon chain with an alcohol group coming off the first carbon and an amine group off the second carbon. The major difference between these chemical structures is the different R groups that are attached to the amine group on the second carbon of the ethanolamine group (Figure 1).

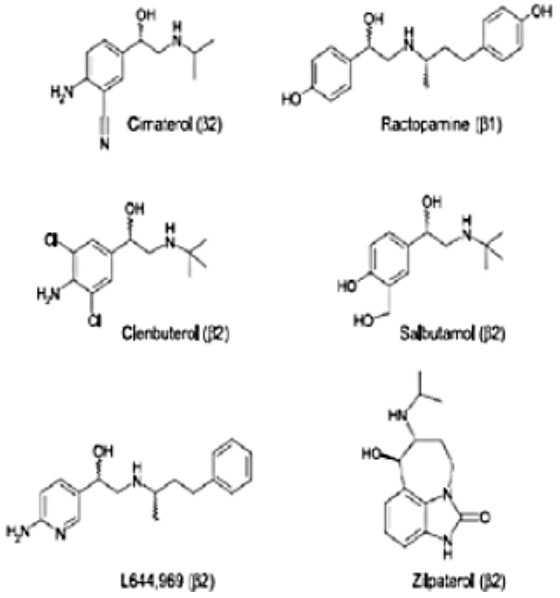

Figure 1 Chemical Structure of the β-AAs cimaterol, clenbuterol, L644,969, RH, salbutamol and ZH. (Pleadin et al., 2012).

B. Physiological Responses in Humans

β-adrenergic agonists were first developed as a means to help patients control their asthma (Crompton, 2006). In humans, β-AAs are part of a group of medications that elicit a response like the naturally occurring catecholamines epinephrine and norepinephrine. In 1956,

the first pressurized metered-dose inhaler (pMDI) was introduced to patients who suffered from asthma (Crompton, 2006). The β-AA isoprenaline was the primary compound in the most popular pMDI; however, it was determined that isoprenaline could be the possible cause of an increase in mortality in asthma patients (Crompton, 2006). Salbutamol was identified as a replacement for isoprenaline. Today, salbutamol remains one of the most frequently prescribed short-acting β-agonists (SABA). In the late 1980s, the first long-acting β-agonist (LABA), salmeterol, was introduced. When first put into practice, clinicians claimed that salmeterol had clinically significant anti-inflammatory effects along with long bronchodilator action that lasted up to 12 hours (Twentyman et al., 1990). These β-AAs continue to be the main drug in inhalers for asthma patients today. The β-AAs elicit a response with the β-AR and relax constricted muscles around the airways, allowing the person to breathe easier.

It has been reported that β-AAs could act as a potential treatment for patients that have muscle wasting disorders due to paralysis, muscular dystrophy and burn- and cancer-induced cachexia (Yang and McElligot, 1989). Use of β-AAs has been reported to increase muscle mass, reduce fat deposition, and reduce recovery time due to injuries (Yang and McElligot, 1989). Increases in muscle mass and reduction in fat deposition are precisely the reason the animal agriculture industry is interested in using β-AAs.

II. History of Beta-Agonists in Livestock

Cunningham (1965) reported the possibility of altering animal growth by administering various agents such as caffeine, epinephrine, theophylline, and nicotine in swine. These agents presented either a direct or indirect effect on changing the intracellular concentration of cyclic adenosine monophosphate (cAMP). Cyclic AMP is incorporated into the actions of many hormones (i.e. growth hormone, epinephrine, and norepinephrine) on cellular physiological

processes by acting as a second messenger thus resulting in the activation or inactivation of certain enzymes when phosphorylated (Mersmann, 1998).

Once it was discovered in humans that β-AAs could be used as a treatment method for patients with muscle wasting diseases, it was investigated for the use of livestock. One of the first compounds to be developed was clenbuterol. This compound was developed using a non-obese rat model to test the compounds' capability to increase lean muscle growth (Moloney et al., 1991). Clenbuterol administration also resulted in an increase in muscle and decrease in fat on the carcasses of steers (Ricks et al., 1984b) and in lambs (Baker et al., 1984). Other compounds were synthesized (i.e. cimaterol, RH, and salbutamol) and studied in rodents and then livestock species. The response of these compounds varies by species, tissue type, dosage, duration of administration, and age of the animal (Mersmann, 1989; Anderson et al., 1991).

This variation between compounds is why some have never been approved for use in livestock. To this day, the only compounds approved by the Food Drug Administration (FDA) are RH and ZH. Clenbuterol (a very potent compound) was never approved in livestock production in the United States because residues were found in the meat. In Italy, there have been reported cases of clenbuterol poisoning in patients that had eaten meat from supplemented animals (Brambilla et al., 2000). Similar effects have been observed in Spain and France, where individuals consuming liver from animals

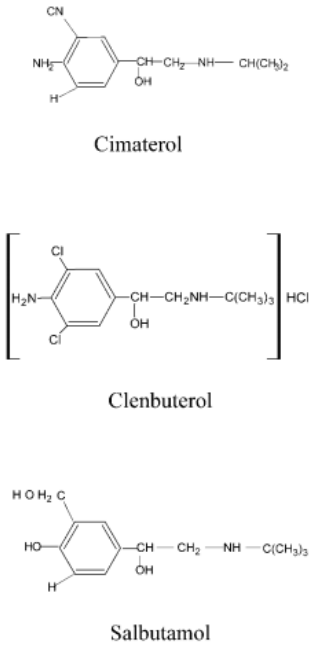

Figure 2 Chemical structures of cimaterol, clenbuterol, and salbutamol (Chen et al., 2005).

illegally-administered clenbuterol displayed symptoms of tremors, headaches, and dizziness (Martinez-Navarro, 1990; Salleras et al., 1995).

Cimaterol was another potent β-AA that was never approved for livestock use. Clenbuterol, cimaterol, and salbutamol all have similar chemical structures (Figure 2) and when fed to cattle could result in residue levels in the kidneys and liver that can pose a threat to human health (Johnson et al., 2014). Since these compounds are not entire cleared from animal tissues, they are not approved for use in livestock production.

A. **Physiological Responses in Livestock**

With the population of the world increasing each year, higher demands have been placed on agricultural research to develop new technologies that will provide food for this expanding population. The United Nations Population Fund has developed three population projections called variants (a high, low, and in between). The medium variant predicts that the world population will grow to almost 10 billion by the middle of this century (UNFPA, 2015). In the livestock industry, β-AAs have been a major focus to researchers since the early 1980s due to the benefits associated with increasing production efficiency. Some of those benefits include but are not limited to: increase in muscle mass, decrease in adipose tissue, improved average daily gain (ADG), and an overall improvement of feed efficiency. This increase in muscle mass and a decrease in fat deposition provide consumers with more lean meat without an increase in inputs. It is important for people to increase their protein intake and consuming lean meat is one of the primary sources of high quality protein.

Several β-AAs have been tested to determine their impact on muscle growth. These β-AAs include clenbuterol, cimaterol and RH. In the early 1980s, data were published on modulation of growth in animals that were fed clenbuterol and cimaterol (Beermann et al., 1986;

Ricks et al. 1984a; Jones et al., 1985). Oral administration of clenbuterol resulted in an increase in muscle mass and a decrease in fat deposition in growing cattle (Eisemann et al., 1988), swine (Ricks et al., 1984a), and sheep (Baker et al., 1984). Early use of β-AAs in swine production was focused on the compound cimaterol. Jones et al. (1985) observed that feed intake reduced linearly in swine as the amount of cimaterol administered increased. Dalrymple et al. (1984) reported no significant effect on feed intake. Dalrymple et al. (1985) and Beermann et al. (1986) reported that administration of cimaterol increased the rate of gain in lambs. In swine, it was observed that administration of the β-AA cimaterol reduced back fat (Dalrymple et al. 1984; Jones et al. 1985). In conjunction, it has also been reported that cimaterol administered to pigs causes an increase in muscle hypertrophy (Jones et al., 1985). In lambs (Baker et al. 1984; Beermann et al. 1986) and in steers (Ricks et al., 1984b) it has been noted that the carcass composition is altered towards more protein accretion and a reduction in fat deposition.

Following the research of clenbuterol and cimaterol in livestock, the other β-AR agonists (such as RH, and ZH) were fed to different species and similar physiological effects were observed in livestock. Studies have reported that other β-AAs (specifically RH and ZH) have similar abilities to increase ADG and G:F as well as affecting several carcass characteristics (Moloney et al., 1990; Chikhou et al., 1993b). Avendaño-Reyes et al. (2006) suggested that the supplementation of ZH and RH resulted in an overall improved feedlot performance of steers based on increases in ADG and G:F. In addition, it was reported that hot carcass weight (HCW) and dressing percentage (DP) were improved with the supplementation of β- AAs. The beneficial effects of β-AAs on growth performance have been observed in cattle, pigs, and sheep.

Montgomery et al. (2009) reported the increases in G:F in feedlot cattle. They also reported that supplementation of ZH resulted in an increased final body weight (BW) of both

steers and heifers. Furthermore, they observed ADG and G:F increased 36% and 28%, respectively, for the ZH supplemented steers and heifers (Montgomery et al., 2009). When comparing the two β-AAs that the Food Drug Administration (FDA) have approved (RH and ZH), ZH tends to show more of an increase in ADG than RH (Avendaño-Reyes et al., 2006). Vasconcelos et al. (2008) observed that ZH-supplemented cattle increased ADG and G:F by approximately 28% when compared to the controls. Literature also states that ZH fed cattle have improved DP compared to RH and non-supplemented cattle (Scramlin et al., 2010). There have been suggested theories to why this phenomenon happens. One possible explanation is the effect ZH has on fat metabolism in components of the animal that do not make up the carcass weight such as the hide or the viscera. This theory is supported by the study by Scramlin et al. (2010) in which they reported cattle supplemented with ZH had a reduction in total weight of kidney, pelvic, and heart (KPH) fat in the carcasses compared to steers fed RH and steers in the control group. In addition, ZH-supplemented steers had reduced average hide weights compared to the RH steers and the control steers (Scramlin et al., 2010).

Zilpaterol hydrochloride supplementation to feedlot cattle, DMI decreases compared to controls (Avendaño-Reyes et al., 2006; Vasconcelos et al., 2008). In summary, β-AA administration results in an increase in muscle mass, decrease in fat, and overall improved feedlot performance in livestock.

III. Development of Beta-Agonists on the market

The multitude of physiological effects controlled by β-AR sparked interest in the biomedical community. This extensive interest led to the synthesis of numerous amounts of organic molecules that bind to β-ARs; some being agonists and others acting as antagonists (Mersmann, 1998). Clenbuterol was the first β-AA to be studied, which is considered a synthetic analog of

epinephrine (Etherton., 2009). Clenbuterol supplementation resulted in an increase in DP, LM, and an overall increase in muscle mass in lambs (Baker et al., 1984) and in cattle (Miller et al., 1988). Cimaterol was studied next as it has a very similar chemical structure to clenbuterol and effects in lambs (Beermann et al., 1986, 1987; Kim et al., 1987) and in steers (Chikhou et al., 1993b). Ractopamine is another compound that possesses β-AA activity and was noted for stimulating growth in swine (Anderson et al., 1987) and in beef cattle (Anderson et al., 1989). Previous research led to the development of ZH. With the development of these synthetic compounds, there are very few that have actually been approved by the FDA for use in livestock.

A. Ractopamine Hydrochloride

Ractopamine hydrochloride was the first β-AA approved by the FDA for use in livestock. Ractopamine can be used as a β-AA for both swine and cattle. In swine, the use of RH was FDA approved on April 25, 2006. Ractopamine has the market name of Paylean® and is manufactured by Elanco Animal Health based out of Greenfield, Indiana. Swine supplemented RH are noted to have an increase in rate of weight gain, improved feed efficiency, and increase in overall leanness of the carcass (Anderson et al., 1987). There are numerous positives to supplementing Paylean® to finishing swine; however, it has also been reported (during the FDA approval trials) that administration of Paylean® is associated with an increased number of injured or lame pigs during marketing (Marchant-Forde et al., 2003). Paylean® is utilized at the concentration of 4.96 to 9.92 mg/kg of feed during the last 35 days prior to harvest. It is also important to note that Paylean® be fed in a diet containing at least 16% crude protein (FDA, 2006a). With administration of Paylean® there is no mandatory withdrawal period before harvest.

In cattle, the use of RH was FDA approved on June 13, 2003 under the tradename Optaflexx®. Optaflexx® is also manufactured by the company Elanco Animal Health. This

product also results in the increase in feed efficiency and weight gain but in feedlot cattle instead of swine. Optaflexx® also results in an increase in live weight (LW), HCW, and DP (observed in the FDA approval study). It has different severities at different doses. Just like Paylean®, Optaflexx® is supplemented as part of a complete feed but at the concentrations of 8.2 to 24.6 grams per ton during the last 28 to 42 days on feed. However; Optaflexx® can also be top dressed at 70 to 400 milligrams per head per day when administered to cattle. There is no withdrawal period for administration of Optaflexx® (FDA, 2009). With the administration of Optaflexx®, there have been no dramatic changes in meat quality. This product has been reported to have no effect on meat color, the degree of marbling, or the percentage of carcasses that grade USDA Choice (Quinn et al., 2008). In addition, Quinn et al. (2008) also observed no significant difference in Warner-Bratzler shear force values between the controls and cattle treated with Optaflexx®. Another study observed that RH-supplemented steers (RH = 4.83 kg/cm^2) had significantly greater shear force values compared to the controls (Control = 4.39 kg/cm^2) (Avendano-Reyes et al., 2006). Overall, supplementation of Optaflexx® results in an increase in feed efficiency and ADG but does not have much of an effect on carcass characteristics.

B. Zilpaterol Hydrochloride

Zilpaterol hydrochloride was approved for supplementation to cattle on August 10, 2006. Its market name is Zilmax® and is manufactured by Merck Animal Health. Zilmax® is supplemented at 8.33 mg/kg on a 100% DM basis for the last 20 days prior to harvest followed by a 3 day withdrawal period. Supplementation of Zilmax® has been shown to increase HCW, LW, DP, and yield grade (Avendano-Reyes et al., 2006; Beckett et al., 2009; Rathmann et al., 2009). Unlike Optaflexx®, there is a reported difference in meat quality in cattle supplemented Zilmax®. Zilmax® supplementation resulted in a lower percentage of carcasses that grade USDA

Prime and Top Choice when compared to control cattle (Elam et al., 2009). In addition, there has been an observed increase in the percentage of cattle grading USDA Select. Not only does Zilmax® affect the marbling scores but it has an effect on tenderness. Studies have shown that cattle supplemented with Zilmax® have increased shear force values (Rathmann et al., 2009; Leheska et al., 2009). Leheska et al. (2009) observed on average a 0.7 kg increase in shear force values in the LM with Zilmax® supplemented beef cattle.

Zilmax® was voluntarily taken off the market as personnel at various abattoirs throughout the United States had noted cattle being tender footed and lame. These accusations were not scientifically proven and were not taken lightly. Several beef packing companies announced they would no longer accept cattle supplemented with Zilmax®. Since packers were no longer accepting these cattle, Merck voluntarily removed their product from the market to do further investigations to the accusations that were made.

IV. Mode of Action of Beta-Agonists

A. Adrenergic Receptors

There are two types of AR: α-adrenergic and β-adrenergic (Mersmann, 1998). In order for β-AAs to elicit a biological effect the β-AA must bind to a β-AR (Hadley and Levine, 2007). These receptors are naturally occurring in the body and the catecholamines epinephrine (EPI) and norepinephrine (NEPI) are the naturally occurring ligands that bind to these receptors. Epinephrine is a ligand for both of the adrenergic agonist receptors but the net response to epinephrine depends on the presence of the receptors. One of the observed differences between α-AR and β-AR is the type of G-coupled protein to which each binds. The β-AR is important in regulating cell metabolism by coupling with the Gs-protein. The α-AR has an opposing effect as it is coupled with the Gi-protein or inhibitory protein (Johnson et al., 2014).

In the livestock industry β-ARs are the receptors of focus. The β-ARs can be subdivided even further into β_1-AR, β_2-AR, and β_3-AR whereas α-ARs are subdivided into α_1-AR and α_2-AR. Almost all cells in the mammalian body contain β-AR but the abundance and distribution of each receptor subtype are varied between the different tissues of the same species. This suggests why scientists have observed differences in magnitudes of biological effects across tissues in the same animal (Johnson et al., 2014).

The α-AR subtypes have opposite effects than β-AR. The α_1-ARs are found in blood vessels that serve the kidneys, abdominal viscera, skin, mucosae, and salivary glands (Reece et al., 2015).. Instead of dilating the blood vessels like β-ARs, the α_1-ARs constrict the blood vessels and visceral organ sphincters (Reece et al., 2015). The α_2-ARs receptors are located in the membrane of adrenergic axon terminals, pancreas, and in blood platelets. The α_2-ARs inhibit the release of norepinephrine from the adrenergic terminals, inhibit insulin secretion from the pancreas, as well as promote blood clotting (Reece et al., 2015).

The three subtypes of β-ARs are not present in all tissues but certain receptors are prevalent in some tissues more than others. For example, β_1-AR is primarily located in the heart but can also be identified in the kidneys and some adipose tissue. When binding occurs with the β_1-ARs, the results are an increase in heart rate along with an increase in renin production by the kidneys (Reece et al., 2015). The β_2-ARs have been on every mammalian cell in the body (Mersmann, 1998). The β_2-ARs are located abundantly on blood vessels serving the heart, liver, and skeletal muscle. When bound, numerous responses occur, including dilation of blood vessels and bronchioles, along with relaxation of smooth muscle of the digestive and urinary organs (Reece et al., 2015). The last subtype of β-receptors (β_3-AR) is primarily located in adipose tissue. The β_3-ARs stimulate lipolysis in adipose tissue. Sillence et al. (2005) have

suggested the possibility that a fourth β-AR subtype may exist only in porcine adipose tissue. However, no unique sequence has yet been determined for this suggested receptor whereas, the other three subtypes have been cloned and sequenced (Johnson et al., 2014).

All the subtypes of β-ARs possess seven hydrophobic, transmembrane-spanning domains that form loops both extracellular and intracellular on the surface of most mammalian cells. The β-ARs are also composed of more than 400 amino acids. The binding of ligands occur on the extracellular portion of the loop which then initiates a conformational change of this membrane-bound receptor. These receptors are part of a large family of G-coupled protein receptors that elicit a response by binding to Gs proteins. Once the β-AR is coupled with a Gs protein, the enzyme adenylate cyclase is activated to cause synthesis of cyclic adenosine monophosphate (cAMP). Cyclic adenosine monophosphate is responsible for regulation of the catalytic subunit protein kinase A (PKA). Protein kinase A is known for being vital to many biological functions as it results in the phosphorylation of key enzymes (Mersmann, 1998). This phosphorylation can lead to either activation or inactivation of various enzymes. In addition, PKA targets transcription factors that regulate the transcription of DNA to mRNA. For example, the cAMP response element binding protein (CREB) is phosphorylated by PKA. The CREB binds to a cAMP response element which then stimulates the transcription of the regulatory part of that gene (Mersmann, 1998). This increase in transcriptional activity provides the mechanism for β-AR agonist-mediated transcription of many other genes.

Protein kinase A not only targets phosphorylation within the extracellular domains but it also targets intracellular domains. In the intracellular domains, phosphorylation causes the uncoupling of the β-AR from the Gs protein. This uncoupling causes the inactivation of the receptor, also known as desensitization (Mills and Mersmann, 1995).

B. Impact on Muscle

In beef cattle, muscle hypertrophy is the result of increased protein synthesis and/or reduced protein turnover (Mersmann, 1998). Postnatal muscle growth has been a major focus related as β-AAs effects are the increase in muscle mass. Skeletal muscle hypertrophy is obtained by an expected increase in protein accretion, a decrease in protein degradation, or a combination of both (Yang and McElligot, 1989; Moloney et al., 1991; Mersmann, 1998). There are limited data on measuring protein degradation; instead research has focused on protease activities (specifically calpains) in muscle from animals treated with β-AAs. Researchers have reported that several protease activities are often reduced, or the protease inhibitors (calpastatins) are increased by β-AAs administration (Wang and Beermann, 1988; Kretchmar et al., 1990, Koohmaraie et al., 1991; Bardsley et al., 1992; Sainz et al., 1993).

With supplementation of β-AAs, a rapid growth in muscle is observed. For example, ZH administration results in an average of 11- 15 kg of more muscle on the carcass compared to the cattle not given ZH (Merck Animal Health, 2015). The duration of administration also has an effect on the response of increases in muscle growth. With ZH, it has been reported that supplementation past the recommend 20 days does not have any significant effect on carcass measurements (Vasconcelos et al., 2008).

Another impact β-AA administration has on muscles is the change in muscle fiber type. Studies have indicated that type II fibers (glycolytic) fibers are more likely to be altered with β-AA (cimaterol) administration than type I (oxidative) fibers (Beermann et al., 1987; Kim et al., 1987). Baxa et al. (2010) indicated that ZH increased the abundance of mRNA of myosin heavy chain IIX in cattle which would ultimately result in an increase in larger fiber diameters. In sheep, the size of type II fibers has consistently increased by 10-50% with β-AA administration

(Beermann et al., 1987; Kim et al., 1987). The effects of alterations in type I fibers are more varied than type II. In sheep, Kim et al. (1987) reported that type I fibers were not affected by β-AA administrations whereas Beermann et al., (1987) reported that type I fibers increase in size.

C. Impact on Fat

Ricks et al. (1984b) have termed β-AAs (specifically clenbuterol) as repartitioning agents due to the fact β-AA shift nutrients towards muscle growth and away from adipose deposition. The reduction in fat has been well documented across multiple livestock species with the various β-AAs (Ricks et al., 1984a; Beermann et al., 1986; Cromwell et al., 1988; Anderson et al., 1991; Moloney et al., 1990; Elam et al., 2009). Reduction in adipose is not limited to any specific depot. Reduced adipose depositions has been reported in subcutaneous, visceral, inter- and intra-muscular depots (Quirke et al., 1988; Moloney et al., 1990; Leheska et al., 2009; Rathman et al., 2009). Reduction in kidney, pelvic and heart fat (KPH), 12^{th} rib back fat, and marbling score have been the most documented incidences of alterations in fat. The reduction in marbling score has an impact on the quality grades of beef carcasses (Elam et al. 2009).

The reduction in fat is a result of β-AAs activating a hormone-sensitive lipase that stimulates degradation of the triglycerides in the adipocytes into glycerol and free fatty acids (Fain and Garcia-Sainz, 1983). Other studies have suggested that chronic treatment of β-AAs block lipogenesis (Smith et al., 1987; Liu et al., 1988) through the loss of key signaling enzymes (Steinberg, 1976). However, contradictions between agonists and species have been reported on the effect on lipogenesis. Agonists that cause reduction in adipose and bind to the β-ARs may result in minimal effects on lipid metabolism measured *in-vitro* within the same species (Spurlock et al., 1993; Mills and Mersmann, 1995). The administration of RH has been reported to increase lipolysis and decrease lipogenesis in swine (Merkel et al., 1987; Liu et al., 1988)

while other compounds like clenbuterol (Mersmann, 1987a) only affect the lipolytic rate. Zilpaterol hydrochloride is suggested to have a larger repartitioning effect on fat metabolism than RH (Scramlin et al., 2010).

It has also been suggested that an elevation of plasma nonesterified fatty acid concentration after administration of a β-AA results in the stimulation of the adipocyte lipolytic system. In swine, several β-AAs elevate the plasma nonesterified fatty acid concentrations (Mersmann, 1987b). Blum and Flueckiger (1988) along with Eisemann et al. (1988) observed the same response in cattle.

V. Impact on Carcass Traits

A. Carcass Characteristics

The impact of administration of β-AAs on carcass characteristics is well documented and is where the most noticeable alterations occur. These alterations are evident across a majority of β-AAs; the severity depends upon the β-AA and amount that is used. One of the most prevalent effects of the use of β-AAs in livestock is the increase in HCW. An increase in HCW could result in an increase in yield grade 1 and 2 carcasses due to β-AA administration causing a decreased 12^{th} rib fat and increased LM area size. A lower yield grade is desirable in carcass as it represents a higher percentage of boneless closely trimmed retail cuts. Beckett et al. (2009) observed an increase in USDA stamped yield grade 1 and 2 carcasses in conjunction with a reduction in yield grade 3 and 4 when feeding ZH. In another study, yield grades were decreased in ZH carcasses compared to the RH and controlled carcasses (Scramlin et al., 2010). These observed changes in yield grade are in direct correlation to the four components of the yield grade equation (12^{th} rib fat, HCW, LM area, and KPH). With β-AA administration, an increase

in LM area and decreases in the fat at the 12th rib and KPH cause the observed increase in lower numerical yield grades.

Zilpaterol hydrochloride supplementation is characterized as having a strong effect on the carcass cutout. Rathmann et al. (2009) observed that 22 of the 33 subprimal cuts from a beef carcass displayed a positive difference (meaning an increase in lean yield) between the control and ZH-supplemented cattle. Although research shows that ZH elicits a response of an increase in weight within every whole primal region of the carcass, the most consistent area affected is the round (Rathmann et al., 2009). Rathmann et al. (2009) also observed an increase in the percentage yield for every subprimal cut from the round in ZH fed steers. The LM is another muscle that consistently increases in size with ZH supplementation; however this is not true for all β-AAs. The LM area is larger in ZH fed cattle whereas RH fed steers do not differ from the control steers (Avendaño-Reyes et al., 2006). This increase in skeletal muscle tissue is the most consistent biological effect of ZH supplementation and this confirms many of the well documented carcass trait changes that have been observed (Becket et al. 2009; Leheska et al., 2009; Montgomery et al., 2009; Rathmann et al., 2009).

Another factor that affects yield grade values is the amount of fat on a carcass. It has been observed that the 12th rib back fat is reduced along with other adipose tissue depots decreasing in size with the feeding of β-AAs (Quirke et al., 1988; Moloney et al., 1990; Leheska et al., 2009; Rathman et al., 2009). Ricks et al. (1984b) conducted a study to determine the effects of clenbuterol fed at different concentrations in steers on fat and muscle deposition. Clenbuterol fed at 10 and 500 mg/hd/d reduced KPH by 23 and 33% and reduced 12th rib back fat by 35 and 42%, respectively. This result shows that not only does the reduction of fat content occur subcutaneously but occurs in internal depots as well.

B. Meat Quality

The use of β-AA has been important in increasing production of a lean source of animal protein; however, this increase in yields could potentially change the palatability and quality of the product. Research has shown that when β-AAs are fed meat quality grades tend to decrease. When ZH is fed, studies have shown that there is a reduction in marbling score. Elam et al. (2009) observed that the percentage of carcasses that graded USDA Prime and Top Choice were lower in the ZH-fed cattle. In direct correlation, there was an increase in the percentage of cattle that graded USDA Select. As the duration of ZH feeding increased these effects continued to increase or decrease in a linear fashion (Elam et al., 2009).

Not only does the supplementation of β-AAs have an effect on the marbling score but differences in tenderness have been identified. In general, β-AAs have shown an increase in Warner-Bratzler Shear Force (WBSF) values when compared to the controls. Some β-AAs seem to have greater changes on WBSF than others. This was evident when comparing clenbuterol, RH and ZH-fed cattle to the control group. Strydom et al. (2009) discovered that clenbuterol had the greatest effect on WBSF especially on the *longissimus lumborum* (LL) followed by ZH. Clenbuterol resulted in greater WBSF values. With aging there was a similar decrease in WBSF observed in the controls as well as the ZH and RH-fed cattle. Clenbuterol, however, resulted in a significantly lower reduction WBSF than other treatments during aging (Strydom et al., 2009). In general, ZH supplementation results in an increase in WBSF values across all aging periods compared to control cattle (Rathmann et al., 2009). Quinn et al. (2008) observed no significant difference in shear force values in cattle treated with RH. Leheska et al. (2009) observed that ZH-fed steers increased WBSF values by 22% and heifers increased by 24%, respectively.

There are a few studies that had a trained sensory panel to determine if β-AA- treated groups displayed a change in tenderness or juiciness that consumers could identify. Overall tenderness in one sensory panel decreased 11% and flavor intensity scores decreased by 4% when cattle were fed ZH (Leheska et al., 2009). Leheska et al. (2009) mirrored what Hilton et al. (2009) observed. Hilton et al. (2009) observed the same decrease in the trained sensory panel juiciness, tenderness, and the beef flavor intensity.

VI. Impact on Animal Well Being

There are currently very little data present on the use of β-AAs associated with a compromises animal welfare. Within the past couple years there have been observed abnormalities in the mobility of cattle upon arrival at the abattoirs. These reported incidences have generated concern in the beef industry. Personnel at various abattoirs throughout the United States observed cattle being nonambulatory or slow and having difficulty moving as well as cattle that appeared to have sloughed hoof walls which has raised concerns (Thomson et al., 2015). Some individuals believe there is a relationship between these mobility issues and the use of the β-AA ZH. There are insufficient data to establish an actual causal relationship between the two. It has been suggested that the cattle supplemented with ZH are at a greater risk in developing the mobility issues at the abattoirs (Thomson et al., 2015).

Thomson et al. (2015) suggested that many of the cattle that present mobility issues upon arrival at the abattoirs have clinical signs that are similar to those of pigs with fatigued pig syndrome (FPS). Fatigued pig syndrome-affected pigs become nonambulatory without any noticeable trauma, injury, or disease and the pigs refuse to move (Marchant-Forde et al., 2003). This syndrome is associated with many factors including; but not limited to, stressful handling and the supplementation of the β-AA RH in doses that are on the higher end of the approved

range. Thomson et al. (2015) hypothesized that those cattle upon arrival at the abattoirs with mobility problems may have a similar condition as FPS due to all the clinical similarities. A study done by Marchant-Forde et al. (2003) suggested that FPS is associated with finishing pigs fed RH. Pigs had elevated heart rates and increased concentrations of circulating catecholamines such as epinephrine and norepinephrine, which resulted in behavioral changes that made handling the pigs more difficult at the time of transport to the abattoirs and less tolerant of stress. Following this study, the manufacturer of RH reduced the recommended dose and added a caution on the label that the use of RH may result in an increased risk of injured or fatigued pigs (Elanco, 2016). This association of FPS with the use of RH leads to speculation that β-AAs could have the same effect on cattle resulting in FCS.

It has been suggested that ZH supplementation could be a compounding factor of "muscle fatigue." Studies have reported that ZH enhances the growth of the "fast-twitch" or white muscle fibers (Johnson et al., 2014). The fast twitch muscle fibers are used for short durations of muscle contractions (phasic). These muscle fibers are not used for endurance so they fatigue more easily than the other muscle fiber types. Baxa et al. (2010) observed that when cattle were fed ZH there was a differential response in the mRNA abundance of myosin heavy chain. This resulted in the transition away from slower twitch fibers to faster twitch fibers which could ultimately be the source of increase in skeletal muscle fiber diameters in carcasses that are fed ZH. This transition to white muscle fibers could be the cause for the observation of muscle fatigue when cattle are placed under stressful situations. White muscle fibers are phasic and therefore are more prone to fatigue. If cattle are being stressed and they have transitioned to more white muscle fibers due to β-AA supplementation then the muscles are expected to fatigue faster.

Alterations in body temperature can be indicative of negative physiological functions and could lead to potential animal welfare issues. Environmental temperatures are vital in regulating core body temperature which promotes better productivity in livestock. When livestock are under heat stress their body temperatures become elevated which could ultimately hinder overall production. A study by Marcias-Cruz et al. (2010) used ewe lambs to look at a possible correlation between body temperature and ZH supplementation during heat stress conditions. Temperatures where collected from the head, rump, belly, and right flank of the lambs. Marcias-Cruiz et al. (2010) observed that lambs fed ZH resulted in higher temperatures in the belly and flank whereas there were no differences in the head and rump. It was suggested that the increase in temperature could be a result to changes in the rumen and intestinal environment when ZH was fed.

Another trial (Boyd et al. 2015) examined the effects of shading and feeding ZH to finishing steers. What Boyd et al. (2015) discovered was contradictory to their theory that ZH supplementation increases the heat load on the animal. The average and maximum body temperature was lower in the cattle fed ZH than the controls, even with the different housing. There were two types of housing in this trial- open lot and shaded. The results of the control cattle were what they expected and the shaded cattle had lower average and maximum body temperature than the open lots. With feeding of ZH they observed the very opposite. The cattle in the open lots had lower average and maximum body temperature than the shaded cattle. It has been suggested that the addition of a β-AA could result in an increase in blood flow to skeletal muscle and adipose tissues (Mersmann, 1998). Therefore, it could be speculated that more blood moving away from the body's core towards muscle and fat could aid in cooling the animal and result in decreased body temperatures through conductive heat loss (Boyd et al., 2015).

When analyzing stress, the steroid hormone cortisol is often measured. Cortisol is considered an adrenocortical steroid and due to the hyperglycemic effect; cortisol-like substances are given the name glucocorticoids (Reece et al., 2015). The hyperglycemic effect occurs due to glucocorticoids reducing the affinity of insulin in certain cells. Glucocorticoids also serve many other metabolic functions in the body which include alterations in lipid, protein and carbohydrate metabolism. Cortisol is known as the "stress hormone" and is in higher concentrations when an individual is under stress. Cortisol provokes the cell to produce glucose from proteins and fatty acids through the process of gluconeogenesis. Ultimately, cortisol saves the glucose for the brain and forces the body to use other alternatives for energy. In addition, cortisol increases blood pressure which results in an increase in blood flow to distribute the glucose and other nutrients as fast as possible to the cells (Reece et al., 2015).

There are limited data on the correlation of cortisol concentrations and β-AA. Marchant-Forde et al. (2003) observed in pigs fed RH that there was no significant difference between the RH fed group and controls in the percentage change of cortisol concentrations from the baseline, even when responding to transportation stress. Another study indicated similar results, where plasma cortisol concentrations were not affected by β-AAs use in lambs (Li et al., 2000). Bruckmaier et al. (1992) conducted research on the responses of calves to exercise during feeding the β-AA clenbuterol and observed cortisol concentrations did not change.

VII. Blood Variable Changes due to Beta- Agonists

There is very limited information on the effects of β-AAs on the metabolic profile of livestock and what is available is very inconsistent.

A. Glucose

Glucose is an important component of proper cell function. It provides cells with a source of energy. Blood glucose concentrations in association with β-AAs are not consistent in the literature. Researchers observed that whole-blood concentrations of glucose decreased with the supplementation of ZH over time while controls remained constant in cattle supplemented with ZH (Bibber-Krueger et al., 2015). It is suggested that ZH administration may result in the secretion of stored red blood cells in the spleen via activation of the β-ARs, which would result in an increased number of cells in circulation to metabolize glucose (Bibber-Krueger et al., 2015). Evaluating clenbuterol, Eisemann et al. (1988) observed an initial increase in glucose, however, after day nine of feeding clenbuterol, glucose concentration was not different between treatments. These results suggest there is the initial response of glucose metabolism to the supplementation of the β-AA but cells eventually become desensitized and there is no longer a response. Another study conducted with feedlot lambs observed no effects on blood glucose with administration of RH or ZH (López-Carlos et al., 2010). A study observing the effects of RH on genetically obese and lean pigs reported no difference in glucose concentration between the groups (Yen et al., 1990).

B. Insulin

Insulin is secreted by the pancreas and is vital in the regulation of blood concentration of glucose. Effects of β-AAs on insulin concentrations have varied. O'Conner et al. (1991) observed in lambs that concentrations of insulin were elevated after 2 hours of cimaterol supplementation whereas in 3 to 6 weeks insulin concentrations decreased. O'Conner et al. (1991) suggested that the acute response in the elevation of insulin was a result of stimulation of glycogenolysis and lipolysis by cimaterol. It has also been suggested that the repartitioning

effects of β-AAs may be caused by opposing effects on insulin sensitivity in adipose versus muscle tissues (Anderson et al., 1991). Insulin is associated with increased protein synthesis and decreased proteolysis in muscle and increased lipid synthesis and decreased lipolysis in adipose, the inhibitory effects of insulin on adipose and the enhancement on muscle tissue would repartition nutrients towards lean tissue growth (Anderson et al.,1991). Liu and Mills (1990) observed a reduction in insulin sensitivity in adipocytes in pigs supplemented with RH. However, Beermann (1987) reported no change in insulin sensitivity in lambs despite the fact that insulin concentrations decreased. In summary the effects of β-AAs on insulin concentrations are varied.

C. Blood Urea Nitrogen

Blood urea nitrogen (BUN) is a measurement of the amount of nitrogen found in the blood that comes from the waste product of urea. Urea is made when protein is broken down in the body and secreted from the liver. In the literature BUN is consistently lower in animals administered a β-AA. Steers administered clenbuterol presented depressed BUN concentrations (Ricks et al., 1984b). In correlation BUN concentrations, another study revealed feeding cattle ZH resulted in decreased plasma urea nitrogen (PUN) concentrations (Bibber-Krueger et al., 2015). Decreased BUN or PUN concentrations are indicative of increased nitrogen retention. Increased nitrogen retention is a reflection of decreased protein catabolism in skeletal muscle when β-AAs are administered (Bibber-Krueger et al., 2015). It has been a general finding that administration of β-AAs results in an increase in nitrogen retention which correlates to muscle accretion (specifically the increase in carcass weight and LM area) in ruminants (Williams et al., 1987).

D. Creatinine

Creatinine is a product of normal functioning muscle metabolism and is considered a measurement of muscle mass. It is produced from the breakdown of creatine. According to Istasse et al. (1990) there is a positive correlation between creatinine concentrations and carcass weight, DP, and the proportion of lean mass in the carcass with administration of β-AAs. The literature for creatinine is well documented in saying that with the administration of a β-AA there is an increase in serum concentrations of creatinine (Chikhou et al., 1993a, and Quirke et al., 1988). It has been suggested that the alterations in the creatine pool are most likely the result in the changes in total muscle mass (Perrone et al., 1992).

E. Creatine Phosphokinase

Creatine phosphokinase (CPK) or creatine kinase is a catalyst for the reversible reaction of creatine to phosphocreatine in which the by-product is creatinine. Cattle fed ZH have increased concentrations of CPK which was observed in many studies including the FDA approval studies (FDA, 2006b). Thompson et al. (2015) evaluated two cattle from a shipment of 200 head that were fed ZH in accordance with the label directions and markedly increased concentrations of CPK from the reference ranges were observed. However, these two cattle evaluated were considered downer cattle and other cattle maintained in a typical feedlot setting with minimal stress did not differ significantly between the β-AA fed cattle and the controls. The CPK concentrations are increased in swine that are supplemented with ractopamine (Athayde et al., 2013). Elevated concentrations of CPK would be expected in cattle fed β-AAs due to the increase in muscle mass. The creatine pool increases with increasing muscle size, therefore, more CPK is required to catalyze the reaction. Since β-AA-fed cattle have documented increases in

creatinine, it is known CPK also increased to catalyze the reaction to produce creatinine as a by-product.

F. Proteins

Plasma is composed of 91-92% water and the rest is solid constituents. Of these constituents, plasma proteins are the most abundant. The two major classes of proteins in the plasma are albumin and globulin. The function of albumin is to regulate and maintain osmotic pressure of the blood and to act as a transporter of various substances (Reece et al., 2015). Furthermore, albumin is associated with bicarbonate and phosphate as a buffer for the extracellular fluid. Globulins main function is in immune response (Reece et al., 2015). Together, albumin and globulin make the total protein concentration in the blood. There is limited information on administration of β-AAs and protein concentrations. A study by Ricks et al. (1984b) observed no significant alterations in albumin and total protein in steers administered clenbuterol.

G. Lactate

When cells have a high demand for glucose utilization lactate is produced. Lactate is produced when pyruvate is reduced during glycolysis due to the insufficient supply of oxygen, like when skeletal muscles are contracting (Reece et al., 2015). Blum and Flueckiger (1988) observed in calves an increase in lactate concentrations up to 10 hours after the administration of the β-AAs. Eisemann et al. (1988) presented similar findings when administrating clenbuterol to steers. This increase in lactate suggests a possible increase in peripheral glycolysis. Furthermore, ZH supplementation may have an increase in the demand for glucose by stimulating glycogenolysis and glycolysis (Bibber-Krueger et al., 2015). Therefore, this would result in an increase of lactate concentrations.

H. β-hydroxybutyrate

Skeletal muscles derive a significant amount of their energy requirements from oxidation of ketone bodies into acetylCoA (Reece et al., 2015). Beta-hydroxybutyrate is one of those ketone bodies. It is produced from the metabolism of non-essential fatty acids via the liver. Elevated concentrations are usually indicative of a negative energy balance. Both Bibber-Krueger et al. (2015) and Eisemann et al. (1988) observed no significant differences when a β-AA was administered to cattle. These observations suggest that β-hydroxybutyrate is being metabolized efficiently enough to avoid accumulation in the blood (Bibber-Krueger et al., 2015).

I. Minerals

Minerals such as calcium, phosphorus, sodium, and potassium are essential for many cellular functions. The major function of calcium is in skeletal muscle and bone formation along with initiating muscle contraction. Phosphorus is involved in every major metabolic pathway in the body due to it being a component of phospholipids and adenosine triphosphate (ATP). Sodium has a primary function of acid-base balance of the body. Lastly, the major function of potassium is determining the resting potential of a membrane (Reece et al., 2015). This being said, there are extremely limited data published on the connection between mineral concentrations and the administration of β-AAs in livestock. Ricks et al. (1984b) reported no obvious abnormalities with calcium and phosphorus concentrations in steers administered clenbuterol. It has been observed in horses that calcium and potassium were elevated and sodium was decreased in the ZH group (Wagner et al., 2008). The authors were unable to give an explanation as to why this phenomenon happened.

J. Liver Enzymes

Liver enzyme concentrations are used as an indicator of tissue damage in the liver. There is very little literature on the alteration of liver enzyme concentrations when administered β-AAs. Ricks et al. (1984b) reported no abnormalities in the enzyme alkaline phosphatase in Hereford steers administered clenbuterol. Another study by Chikhou et al. (1993a) observed elevated concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) with β-AA feeding. However, the concentrations of these enzymes were still within the range of normal healthy cattle. In addition, these studies revealed neither liver nor heart damage occurred in the animals. There have been elevations in some liver enzymes observed with administration of β-AAs but concentrations are still within the biological range for healthy animals.

VIII. Conclusion

In conclusion, the most positive attributes in the use of β-AAs are an increase in feed efficiency, ADG, DP, HCW, LM area, coupled with a decrease in fat deposition. These characteristics are vital to continue providing consumers with a viable animal protein source. Harvesting animals with more lean meat is vital in reaching consumers demands; however, the use of β-AAs raises the concern of animal well-being. They must be fed at the appropriate concentrations or we might see more lameness in treated animals. Ractopamine-supplemented pigs have an increase in fatigue during handling (Marchant-Forde et al. 2003). Some believe (Thompson et al., 2015) we are seeing similar effects with cattle supplemented with ZH. However, the changes that are observed in the animals metabolically are in direct correlation with protein accretion which ultimately leads to the increase in muscle mass. All other metabolic parameters are not changing drastically enough to constitute any animal well-being concerns.

The objective of the present study was to conduct a very intensive and controlled trial to evaluate the impact of ZH supplementation on hematological as well as serum biochemistry profiles.

Materials and Methods

Experimental design

All the experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Nebraska (IACUC #902) along with being in compliance with the *Guide for the Care and Use of Agricultural Animal in Research and Teaching* Third Edition (2010).

Twenty crossbred yearling heifers (556±7 kg initial BW) utilized for this study were obtained from the University of Nebraska Agricultural Research and Development Center feedlot in Ithica, NE.

Heifers were allowed a 3 week acclimation period while being maintained in the feedlot. During this time, heifers were haltered and adapted to being restrained in a tie stall environment and exposed to human interaction. After the 3 week acclimation period, heifers were randomly assigned to one of the two treatment groups: 1) control (CON; n=10): fed a finishing diet without ZH (Table 1.); and 2) zilpaterol hydrochloride (ZH; n=10): fed the same finishing diet as the controls except supplemented with ZH (Zilmax®; Merck Animal Health) at 8.33 mg/kg BW on a DM basis. For the supplementation 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 of BW on a DM basis. To ensure similarity between the two diets, five percent of the CON diet was replaced with fine ground corn. Heifers were fed once daily at 0800 h for 20 days. Prior to feeding, all supplements were individually mixed into each heifer's daily allotment. After 20 days the ZH was withdrawn from the diet for 3 days and the five percent high- moisture corn was returned to both diets to replace the five percent ground corn

supplement. The study was designed to follow approved industry procedures and feed ZH for 20 days followed by a 3 day mandatory withdrawal prior to being shipped to an abattoir.

Eight days prior to the beginning of ZH supplementation, heifers were relocated from the feedlot to the University of Nebraska-Lincoln Agricultural Research and Development Center Nutrition Dairy Barn in Ithica, NE. The barn had individual stalls that were equipped with individual bunks, automatic waterers, and a dairy mattress underneath. Prior to the heifers being moved into the barn, the first ten stalls on each side of the alleyway were randomly assigned to a treatment, but were blocked by treatment group (two heifers per block) to ensure that no CON shared a water with a ZH heifer. The heifers were maintained in individual tie stalls which were 1.34 meters wide by 1.84 meters long for the duration of the trial. To help with cleanliness, pine shavings were added on top of the dairy mattress and replaced as needed.

Environmental temperature and relative humidity data were collected from inside the barn by four HONO U23 Pro v2 temperature/relative humidity data loggers (U23, Onset, Bourne, MA, USA). All data from the probes were averaged and compiled to provide an overall environmental temperature and relative humidity. During the trial, the average environmental temperature was 9.4º C ± 2.9ºC and the average relative humidity was 57.7% ± 5.2%. In addition, the average temperature humidity index was 20.7 ± 2.3 in the barn.

Catheterization Process

Each heifer was fitted with an indwelling jugular catheter (Burdick Sanchez et al., 2013) 24 hours prior to the start of each blood collection period (d -3, 12, and 20 of the trial). To insert the indwelling jugular catheters, heifers were restrained in a working squeeze chute. Before making the incision, Lidocaine Hydrochloride Jelly USP (Akorn, Inc., Lake Forest, IL, USA) was administered directly on the incision site. An incision roughly 2-3 cm long was made in the

skin for more accessibility of the jugular vein. The indwelling jugular catheters consisted 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.). The tubing was inserted into the jugular vein via a 14-gauge by 5.08-cm thin-walled stainless steel biomedical needle (o.d.= 3mm). The catheter was secured using tag cement and a 2.08-cm wide porous surgical tape at the incision site. After the catheter was secured, vet wrap (Vetrap™; 3M Animal Care Products, St. Paul, MN, USA) was wrapped around the entire neck of each heifer to ensure the catheter was stable in the incision site. There was a portion of the tubing that was not inserted into the heifers that served as an extension of the cannula that allowed for easier collection of blood samples (Burdick Sanchez et al., 2013). After being fitted with their jugular catheters, the heifers were returned to their respective tie stall. One animal developed a sore underneath the halter and another had a catheter failure on day 13 and 21, respectively.

Sample collection was divided into three very intensive blood collection periods. The first period consisted of 3 days prior to the supplementation of ZH (days -2, - 0) and the four days following the beginning of supplementation (days 1-4). The second collection period was during the middle of ZH supplementation which consisted of days 13 through 16. The third period consisted of three days (days 21-23); which was the three day mandatory withdrawal period.

During the three intensive blood collection periods, 18 mL of blood was collected in Sarstedt tubes containing no additive (Sarstedt, Inc., Newton, NC USA) at 4 hour intervals (0000, 0400, 0800, 1200, 1600, and 2000 hours) from each heifer daily. The blood samples were allowed 30 minutes to clot at room temperature prior to being centrifuged at 1500 x *g* for 20 minutes at 4º C. Once the serum was isolated it was stored at -80º C until analysis. At 0800 and

2000 hours, an additional 9 mL of blood was collected for the Large Animal Chemistry Profile analysis. These samples were collected, separated, and shipped to the Kansas State Veterinary Diagnostic Laboratory.

Daily, at 1200 hour, a 3 mL blood sample was collected in EDTA Vacutainer® tubes (Fisher Scientific, Pittsburg, PA USA) and were immediately analyzed for complete blood cell (CBC) counts utilizing a ProCyte Dx Hematology Analyzer (IDEXX Laboratories, Westbrook, ME USA).

Tissue Collection

Beginning on day 25 of the trial, the heifers were harvested at the Loeffel Meat Laboratory located at the University of Nebraska-Lincoln. The heifers were randomly assigned to be harvested on one of the three harvest days (days 25, 26, and 27 of the trial) and within days, harvest order was alternated on the basis of treatment, resulting in extended withdrawal time for heifers harvested on different days (4, 5, and 6 d after ZH supplementation). After evisceration, samples were obtained from the liver, *Longissimus muscle* (*LM*) directly above the 13^{th} rib, and the *Biceps Femoris* (*BF*) from the outer center of the muscle. These samples were collected within 45 minutes of stunning, flash frozen in liquid nitrogen, and stored at -80º C until vitamin E analysis.

Serum Analysis

Samples were shipped on dry ice and overnighted to the Kansas State University Veterinary Diagnostic Laboratory (Manhattan, KS 66506) for a Large Animal Chemistry Profile analysis utilizing laboratory methods that are approved by the American Association of Veterinary Laboratory Diagnosticians (Visalia, CA). This profile consisted of serum concentrations of glucose (mg/dL), blood urea nitrogen (BUN, mg/dL), creatinine (mg/dL), total

protein (g/dL), albumin (g/dL), globulin (g/dL), total calcium (mg/dL), phosphorus (mg/dL), sodium (mmol/L), potassium (mmol/L), chloride (mmol/L), bicarbonate (mmol/L), anion gap ((calculated as ($[Na^+] + [K^+]) – ([CL^-] + [HCO_3^-]$); mmol/L)), sodium : potassium ratio, creatine phosphokinase (CPK, U/L), aspartate transaminase (AT, U/L), alkaline phosphatase (AP, U/L), γ- glutamyltransferase (GG, U/L), and sorbitol dehydrogenase (SD, U/L).

A second set of serum samples were shipped overnight on dry ice to the Iowa State University Veterinary Diagnostic Laboratory (Ames, IA 50011) for analysis of vitamin E concentrations. Concentrations of vitamin E were determined by a high performance liquid chromatography (HPLC) with UV detection set at 292 nm. In summary, 0.5 mL of serum was pipetted into 15 mL screw top tubes and is then spiked by adding 100 µL of 0.02 µg/µL Vitamin E acetate standard for a 2 ppm spike. Next, 2 mL of 95% ethanol was added to each tube and vortexed. For extraction of Vitamin E, 4 mL of 95/5 hexane/chloroform was added to each tube. Tubes were centrifuged for 5 minutes at 1500 rpm. Once centrifuged, 2mL of the top solvent layer (the 95/5 layer) was removed and concentrated in order for analysis by HPLC using UV at 292 nm. Vitamin E concentrations were reported as ppm and the detection limit for vitamin E is 0.5 ppm.

Serum cortisol concentrations were determined by utilizing the DetectX® cortisol immunoassay kit (Arbor Assays, Ann Arbor, MI) in duplicate samples. This kit measures total cortisol and the cortisol standard is provided to generate a standard curve. In a 96-well plate, 50 µL of samples or standards were pipetted into each well. Next, 75 µL of assay buffer was placed into the non-specific binding (NSB) wells and 50 µL of assay buffer was pipetted into wells to act as a maximum binding well. Next, 25 µL of the cortisol conjugate was added to each well followed by adding 25 µL of cortisol antibody to each well, except for the NSB wells. Plates

were placed on a plate shaker for 1 hr and afterwards were washed 4 times with 300 µL of wash buffer. Next, 100 µL of the TMB substrate was added to each well and allowed to incubate at room temperature for 30 minutes before 50 µL of stop solution was added. Then plates were read using a plate reader at 450 nm. The minimum detectable concentration was 45.4 ng/mL and the intra- and inter- assay coefficients of variation were 18.6% and 16.0%, respectively. To determine the serum concentration of cortisol, the unknown samples were compared to a standard curve of known cortisol concentrations. Cortisol concentrations were recorded as ng/mL.

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

Serum concentrations of lactate were determined by using duplicate samples in a 96-well plate format and by utilizing the lactate assay kit (MAK064; Sigma-Aldrich, St. Louis, MO, USA). In summary, plates were incubated at 37º C for 30 minutes followed by using a plate reader set at 570 nm to record the results. To determine the concentration of lactate, unknown samples were compared to a standard curve of known lactate concentrations. Lactate concentrations were recorded as ng/µL.

Concentrations of serum lactate dehydrogenase were determined by utilizing a lactate dehydrogenase activity assay kit (MAK066; Sigma-Aldrich, St. Louis, MO, USA) in duplicate samples in a 96-well plate format. Each plate was incubated for 37ºC for 2 minutes followed by using a plate reader set at 450 nm to record the initial reading. Plate readings were taken every 3

minutes after the initial reading at 450 nm until the most active unknown sample was greater than the highest standard. The final measurement that was utilized for calculating the enzyme activity was the value before the most active sample that is near or exceeds the end of the linear range of the standard curve. To determine the lactate dehydrogenase concentrations, the unknown samples were compared to a standard curve of known concentrations. Results were recorded in milliunits/mL.

Serum concentrations of β-hydroxybutyrate were determined by utilizing a β-hydroxybutyrate assay kit (MAK041; Sigma-Aldrich, St. Louis, MO, USA) in duplicate samples in a 96-well plate format. Each plate was incubated for 37ºC for 30 minutes followed by using a plate reader set 450 nm. To determine serum β-hydroxybutyrate concentrations, the unknown sample was compared to a standard curve of known β-hydroxybutyrate concentrations and results were recorded in ng/µL.

Tissue Analysis

Liver, LM and BF samples were sent overnight on dry ice to the Iowa State University Veterinary Diagnostic Laboratory for analysis of vitamin E concentrations in the tissue samples. In summary, two 5 g samples of tissue (liver, LM or BF) were weighed for vitamin E analysis and placed into 50 mL screw top tubes with Teflon™ lined screw caps. Samples were spiked with 5 ppm vitamin E. Next, 1 g of Celite™ was added to each tube and vortexed until homogenized. Then 10 mL of 95% ethanol followed by 20 mL of 95/5 hexane/chloroform were added to each tube. After fully mixing, 5mL of the top solvent layer was removed and concentrated. Concentrated samples were analyzed by HPLC using UV set at 292 nm. Data were recorded as ppm.

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS specific for repeated measures (SAS Inst. Inc., Cary, NC USA). The model included the fixed effects of treatment, time, and treatment x time interaction. Each heifer within the treatment was included as the experimental unit. If there was a significant difference in treatment or the treatment x time interaction, data were further analyzed separately in the three bleeding periods: baseline (days -2 to 4), middle of supplementation (days 13 to 16), and the withdrawal period (days 21 to 23). This was used to analyze all the serum metabolites.

For tissue samples, data were analyzed with treatment included as the fixed effect and heifer within treatment was the experimental unit. Specific treatment comparisons were made using the PDIFF option in SAS, with $P \leq 0.05$ considered significant and $0.05 \leq P \leq 0.10$ were considered a tendency. All data are reported as LSM±SEM.

Table 1. Composition of finishing diets fed to finishing heifers as a percent of DM basis

	Treatment	
Item	**Control**	**ZH**
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]The control supplement contained fine ground corn only. The 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).

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Evaluation of hematological and serum biochemical profiles associated with the supplementation of Zilpaterol Hydrochloride to feedlot heifers.

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Prepared using the guide for authors for the Journal of Animal Science

ABSTRACT: The objective of this study was to evaluate hematological and serum biochemical profiles associated with supplementation of zilpaterol hydrochloride (ZH) to feedlot heifers. Heifers (n=20; 556±7 kg BW) were randomly assigned to one of two treatment groups: 1) Control (CON: no ZH supplementation), or 2) Zilpaterol (ZH: supplemented ZH at 8.33 mg/kg per feed on a DM basis). Heifers in the ZH treatment group were supplemented with ZH for 20 d, followed by a 3 d withdrawal period. The trial was conducted over a 25 d period (-2 to 23 d, ZH supplementation beginning on d 1), with three serial blood collection periods: -2 to 4 d, 13 to 16 d, and 20 to 23 d (withdrawal period). Blood samples were collected and serum was separated then stored at -80ºC until analyzed for serum biochemical profile. Each d, at 1200 h a whole blood sample was collected in 4- mL tubes for hematological analysis. Heifers were harvested and tissue samples were collected from the liver, *longissimus* muscle (LM), and *biceps femoris* (*BF*) for analysis of vitamin E. There was a treatment effect for serum concentrations of calcium ($P = 0.008$) with concentrations being greater in CON heifers than when compared with ZH. A treatment effect was observed for serum concentrations of bicarbonate ($P = 0.03$), with ZH having greater concentrations than CON heifers. A treatment x time interaction was observed for glucose ($P = 0.02$), BUN ($P < 0.001$), Cl ($P = 0.04$), CREAT ($P \leq 0.001$), and CK ($P \leq 0.001$). Overall CREAT, CK, and Cl serum concentrations were greater in ZH compared to CON group whereas glucose and BUN concentrations were decreased in the ZH treated heifers. There were no differences in the liver enzymes aspartate transaminase (AST; $P = 0.08$) and sorbitol dehydrogenase (SBD; $P = 0.46$), however; there was a treatment x time interaction for alkaline phosphatase (ALP; $P < 0.001$) and γ-glutamyltransferase (GGT; $P = 0.004$). Overall, concentrations of ALP were increased in CON compared to ZH heifers. Overall, GGT concentrations were greater in ZH treated heifers. In the CBC, there was an overall treatment

effect (*P* = 0.02) for hematocrit, where ZH had increased hematocrit when compared to the CON heifers. There was no differences in vitamin E concentrations in the liver (*P* = 0.28), *LM* (*P* = 0.44), and *BF* (*P* = 0.44*).* These data suggest that in this controlled environment, ZH supplementation did result in alterations of the serum biochemical and hematological profiles, primarily in variables associated with alterations in protein accretion x(increased CREAT and CK along with decreased BUN). However, alteration in serum biochemical and hematological profiles observed within the constraints of this study do not appear to have a significant impact on the well-being of feedlot heifers.

Keywords: complete blood cell count, serum profile, β-agonist, cattle

INTRODUCTION

Zilpaterol hydrochloride (**ZH**; Zilmax®, Merck Animal Health, Summit, NJ) is a β_2-adrenergic receptor agonist (β-**AA**) that has been utilized in the US beef since 2007 (Schmidt and Olsen, 2007). The use of ZH has been reported to increase ADG (Montgomery et al., 2009) while decreasing DMI, resulting in an overall increase in feed efficiency (Mersmann, 1998; Avendaño-Reyes et al., 2006). Furthermore, supplementation of ZH has been reported to increase lean muscle mass and decrease adipose deposition. As a β-AA, ZH is binds to 7-transmembrane G-protein-coupled receptors (Mersmann, 1998). These receptors are part of a large family of G-coupled protein receptors that elicit a response by binding to Gs proteins. Once coupled with a Gs protein, the enzyme adenylate cyclase is activated to cause cyclic adenosine monophosphate (cAMP) to be synthesized (Mersmann, 1998). The synthesized cAMP is responsible for the regulation of the catalytic subunit protein kinase A (PKA). Protein kinase A is responsible for many biological effects as it results in the phosphorylation of key enzymes (Mersmann, 1998).

In 2013, it was suggested that there was an association between ZH supplementation and reports of slow moving and lame cattle upon arrival at the abattoirs (Thomson et al., 2015). Furthermore, Longeragan et al. (2014) reported a suggested association related to ZH supplementation and increased cumulative incidence (risk) and incidence of rate of death in feedlot cattle. Further, it has been hypothesized that a novel fatigue syndrome in finished cattle could be related to ZH supplementation (Thomson et al., 2015). With these reports of suggested associations to ZH supplementation, the objective of this study was to conduct an intensive and controlled trial to evaluate alterations in the hematological and serum biochemical profiles of feedlot cattle supplemented ZH.

MATERIALS AND METHODS

Experimental Design

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

Twenty English- influenced crossbred yearling ovariectomized heifers (556± 7 kg initial BW) from the University of Nebraska Agricultural Research and Development Center (Ithica, NE) feedlot were utilized for the study. While being maintained in the feedlot, heifers were acclimated to being halter tied and exposed to frequent human interaction to allow the heifers to acclimate to the environment more efficiently and reduce stress when placed into the tie stall barn during the study. This acclimation period occurred 3 wk prior to the start of the study.

At the conclusion of the adaptation period, heifers were randomly assigned to one of two treatment groups (n=10 per treatment): 1) Control (CON): heifers received a finishing diet with no ZH supplementation (Table 1); 2) zilpaterol hydrochloride (ZIL): heifers received a finishing diet with ZH (Zilmax®, Merck Animal Health) supplementation at a 8.33 mg/kg BW on a DM basis. For delivery of ZH, 5% of the high-moisture corn in the finishing diet was replaced with 4.9853% fine-ground corn containing ZH at 0.0147%. For the CON diet, 5% of the high-moisture corn was replaced with fine ground corn to ensure similarity between to two diets. Supplements were individually mixed into each heifer's daily allotment prior to feeding. Heifers were fed once daily at 0800 for the entire 26 d feeding period, including the 3 d withdraw period. On day 20, the 5% high-moisture corn was returned to the diet of all heifers (ZH and Controls) for the required 3 d mandatory withdrawal period to replace the 5% ground corn supplement.

The design of the trial followed common industry feeding practices for feeding ZH for the 20 d period followed by the 3 d mandatory withdrawal before heifers would be shipped to the abattoir.

Eight d prior to the start of the trial, the heifers were transported to the University of Nebraska Agricultural Research and Development Center Nutrition Barn (Ithica, NE). The Nutrition Barn was equipped with individual bunks, automatic waters, and dairy mattresses. Prior to heifers being relocated into the barn, each heifer were blocked by treatment groups to tie stalls (two heifers per block) to ensure that no CON heifer shared a waterer with a ZH heifer. Location of blocks within the tie stall barn was randomly assigned. For the duration of the trial, heifers where maintained in their individual tie stalls.

Due to the duration of the study (25 d), blood collection was separated into three collection periods: serum collection period1 (SCP1; d -2 thru 4 d post ZH supplementation), serum collection period 2 (SCP2; d 13 – 16), and serum collection period 3 (SCP3; d 21 – 23). Twenty- four h prior to the start of each serum collection period, heifers were fitted with an indwelling jugular catheter (Burdick Sanchez et al., 2013). For the jugular catheterization, heifers were placed in a restraining chute for 5 to 10 min. A 2–3 cm incision was made into the skin above the jugular vein for easier access. Catheters consisted of 30.48 cm of sterile Tygon® tubing (AAQ4113; US Plastics, Lima, OH, USA; 1.27 mm i.d. and 2.286 mm o.d.) and were inserted into the jugular vein using a 14- gauge by 5.08-cm thin- walled stainless steel biomedical needle (o.d. =3 mm). Stabilization of the catheter was achieved by using tag cement and a 2.08-cm wide porous surgical tape around the incision site. The entire neck region of each heifer was wrapped with vet wrap (Ventrap™; 3 M Animal Care Products, St. Paul, MN, USA) to that the catheter injection site was stable. The remaining portion of the tubing not inserted into the jugular served

as the extension portion of the cannula that was utilized for collection of the blood samples (Burdick Sanchez et al., 2013).

Environmental temperature and relative humidity data were collected from inside the barn by four HONO U23 Pro v2 temperature/relative humidity data loggers (U23, Onset, Bourne, MA, USA). All data from the loggers were averaged and compiled to provide an overall environmental temperature and relative humidity. During the trial, the average environmental temperature was 9.4º C ± 2.9ºC and the average relative humidity was 57.7% ± 5.2%. In addition, the average temperature humidity index was 20.7 ± 2.3 in the barn.

For each day of SCP1, SCP2, and SCP3, 18mL of blood was collected at 0800 and 2000 h in Sarstedt tubes containing no additive (Sarstedt, Inc., Newton, NC USA) for serum analysis. All blood samples were allowed to clot for 30 min at room temperature prior to being centrifuged at 1500 x *g* for 20 min at 4^{o}C. The isolated serum samples were stored at -80^{o}C until analysis. An additional 4 mL blood sample was collected in 4- mL Vacutainers® containing EDTA (Fisher Scientific, Pittsburg, PA USA) at 1200 h and immediately analyzed for complete blood cell (CBC) counts using a ProCyte Dx Hematology Analyzer (IDEXX Laboratories, Westbrook, ME USA).

At the conclusion of the study, heifers were transported (58 km) to the Loeffel Meat Laboratory located at the University of Nebraska – Lincoln and harvested. Due to limitations to the capacity of the Loeffel Meat Laboratory, heifers were harvested of a 3 d period (25, 26, and 27 d). Heifers were randomly assigned to one of the three harvest d, with an equal number of heifers from each treatment group harvested on each day. Order of harvest was also alternated, within days on the basis of treatment. After evisceration, tissue samples were collected from the LM (directly above the 13th rib, 3/4th the lateral length of the LM), *biceps femoris* (*BF;* outer

center of the muscle) and liver. Tissue samples were collected and immediately flash frozen in liquid nitrogen and stored at -80 ^{o}C until analyzed for vitamin E.

Two heifers from the ZH treatment group were removed during the trial. One heifer developed an abrasion underneath the halter and one heifer due to loss of patency of jugular catheter on d 13 and 21. This resulted in 10 heifers in the CON treatment and 8 heifers in the ZH treatment group.

Serum Analysis

Serum samples were shipped overnight on dry ice to the Kansas State Veterinary Diagnostic Laboratory (Manhattan, KS 66506) for a Large Animal Chemistry Profile. The Large Animal Chemistry Profile analyzed glucose (mg/dL), blood urea nitrogen (BUN, mg/dL), creatinine (mg/dL), total protein (g/dL), albumin (g/dL), globulin (g/dL), total calcium (mg/dL), phosphorus (mg/dL), sodium (mmol/L), potassium (mmol/L), chloride (mmol/L), bicarbonate (mmol/L), anion gap (mmol/L), Na:K Ratio, creatine kinase (CK, U/L), aspartate transaminase (AST, U/L), alkaline phosphatase (ALP, U/L), γ- glutamyltransferase (GGT, U/L), and sorbitol dehydrogenase (SBH, U/L) utilizing methods that are approved by the American Association of Veterinary Laboratory Diagnosticians (Visalia, CA).

In addition, serum samples were shipped overnight on dry ice to the Iowa State University Veterinary Diagnostic Laboratory (Ames, IA 50011) for Vitamin E concentration analysis. Vitamin E concentrations were determined by a high performance liquid chromatography (HPLC) with UV detection test. Briefly, 0.5 mL of serum was pipetted into 15 mL screw top tubes and was then spiked by adding 100 µL of 0.02 µg/µL Vitamin E acetate standard for a 2 ppm spike. Then 2 mL of 95% ethanol to each tube was added and vortexed. For the extraction of Vitamin E, 4 mL of 95/5 hexane/chloroform was added to each tube. Tubes

were centrifuged for 5 min at 1500 rpm. Then 2 mL of the top solvent layer (the 95/5 layer) was removed and concentrated in order for analysis by HPLC using UV at 292 nm. Concentrations of Vitamin E were recorded as ppm. The detection limit for Vitamin E is 0.5 ppm.

Cortisol concentrations were determined by the DetectX® cortisol immunoassay kit (Arbor Assays, Ann Arbor, MI) in duplicate samples, according to the manufacturer's instructions. Cortisol concentrations in the samples were determined by comparison to a standard curve of known cortisol concentrations. In a 96-well plate, 50 µL of samples or standards were pipetted into each well. Next, 75 µL of assay buffer was placed into the non-specific binding (NSB) wells and 50 µL of assay buffer was pipetted into wells to act as a maximum binding well. Next, 25 µL of the cortisol conjugate was added to each well followed by adding 25 µL of cortisol antibody to each well, except for the NSB wells. Plates were placed on a plate shaker for 1 h and afterwards were washed 4 times with 300 µL of wash buffer. Next, 100 µL of the TMB substrate was added to each well and allowed to incubate at room temperature for 30 minutes before 50 µL of stop solution was added. Then plates were read using a plate reader at 450 nm. The minimum detectable concentration of cortisol was 45.4 ng/mL and the intra- inter- assay coefficients of variation were 18.6% and 16.0%, respectively. Cortisol concentrations were recorded as ng/mL.

Insulin concentrations were determined by utilizing a bovine-specific insulin ELISA according to the manufacturer's instructions (Cat # 80-INSBO-E01; Alpco Diagnostics, Salem, NH). The minimum detectable concentration of insulin was 0.1 ng/mL and the intra- and inter-assay coefficients of variation were 11.8% and 17.9%, respectfully. Concentrations of insulin were recorded as ng/mL.

Serum lactate concentrations in the serum were determined in duplicate samples utilizing a lactate assay kit according to the manufacturer's instructions (MAK064; Sigma-Aldrich, St. Louis, MO, USA). To determine the concentration of lactate, unknown samples were compared to a standard curve. Concentrations of lactate were recorded as ng/µL.

Serum lactate dehydrogenase (LDH) were determined in duplicate samples by utilizing a LDH activity assay kit (MAK066; Sigma-Aldrich, St. Louis, MO, USA). Plates were incubated for 37º C for 2 minutes followed by using a plate reader set at 450 nm to record the initial absorbance. After the initial reading, subsequent absorbances were measured every 3 min at 450 nm until the most active unknown sample 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 for the standard curve. To determine the concentration of LDH, the unknown samples were compared to a standard curve of known concentrations. Concentrations of LDH were recorded as milliunits/mL.

Serum concentrations of β-hydroxybutyrate were determined by utilizing a β-hydroxybutyrate assay kit according to the manufacturer's instructions (MAK041; Sigma-Aldrich, St. Louis, MO, USA) in duplicate samples. To determine β-hydroxybutyrate concentrations, the unknown sample was compared to a standard curve of known β-hydroxybutyrate concentrations and results were recorded in ng/µL.

Tissue Analysis

Liver, LM, and BF samples were shipped overnight while on dry ice to Iowa State University's Veterinary Diagnostic Laboratory (Ames, IA 50011) for analysis of vitamin E concentrations, as described above.

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS specific for repeated measures (SAS Inst. Inc., Cary, NC). The model included treatment, time, and treatment x time interaction for the fixed effects. Heifer within treatment was included as the experimental unit. If there was a significant difference in treatment or the treatment x time interaction, data were further analyzed separately in the three blood collection periods: d -2 to 4 (baseline, supplementation starting on 0 d), d 13 to 16 (middle of supplementation), and d 21 to 23 (mandatory withdrawal period).

For tissue samples, data were analyzed with treatment included as the fixed effect and heifer within treatment was the experimental unit. Specific treatment comparisons were made using the PDIFF option in SAS, with $P \leq 0.05$ considered significant and $0.05 \leq P \leq 0.10$ were considered a tendency. All data are reported as least square means ± SEM.

RESULTS

Serum Metabolites

When data from the large animal chemistry profile were initially analyzed in separate 0800 and 2000 h, glucose was the only variable that was significantly different in the 0800 h from the 2000 h. Therefore, glucose was analyzed into separate 0800 and 2000 h samples. All other variables were analyzed with 0800 and 2000 h together. For 0800 h, there was no treatment x time interaction ($P = 0.68$) or treatment effect ($P = 0.46$), however, there was an overall time effect ($P \leq 0.001$; Figure 1A). For 2000 h, there was no overall treatment x time interaction ($P = 0.30$). There was a treatment ($P = 0.0018$) and a time ($P \leq 0.001$) effect for 2000 h serum concentrations of glucose (Figure 1B). Glucose concentrations were decreased in ZH heifers compared to the CON (74.9 ± 1.35 mg/dL vs 81.9 ± 1.34 mg/dL, respectively) Glucose

was further analyzed into the 3 collection periods. There was a treatment ($P \leq 0.04$) and time ($P \leq 0.004$) effects observed in all 3 collection periods at 2000 h.

For serum BUN concentrations, an overall treatment x time interaction ($P < 0.001$) was observed (Figure 2). When further analyzed as the 3 collection periods there was a treatment x time interaction observed in period 1 ($P = 0.05$) and period 2 ($P = 0.002$), but not in period 3 ($P = 0.53$). After initiation of supplementation on d 0 in period 1, BUN concentrations were greater in CON than ZH heifers at h 36. During period 2, BUN concentrations were greater in CON heifers compared with ZH heifers at each time point. Serum BUN concentrations were affected by treatment ($P = 0.01$) in the final collection period. Specifically, BUN concentrations were greater in CON when compared with ZH heifers (12.8 ± 0.67 mg/dL vs 10.0 ± 0.74 mg/dL, respectfully).

A treatment x time interaction ($P \leq 0.001$) was observed for serum concentrations of creatinine (Figure 3). However, there were no treatment x time interactions when each collection period was analyzed independently (period 1: $P = 0.58$, period 2: $P = 0.69$, period 3: $P = 0.65$). Serum creatinine concentrations were affected by treatment in period 2 ($P = 0.001$) and period 3 ($P = 0.003$). Specifically, serum creatinine concentrations were greater in ZH compared with CON heifers (period 2: 1.4 ± 0.06 mg/dL vs 1.07 ± 0.06 mg/dL; period 3: 1.5 ± 0.07 mg/dL vs 1.1 ± 0.07 mg/dL, respectfully). In conjunction with the treatment effect, there was a time effect for each collection period ($P \leq 0.02$). For serum concentrations of CK, a treatment x time interaction ($P \leq 0.001$) was observed. When analyzed within the 3 collection periods, there was no treatment x time interaction ($P = 0.28$) for period 1, while there were a treatment x time interactions in period 2 ($P \leq 0.001$) and period 3 ($P = 0.002$; Figure 4). Concentrations of CK

remained greater in ZH treated heifers in both periods 2 (305.8 ± 30.6 U/L vs 135.9 ± 29.5 U/L) and 3 (331.6 ± 36.2 U/L vs 169.9 ± 32.4 U/L).

There were no overall treatment effects ($P \geq 0.39$) nor treatment x time interactions ($P \geq 0.18$) for serum concentrations of total protein, albumin, globulin, or anion gap (Table 2). However, there was an overall time effect observed for these variables ($P \leq 0.001$).

In regards to mineral concentrations in the serum, there were no overall treatment x time interaction for serum concentrations of calcium ($P = 0.26$) and phosphorus ($P = 0.65$), but there was an overall treatment effect ($P = 0.008$) and time effect ($P \leq 0.001$) for serum calcium concentrations (Table 2). Specifically, concentrations of calcium were greater in CON heifers, when compared to ZH heifers (9.8 ± 0.07 mg/dL vs 9.5 ± 0.07mg/dL). There was no treatment effect for serum concentrations of phosphorus ($P = 0.90$), however there was a time effect ($P \leq 0.001$).

There was no overall treatment x time interaction for serum concentrations for potassium ($P = 0.20$) but there was a tendency for sodium ($P = 0.07$) and Na:K ratio ($P = 0.06$; Table 2). Additionally, there were no treatment effects for serum concentrations of potassium ($P = 0.16$), sodium ($P = 0.21$) and Na:K ratio ($P = 0.11$). However, there was an overall time effect ($P \leq 0.001$) for these variables. For serum concentrations of chloride, there was an overall treatment x time interaction ($P = 0.04$), however, when looking at the 3 collection periods individually there were no treatment x time interactions observed (Figure 5). In collection period 2, there was a treatment effect ($P = 0.04$), with ZH heifers having greater concentrations of serum chloride compared with the CON heifers (99.2± 0.5 mmol/dL vs 97.6 ± 0.5 mmol/dL, respectfully).

There was no treatment x time interaction ($P = 0.21$), but there were an overall treatment ($P = 0.03$) and time ($P \leq 0.001$) effect on serum concentrations of bicarbonate (Table 2). A

treatment effect was observed in collection period 2 (P=0.01) and period 3 (P=0.03) of the study. Specifically, ZH heifers had greater serum concentrations of bicarbonate compared to the CON heifers (period 2: 25.6 ± 0.22 mmol/dL vs 24.7 ± 0.22 mmol/dL; period 3: 25.9 ± 0.32 mmol/dL vs 24.9 ± 0.29 mmol/dL, respectfully).

Four liver enzymes that are routinely evaluated to diagnosis damage of liver cells were also analyzed in the complete blood serum profile: aspartate transaminase (AST), alkaline phosphatase (ALP), γ- glutamyltransferase (GGT), and sorbitol dehydrogenase (SBH). There was no treatment x time interactions ($P \geq 0.08$) or treatment effects ($P \geq 0.64$) for the liver enzymes AST ($P = 0.08$) and SBH ($P = 0.46$); however there were overall time effects ($P \leq 0.001$; Table 2). There was treatment x time interactions observed for the liver enzymes ALP ($P < 0.001$) and GGT ($P = 0.004$). When looking at the individual collection periods for ALP, period 1 had a treatment x time interaction ($P < 0.001$). Additionally, there were treatment ($P = 0.04$) and time ($P \leq 0.001$) effects in the final collection period (period 3) in which CON (104.7 ± 6.7 U/L) had greater concentrations of ALP when compared to the ZH heifers (82.5 ± 7.5 U/L; Figure 6).

There was no treatment effect ($P = 0.62$) or treatment x time interaction ($P = 0.20$) for serum concentrations of insulin, but there was a time effect ($P \leq 0.001$). There was no treatment x time interaction ($P = 0.75$) or treatment effect ($P = 0.19$) for serum cortisol concentrations. However, there was a time effect ($P \leq 0.001$) on serum cortisol. There were no treatment x time interaction ($P \geq 0.20$) or treatment effect ($P \geq 0.14$) for serum concentrations of lactate, LDH, and β-hydroxybutyrate (Table 2); however, there was an overall time effect ($P \leq 0.001$).

There was no treatment x time interaction ($P = 0.26$) or treatment effect ($P = 0.28$) for serum concentrations of vitamin E (Table 2). Vitamin E concentrations in the tissue samples can

be seen in Table 4. Supplementation of ZH did not affect vitamin E concentrations within the liver ($P = 0.28$), LM ($P = 0.44$), and the BF ($P = 0.44$).

Complete Blood Cell Counts

There were no treatment x time interactions for total white blood cells ($P = 0.46$), red blood cells ($P = 0.58$), hemoglobin ($P = 0.69$), hematocrit ($P = 0.60$), platelets ($P = 0.90$), neutrophils ($P = 0.96$), lymphocytes ($P = 0.17$), and monocytes ($P = 0.66$; Table 3). In addition, there were no treatment x time interactions for the percentage of neutrophils ($P = 0.90$), lymphocytes ($P = 0.63$), monocytes ($P = 0.33$). There was an overall treatment effect ($P = 0.02$) for hematocrit, where the ZH heifers (34.1 ± 0.69) had greater concentrations when compared to the CON heifers (31.5 ± 0.68).

DISCUSSION

Currently there is limited information concerning the use of β-AAs and animal stress. Over the past couple of years there have been observed mobility issues in cattle at abattoirs and this has generated concern in the beef industry. These mobility issues are described as the cattle being nonambulatory or fatigued with some appearing to have sloughed hoof walls, which have been observed upon arrival at the abattoirs (Thomson et al., 2015). For all variables that were measured there were time effects ($P \leq 0.001$) observed, however due to the limited number of samples analyzed over a 24 h period and diurnal patterns of metabolites it would be difficult to describe the pattern in which we see these metabolites change over time. Evaluation of time was not the purpose of this study. The purpose of this study was to evaluate alterations in metabolic and complete blood chemistry profiles with supplementation of ZH and observe treatment and treatment x time interactions.

In cattle, studies utilizing other β-AAs such as clenbuterol and cimaterol have reported differences in protein, lipid, and glucose metabolism (Eisemann et al., 1988). Blood glucose concentrations measured when cattle are supplemented with β-AAs have been inconsistent. No differences in glucose and insulin concentrations were found during cimaterol supplementation of Friesian steers when compared to the control steers (Chikhou et al., 1991). Another study observed that whole-blood concentrations of glucose decreased in cattle that were supplemented with ZH (Bibber-Krueger et al., 2015). The present study had similar results in which serum glucose concentrations were decreased in heifers supplemented with ZH. Similar results were observed in finishing steers, where blood glucose concentrations decreased after d 21 of ZH supplementation (Van Bibber et al., 2010). It has been suggested that the decrease in glucose concentrations are the result of the activation of β-adrenergic receptors that stimulate the secretion of red blood cells in the spleen, thus increasing the number of cells available to metabolize glucose (Bibber-Krueger et al., 2015). In regards to insulin, the current study observed no significant treatment effects in insulin concentrations throughout the duration of the study.

When supplementing with β-AAs, the most noticeable alterations in carcass composition are in the growth of skeletal muscle. This increase in muscle mass is due to β-AA binding to the naturally occurring membrane bound β_2-adrenergic receptors that are primarily located on skeletal muscle and adipose tissue (Mersmann, 1998). When these β-receptors are activated they induce lipolysis and protein accretion (Mersmann, 1998). In the current study, BUN concentrations were decreased in heifers supplemented with ZH, which is indicative of increased nitrogen retention. Increased nitrogen retention is a reflection of decreased protein catabolism in skeletal muscle (Bibber-Krueger et al., 2015). The heifers in the current study were provided

with adequate nutrition to support any additional lean muscle accretion through the duration of the study. The decrease in BUN concentrations is similar to other studies that have utilized various β-AAs. When steers were administered clenbuterol, reduced BUN concentrations were observed (Ricks et al., 1984). In conjunction, Bibber-Krueger et al. (2015) observed decreased plasma urea nitrogen (PUN) in cattle supplemented with ZH when compared to control cattle.

In previous cattle studies utilizing clenbuterol, differences in protein metabolism have been observed (Eisemann et al., 1988). During normal muscle metabolism, creatinine is a product of the breakdown of creatine. Creatinine is generally accepted as a measurement of muscle mass. There is a positive correlation between creatinine concentrations and the increase in HCW, DP, and the proportion of lean mass that is observed when administering β-AAs (Istasse et al., 1990). The increase in HCW, DP, and lean mass are factors that directly correlate to the increase in overall muscle mass. A theory to increases in creatinine production is built around the recognition that changes reflect alterations in the size of the creatine pool in the normal biological system (Perrone et at., 1992). The alterations in the creatine pool are most likely the result in the changes in total muscle mass (Perrone et al., 1992). In agreement with the current study, the administration of β-AAs has caused an increase in serum concentrations of creatinine (Chikhou et al., 1993; Quirke et al., 1988). With increased concentrations of creatinine we would suspect to observed increases in CK concentrations as well. In the current study, concentrations of CK were greater in heifers supplemented with ZH starting at h 36 and remained greater throughout the remainder of the trial when compared to the control heifers. Creatinine phosphokinase is the catalyst for the reaction converting creatine to phosphocreatine (in which a by-product is creatinine). As a result it was expected to have greater concentrations

of CK present to catalyze this reaction. This increase in CK has been well documented, including the FDA approval studies for Zilmax® (FDA, 2006).

Evaluation of blood metabolite concentrations in relation to cattle supplemented with ZH is very limited. Plasma is composed of 91-92% water and the rest is solids constituents. Plasma proteins are the most abundant in these constituents. Albumen and globulin are two major classes of proteins that make up total protein in the serum. In feedlot lambs, the supplementation of ZH or ractopamine hydrochloride (RH) resulted in no alterations in the overall protein status (López-Carlos et al., 2010). In another study, Ricks et al. (1984) observed similar results in which there were no significant alterations in albumin and total protein in steers administered clenbuterol. Relative to this study, there were no significant alterations in albumin, globulin and overall total serum protein.

The evaluation of mineral concentrations in serum of cattle supplemented with ZH is limited. Ricks et al. (1984) observed no obvious abnormalities with calcium and phosphorus concentrations in steers administered clenbuterol. Similarly in this study, there were no significant differences in phosphorus concentrations; however, there was a treatment difference for concentrations of serum calcium. Concentrations of calcium in the serum were lower (P = 0.008) in cattle supplemented ZH. However, the circulating pool of serum concentrations of minerals could be an unreliable measurement on the total body mineral status of an animal due to the significant amount that is stored in bone (Russel and Roussel, 2007).

The electrolyte sodium is a major cation that maintains the volume of the extracellular fluid and the serum concentration of sodium is representative of the total body sodium due to this cation being essentially confined to the extracellular fluid (Russel and Roussel, 2007). No differences for serum concentrations of sodium were expected in the current study as all heifers

had ab libitum access to water. The current study revealed tendencies for a treatment x time interaction for sodium ($P = 0.07$) and Na:K ratio ($P = 0.06$). A major anion in the extracellular fluid is chloride and alterations in serum chloride tend to resemble sodium due to the combined renal reabsorption of sodium and chloride (Russell and Roussell, 2007).

Serum concentrations of bicarbonate were lower in CON heifers when compared to ZH in the present study, however, both treatment groups were within the normal biological reference range of 17-29 mmol/L for cattle (Kaneko et al., 2008). Bicarbonate concentrations are a reflection of the acid-base status of the animal and are related to the total carbon dioxide concentrations (Russell and Roussel, 2007). Eisemann and Huntington (1987) reported that clenbuterol supplemented to cattle resulted in an increase in blood flow and oxygen uptake in the hind-quarters. The greater bicarbonate concentrations observed in ZH heifers could suggest an increase in the oxygen that is available due to the increase respiration rate observed with supplementation of a β_2-AA.

The enzymes ALP, AST, GGT and SBH are a component of clinical chemistry profiles for cattle as they are indicative of damage or injury to the liver. The relationship between these liver enzymes and β-AA supplementation is not well documented. In the current study there were no treatment x time interactions for serum concentrations of SBH or AST. A previous study observed elevated concentrations of AST in Friesian steers supplemented with cimaterol, however, the concentration of AST was still within the range of normal healthy cattle (Chikhou et al., 1993). There were treatment x time interactions observed for ALP and GGT. Normal reference values in cattle for ALP concentrations are 78-132 U/L (Kaneko et al., 2008). Ricks et al. (1984) reported no abnormalities in the liver enzyme ALP in Hereford steers administered clenbuterol. Even though there were alterations in some liver enzyme concentrations in both

previous and current studies, the concentrations of these enzymes were within the biological range for healthy cattle.

During times of stress, serum cortisol concentrations increase to help the body respond to the stressor by stimulating cells to produce glucose or alternative forms of energy and to increase blood flow to distribute glucose and other nutrients to cells as fast as possible (Reece et al., 2015). In the current study, serum concentrations of cortisol were not different between treatment groups. Other studies have reported similar results in different species supplemented with β-AAs (Marchant- Forde et al., 2003; Li et al., 2000). Brukmaier et al. (1992) conducted research on the responses of calves to exercise (stress) during the feeding of clenbuterol and the serum concentrations of cortisol were not different between treatment groups. Thus, serum cortisol does not appear to be influenced by ZH supplementation.

The ketone body β-hydroxybutyrate is produced in the liver by metabolism of non-essential fatty acids and elevated concentrations are indicative of a negative energy balance (Ospina et al., 2010). In the current study, no difference was observed between treatment groups for β-hydroxybutyrate. Eisemann et al. (1988) reported no differences in β-hydroxybutyrate concentrations in steers supplemented with clenbuterol. In addition, Bibber-Krueger et al. (2015) observed no difference in β-hydroxybutyrate concentrations for steers supplemented with ZH. These observations could suggest that β-hydroxybutyrate is being metabolized efficiently enough to avoid being accumulating in the blood (Bibber-Krueger et al., 2015). When cells have a high demand for glucose, lactate is produced by the oxidation of NADH by pyruvate to encourage the continuation of glycolysis to occur (Reece et al., 2015). In the current study, there were no observed differences for serum concentrations of lactate. Previous studies have reported similar results with the supplementation of ZH (Bibber-Krueger et al., 2015; Thomson et al., 2015).

Bibber-Krueger et al. (2015) observed that as days on ZH supplementation increased, the concentrations of lactate numerically decreased. However, the aforementioned study did not include a withdrawal period as in the current study. Previous studies have reported increases in lactate concentrations. Eisemann et al. (1988) observed an increase in lactate concentrations when steers were administered clenbuterol. Another study incorporated P-5369 and Q-2636 into milk replacer for calves and reported similar findings where lactate increased in the β-AA treated group. Differences between this study and others could be explained by measuring during the 3 d withdrawal period only whereas other studies measured during supplementation of the β-AA. Lactate dehydrogenase (LDH) is the glycolytic enzyme that is released into plasma as a result of muscle injury or necrosis (Russell and Roussel, 2007). The biological reference range in healthy cattle for LDH concentrations is 692-1445 U/ L (Kaneko et al., 2008). There was no difference in serum concentrations of LDH in the current study. A case study in horses administered ZH observed increased concentrations of LDH (Wagner et al., 2008). This observation could possibly be explained by differences in species since administration of ZH is not approved to be utilized in horses. Vitamin E is contained in cell membranes and acts as the last line of defense towards lipid oxidation of the membranes (Kaneko et al., 2008). The presence of vitamin E allows cells to replace damaged membrane lipids via normal cell turnover. In the current study, no differences were observed in the serum concentrations of vitamin E in both the serum and the tissue samples. To the knowledge of the authors of the current manuscript, there are no known data on the effect of β-AA's on concentrations of vitamin E.

Evaluation of complete blood cell count (CBC) was measured throughout the duration of the study. Complete blood cell count is used to assess the overall health of the animal and can suggest a potential illness when physical examinations results are vague (Jones and Allison,

2007). No significant differences were observed in the variables included in the CBC except for hematocrit. In the current study, ZH heifers had greater hematocrit when compared to CON heifers. Greater packed cell volume or hematocrit values can be the result of dehydration, asphyxia, or excitement that causes the release of erythrocytes concentrated in the spleen (Reece et al., 2015). While there was a treatment effect on hematocrit, both groups were still well within the normal biological range for healthy cattle. The normal reference range for hematocrit is 24-46% (Jones and Allison, 2007) and the ZH heifers for the current study were 34.1% overall.

In conclusion, there were some alterations in serum metabolite concentrations that were primarily due to the increase in muscle mass that is associated with the supplementation of β-AAs. Additionally, there were alterations in serum concentrations of glucose, however, both treatment groups were above the normal reference range of cattle. Overall the supplementation of ZH had no detrimental effects on the metabolic and blood chemistry of feedlot heifers.

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Table 1. Composition of finishing diets fed to control (CON) and zilpaterol (ZH) heifers as a percent of DM basis during 20 supplementation with a 3-d withdrawal.

	Treatment	
Item	**Control**	**ZIL**
Ingredient (%)		
High Moisture Corn	51.00	51.00
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

[1]Heifers receiving zilpaterol hydrochloride for 20-d period with 3-d withdrawal
[2]Sweet Bran, (Cargill Corn Milling; Blair, NE)
[3]Elanco Animal Health; Greenfield, IN
[4]The 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. Mean Complete Serum Profile values for heifers fed Zilpaterol Hydrochloride (ZH) compared to control (CON) heifers for the each blood collection period and overall for the duration of trial.

Variables	Period 1 (d -2-4)		SEM	P-Value	Period 2 (d 13-16)		SEM	P-Value	Period 3 (d 21-23)		SEM	P-Value	Overall (d-2-23)		SEM	P-Value
	CON[1]	ZIL[2]			CON[1]	ZIL[2]			CON[1]	ZIL[2]			CON[1]	ZIL[2]		
Total Protein (g/dL)	7.19	7.19	0.09	0.99	7.07	7.27	0.14	0.32	7.36	7.38	0.11	0.88	7.2	7.3	0.1	0.58
Albumin (g/dL)	3.56	3.57	0.05	0.85	3.43	3.43	0.07	0.98	3.52	3.39	0.06	0.15	3.5	3.5	0.05	0.71
Globulin (g/dL)	3.63	3.62	0.12	0.94	3.65	3.83	0.14	0.36	3.84	3.99	0.14	0.44	3.7	3.8	0.11	0.54
Calcium (mg/dL)[3]	9.9	9.6	0.07	0.005	9.75	9.46	0.09	0.03	9.82	9.52	0.9	0.04	9.8	9.5	0.07	0.008
Phosphorus (mg/dL)	6.15	6.04	0.18	0.65	6.09	6.27	0.17	0.45	6.21	6.19	0.22	0.97	6.16	6.13	0.15	0.9
Sodium (mmol/dL)	139.7	139.7	0.36	0.88	137.2	139.6	0.59	0.01	139	138.7	0.46	0.65	138.8	139.5	0.39	0.21
Potassium (mmol/dL)	3.97	3.92	0.02	0.18	3.95	3.89	0.02	0.14	4.03	3.99	0.04	0.4	3.97	3.93	0.02	0.16
Bicarbonate (mmol/dL)	25.4	25.7	0.3	0.55	24.7	25.6	0.22	0.01	24.9	25.9	0.32	0.03	25.1	25.8	0.23	0.03
Anion Gap (mmol/dL)[4]	19.9	20	0.21	0.68	20	19.7	0.34	0.56	20.2	19.4	0.34	0.11	20	19.7	0.24	0.39
Na:K	35.3	35.8	0.28	0.19	34.9	35.9	0.25	0.009	34.6	34.9	0.43	0.61	35	35.6	0.25	0.11
Aspartate Transaminase[5]	94.4	101.5	7.67	0.51	112.6	113.2	8.29	0.96	116	107.4	10.6	0.56	104.8	106.9	6.72	0.83
Sorbitol Dehydrogenase[5]	27.5	29.9	5	0.74	41.2	36.1	5.3	0.49	40	31.4	5.48	0.26	34.8	31.9	4.27	0.64
Cortisol (ng/mL)	8.06	7.18	0.6	0.31	8.81	6.96	0.87	0.14	7.58	6.21	0.79	0.21	8.13	6.98	0.6	0.19
Insulin (ng/mL)	0.95	1.02	0.12	0.69	0.66	0.47	0.08	0.12	0.77	0.67	0.11	0.52	0.82	0.76	0.09	0.62
Vitamin E (ppm)[6]									1.74	2.17	0.28	0.28				
β-Hydroxybutyrate (ng/μL)[6]									9.8	10.21	0.43	0.49				
Lactate (ng/μL)[6]									13.45	13.28	0.46	0.78				
Lactate Dehydrogenase (milliunits/mL)[6]									637	714.8	36.9	0.14				

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

[2] Total Calcium, which encompasses ionized calcium, protein bound calcium, and calcium complexed with anions

[3] Anion Gap calculated as: $([Na^+] + [K^+]) – ([CL^-] + [HCO_3^-])$

[4] Serum sodium-potassium ratio

[5] Unit of measurement for all liver enzymes is units per liter (U/L)

[6] Only measured during Period 3 (mandatory withdrawal period associate with supplementation of Zilpaterol Hydrochloride)

Table 3. Mean Complete Blood Cell Count values of heifers supplemented with Zilpaterol Hydrochloride (ZH) and control (CON) heifers for each blood collection period and for the overall duration of the entire trial.

Variables	Period 1 (d -2-4)		SEM	P-Value	Period 2 (d 13-16)		SEM	P-Value	Period 3 (d 21-23)		SEM	P-Value	Overall (d-2-23)		SEM	P-Value
	CON[1]	ZIL[2]			CON[1]	ZIL[2]			CON[1]	ZIL[2]			CON[1]	ZIL[2]		
Red Blood Cells (mil/µL)	6.51	6.61	0.28	0.79	6.15	6.56	0.21	0.17	6.24	6.5	0.25	0.47	6.34	6.54	0.22	0.53
Platelets (K/µL)	297.3	319	30.7	0.61	303.3	301	25.1	0.95	280	311.8	28.2	0.43	294.1	317.3	23.2	0.49
White Blood Cells (K/µL)	9.79	9.74	0.5	0.94	9.4	9.53	0.64	0.89	8.25	9.39	0.64	0.22	9.28	9.61	0.49	0.64
Neutrophils (K/µL)	1.18	1.53	0.3	0.41	1.22	1.46	0.29	0.56	0.78	1.21	0.41	0.46	1.09	1.42	0.22	0.29
%	12	14.8	2.81	0.48	12.5	14.8	2.65	0.55	8.77	11.9	3.65	0.55	11.4	14	1.97	0.34
Lymphocytes (K/µL)	6.54	6.06	0.4	0.4	5.97	5.83	0.49	0.84	5.33	6.09	0.52	0.3	6.1	6.01	0.33	0.84
%	66.6	61.2	2.72	0.19	64.7	61.6	2.62	0.42	64.8	65.8	3.46	0.85	65.8	62.4	2.06	0.27
Monocytes (K/µL)	1.88	1.99	0.16	0.64	1.66	1.67	0.18	0.98	1.85	1.74	0.16	0.62	1.81	1.84	0.4	0.89
%	19.2	20.1	1.49	0.66	18.1	17.5	1.57	0.78	22.4	18.6	1.37	0.06	19.7	19	1.24	0.7
Hemoglobin (g/dL)	11	11.5	0.32	0.24	10.5	11.4	0.2	0.003	10.8	11.4	0.24	0.09	10.8	11.4	0.23	0.06
Hematocrit (%)	32.1	34.3	0.97	0.12	30.7	34.4	0.6	0.0004	31.1	33.8	0.76	0.02	31.5	34.1	0.69	0.02

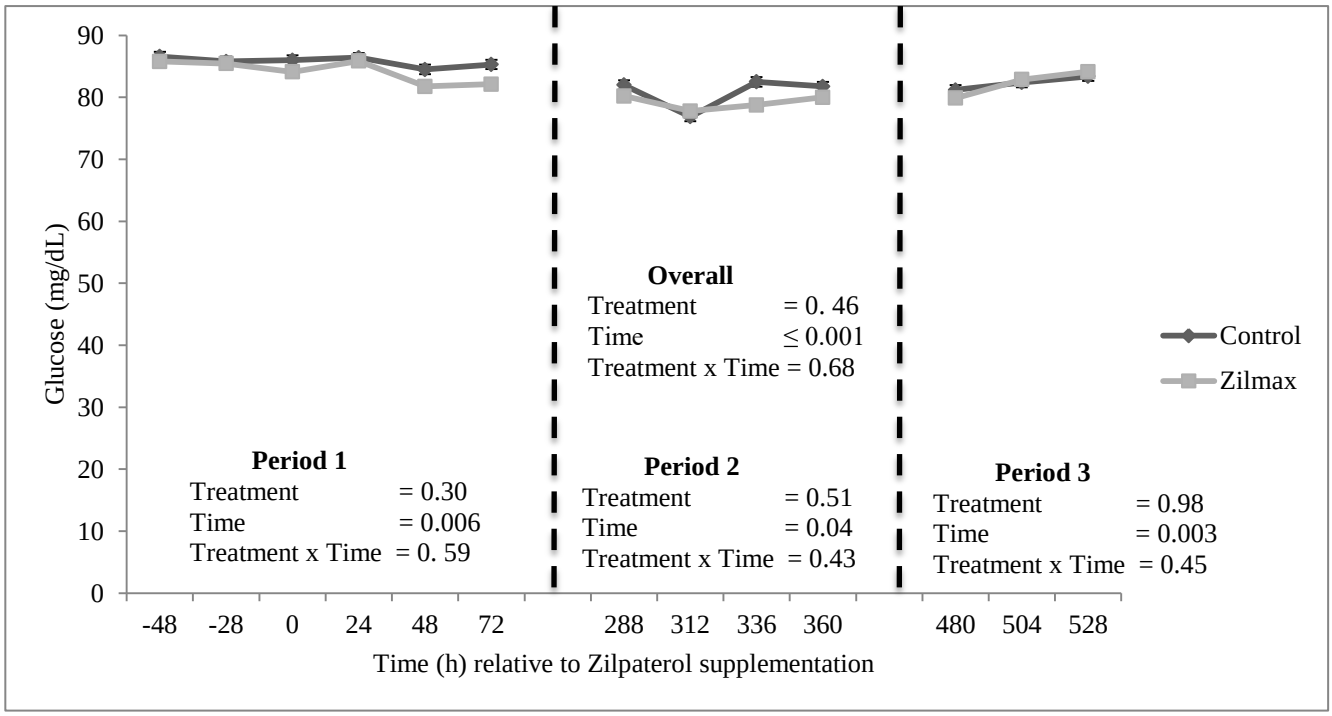
[1] Heifers receiving zilpaterol hydrochloride.

Table 4. Effect of zilpaterol hydrochloride (ZH) supplementation (8.33 mg/kg on a DM basis) on vitamin E in tissue samples following 20-d ZH supplementation.

	Treatment			
Item	**CON**	**ZIL[1]**	**SEM**	**P-value**
Liver	2.56	2.14	0.27	0.28
Longissimus	0.21	0.32	0.09	0.44
Biceps Femoris	0.42	0.32	0.12	0.44

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

(A)



(B)

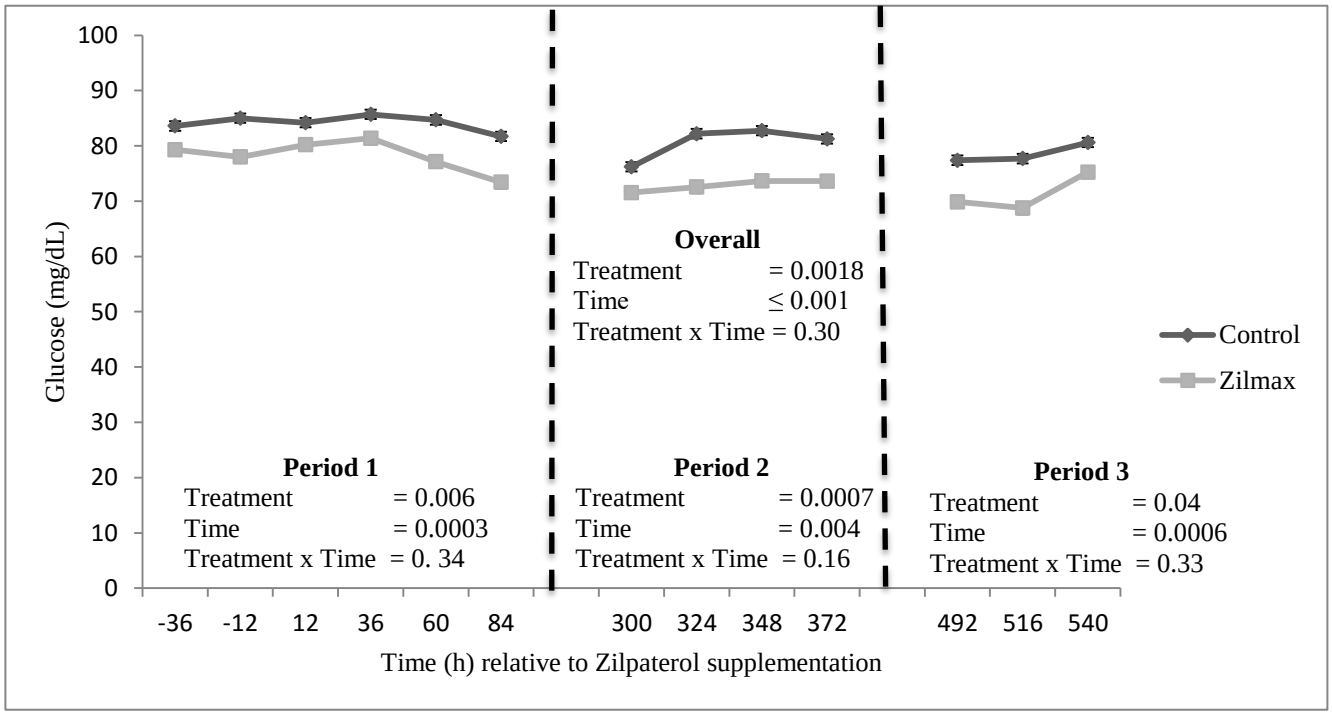


Figure 1. Glucose concentrations during the 20 d supplementation and 3 d withdrawal period of heifers supplemented with Zilpaterol Hydrochloride (ZH) and control (CON) heifers at (A) 0800 and (B) 2000 h

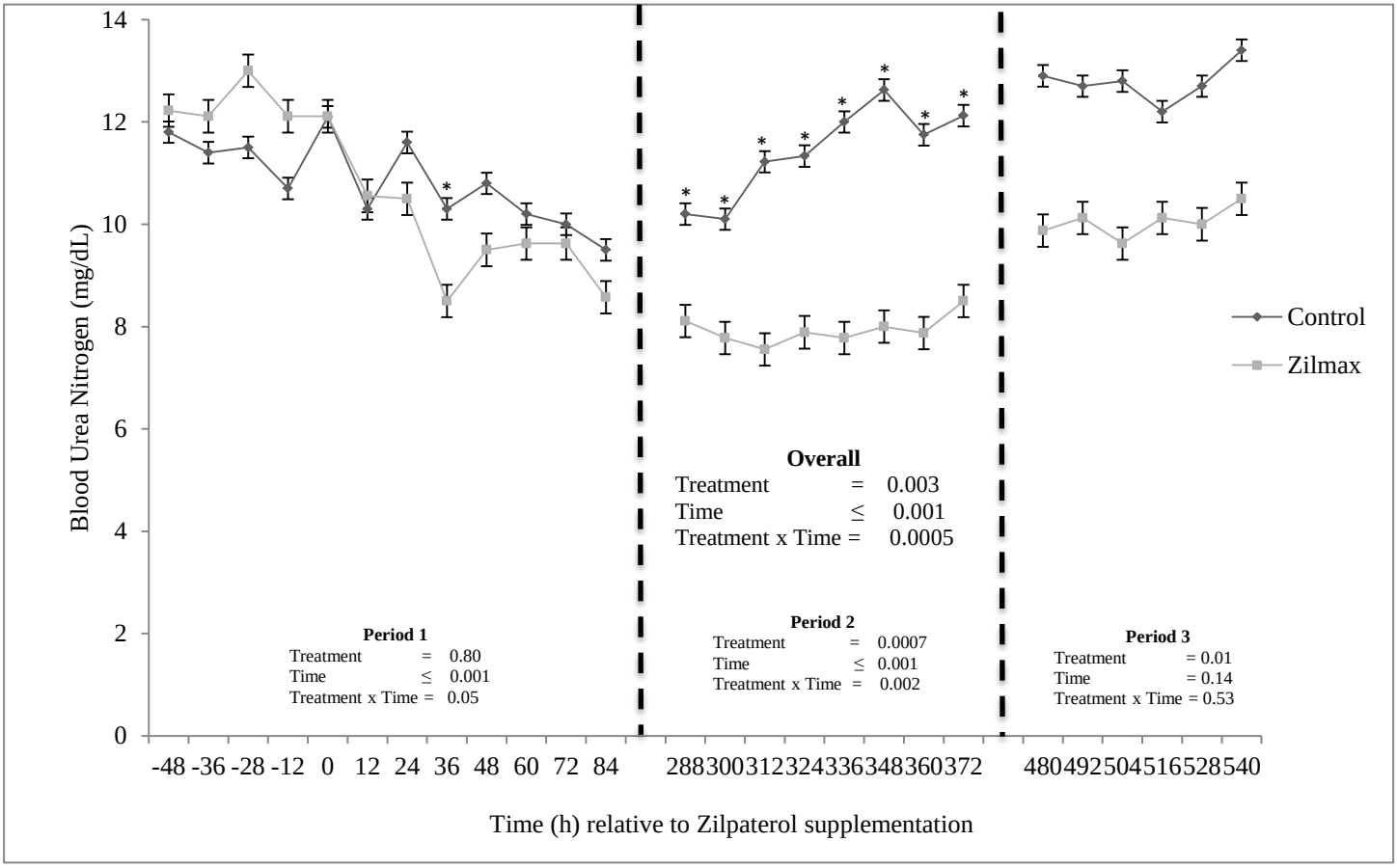


Figure 2. Blood urea nitrogen concentrations during the 20 d supplementation and 3 d withdrawal period of heifers supplemented with Zilpaterol Hydrochloride (ZH) and control (CON) heifers ((* Treatment x Time interaction ($P < 0.05$))

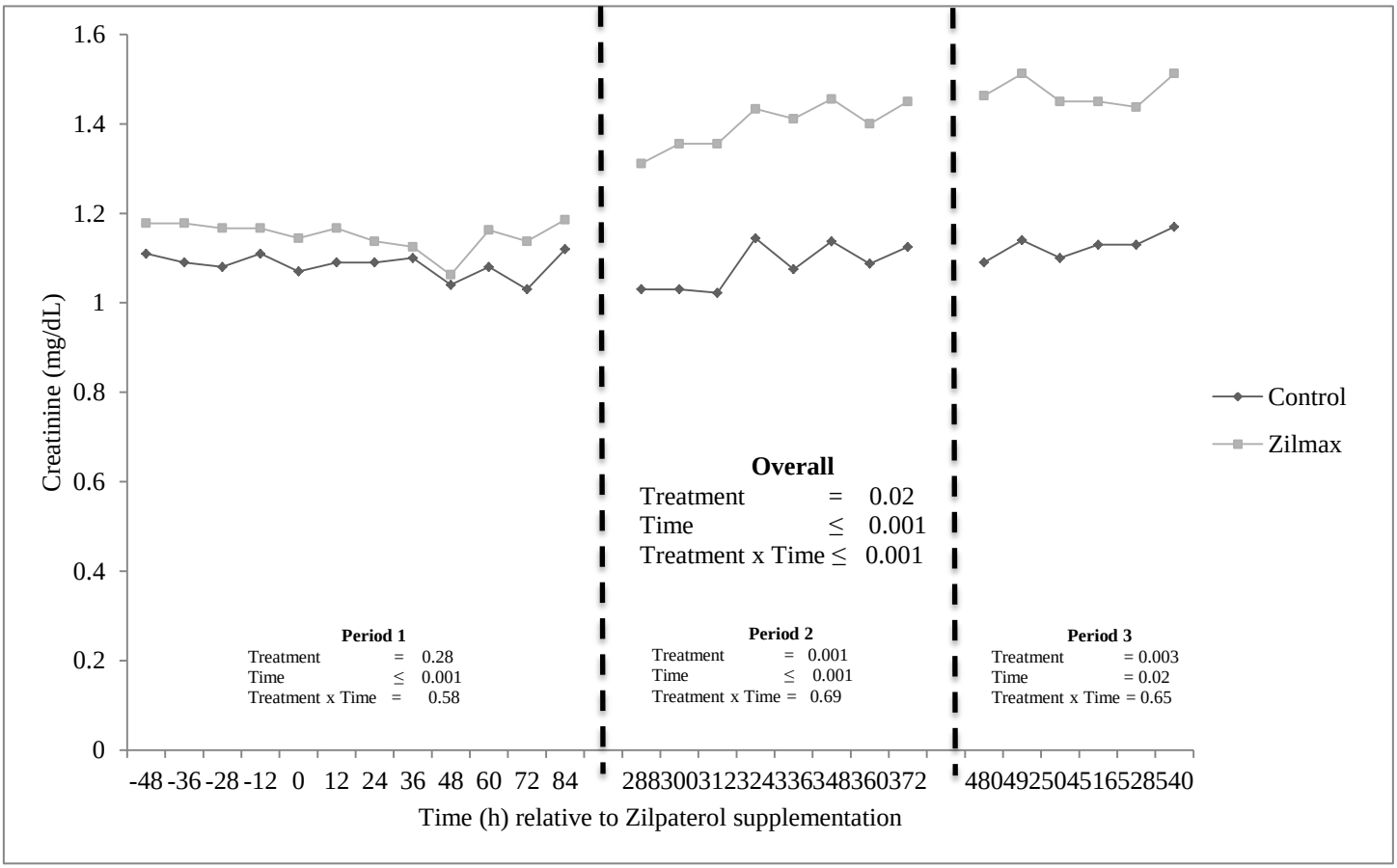


Figure 3. Creatinine concentrations during the 20 d supplementation and 3 d withdrawal period of heifers supplemented with Zilpaterol Hydrochloride (ZH) and control (CON) heifers.

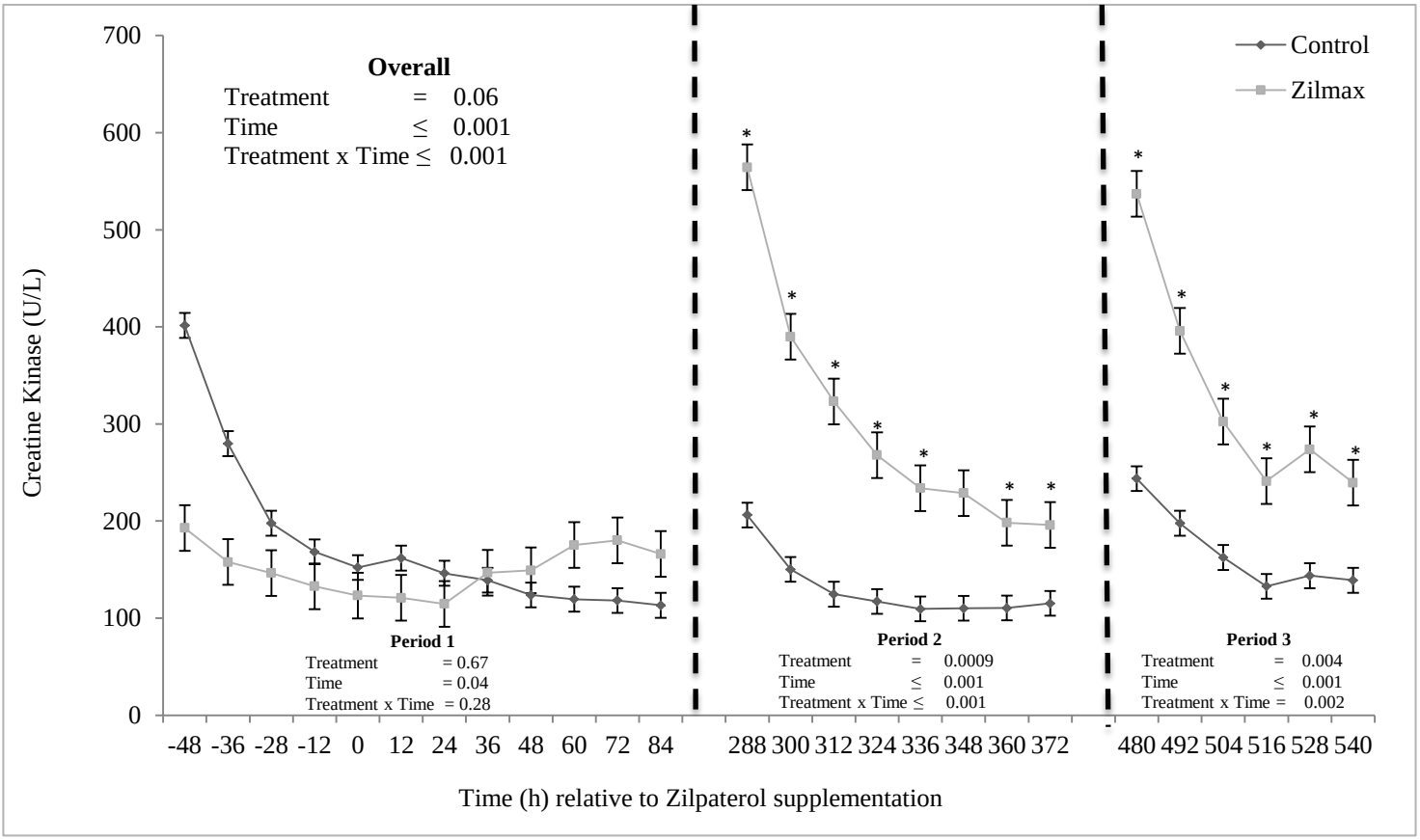


Figure 4. Creatine kinase concentrations during the 20 d supplementation and 3 d withdrawal period of heifers supplemented with Zilpaterol Hydrochloride (ZH) and control (CON) heifers. ((* Treatment x Time interaction ($P < 0.05$))

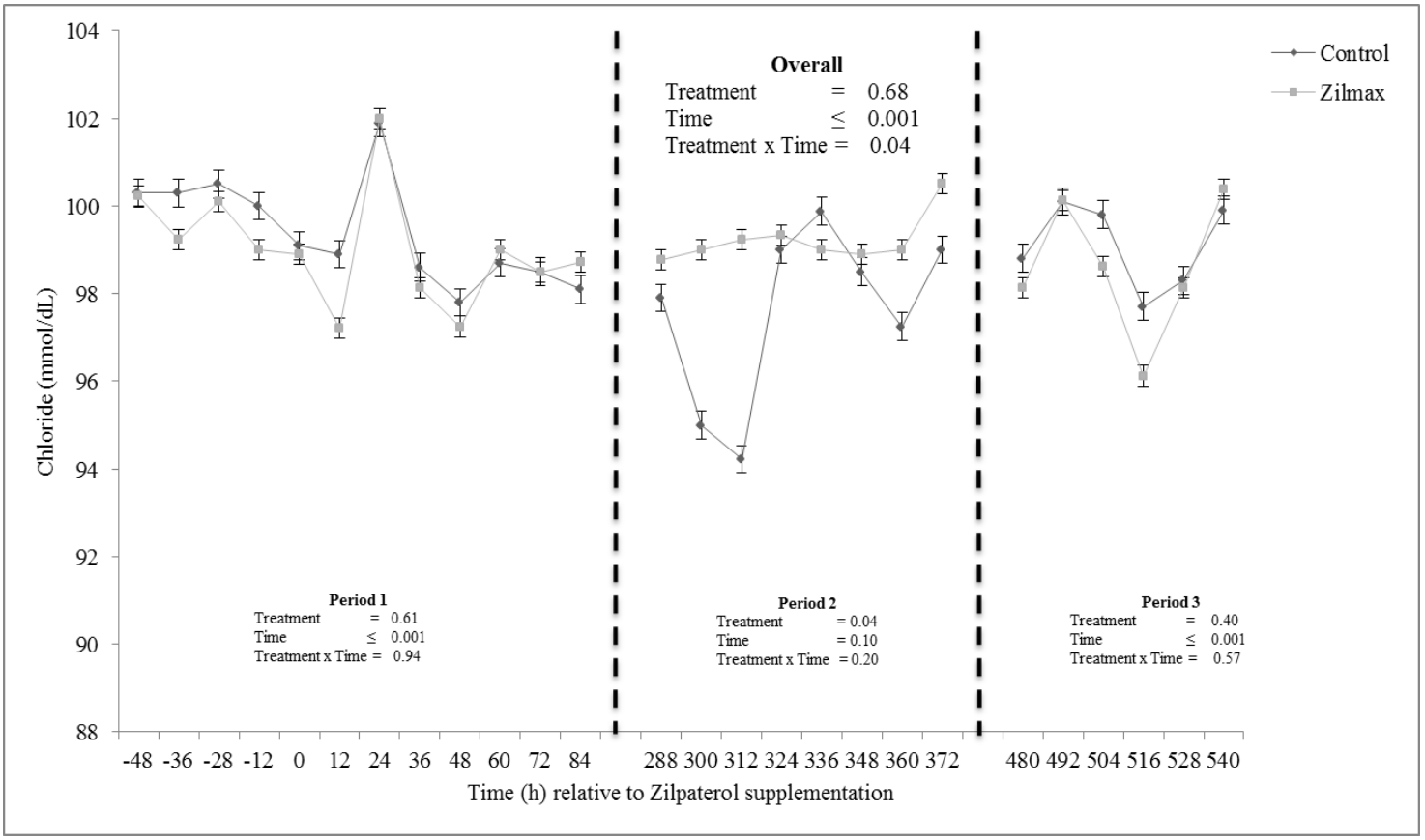


Figure 5. Chloride concentrations during the 20 d supplementation and 3 d withdrawal period of heifers supplemented with Zilpaterol Hydrochloride (ZH) and control (CON) heifers.

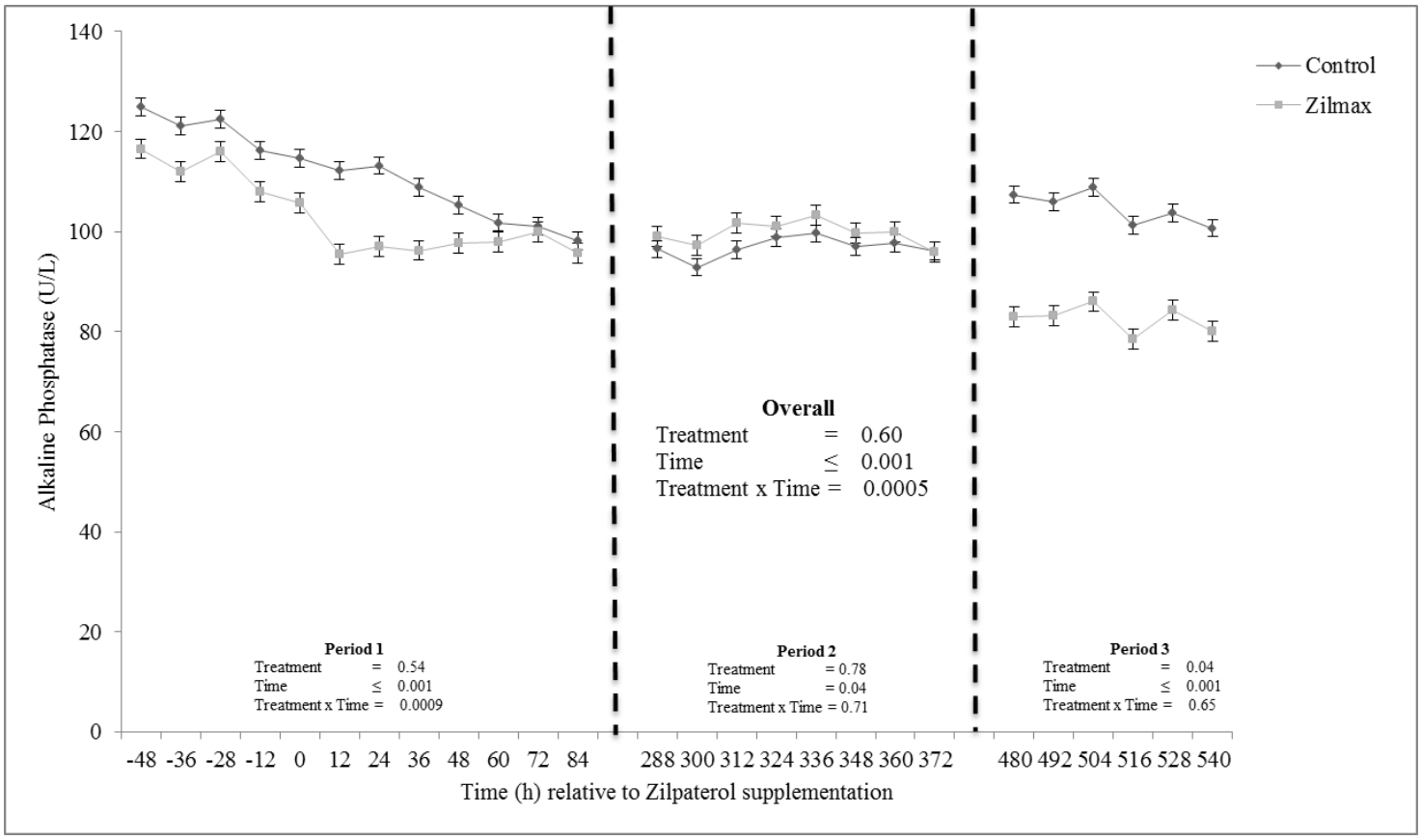


Figure 6. Alkaline Phosphatase concentrations during the 20 d supplementation and 3 d withdrawal period of heifers supplemented with Zilpaterol Hydrochloride (ZH) and control (CON) heifers.

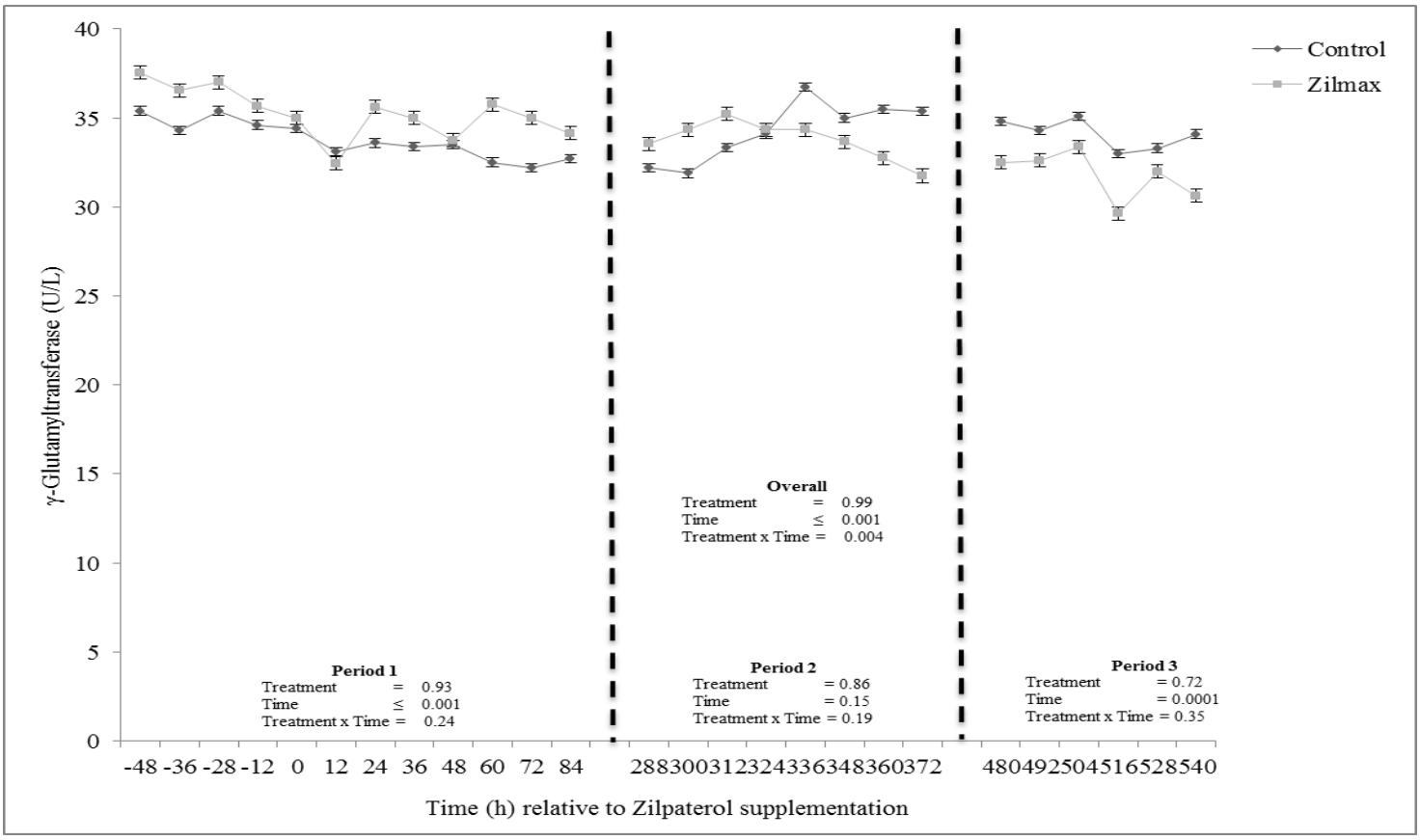


Figure 7. γ- Glutamyltransferase concentrations during the 20 d supplementation and 3 d withdrawal period of heifers supplemented with Zilpaterol Hydrochloride (ZH) and control (CON) heifers.

Suggestions for Future Investigation

With any scientific endeavor, further investigation and questions arise based upon the results for the study. There are a few other avenues of research that could be pursued in relation to the current study. A reason to potentially pursue further investigation would be to gain a better understanding of how the responses observed with β_2-adrenergic agonists are being achieved in the body. Fully understanding the mechanism will give better insight to develop a supplement that could be accepted by the industry in the future.

If Zilmax® would be accepted again, it would be valuable to look at heat stress in cattle supplemented ZH. Ultimately, these heifers were not exposed to stresses like they would be in a feedlot. In the current study, heifers were maintained in a controlled environment. A controlled heat stress could give more insight to if the supplementation of ZH is detrimental to the animal's well-being. Furthermore, cattle supplemented ZH in a feedlot might be valuable as well since in the current study they were maintained in a barn in individual stalls. A big issue with this idea is it would be difficult to collect frequent samples in a feedlot.

Appendices

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 220µl 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 220µl 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 100µl of water to generate a 10mM solution. Mix well via pipetting, then aliquot and store, protected from light, at -20°C.

2. Dilute 10µl of the 10mM β-Hydroxybutyrate Standard Solution with 90µl β-Hydroxybutyrate Assay Buffer to prepare a 1mM standard solution.

3. For 2 plates mix 50µl of the 10mM β-Hydroxybutyrate Standard Solution with 450µl of the β-Hydroxybutyrate Assay Buffer.

4. In 0.6 ml tubes make up the standards according to the following chart:

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

5. Once the standards are made up in the tubes, add 50 µl if the appropriate standard into duplicate wells on the plate.

Preparation of Samples:

Add 8µl of each sample into duplicate wells on the plate.
Add 42µl of Assay Buffer to each well to get a final volume of 50 µl

Assay Reaction:

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

Reagent	Samples and Standards	Blank
β-Hydroxybutyrate Assay Buffer	46 µl	48 µl
β-Hydroxybutyrate Enzyme Mix	2 µl	-
β-Hydroxybutyrate Substrate Mix	2 µl	2 µl

For 1-96 well plate, mix:

- 4600 µl of β-Hydroxybutyrate Assay Buffer
- 200 µl of β-Hydroxybutyrate Enzyme Mix
- 200 µl of β-Hydroxybutyrate Substrate Mix

2. Add 50 µl 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.

3. 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 β-Hydroxybutyrate:

$S_a/S_v = C$
S_a = Amount of β-Hydroxybutyrate in unknown sample (nmole) from standard curve.
S_v=Sample volume (μl) added into the wells
C=Concentration of β-Hydroxybutyrate in sample

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 µl 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 µl Lactate Standard (100 nmole/µl) and dilute with 990 µl of Assay Buffer to result in 1 ml of Diluted Standard (1 nmole/µl).

2. In 0.6 ml tubes make up the standards according to the following chart:

Diluted Standard	Lactate Assay Buffer (µl)	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 µl of the appropriate standard into duplicate wells on the plate.

Preparation of Samples:

1. **Sa**mples must be deproteinized using a 10 kDaMWCO spin filter.

2. Add 500µl 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 µL	4.600 ml
Lactate Enzyme Mix	2 µL	200 µL
Lactate Probe	2 µL	200 µL

2. Add 50 µl 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 µl of Lactate Assay Buffer, mix well

3. Take 10 µl of Lactate Standard and reconstitute with 990 µl of Assay Buffer to get your 1 ml of Diluted Standard (1nmole/µl).

4. Prepare standards according to the previous chart. (This should give enough standard for 2 plates)

5. Place 50 µl of each standard into duplicate wells on the plate. (Do not dilute with assay buffer).

6. Add 3 µl of sample to each duplicate well.

7. Add 47 µl 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 x 89.07 ng/nmole = ng/µL

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

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 µL 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 (µl)	LDH Assay Buffer (µl)	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 µL of the appropriate standard into duplicate wells on the plate.

Preparation of Samples:

1. Dilute samples in tubes off of the plate :
   - 2 µL 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 $(A_{450})_{initial}$. Note: It is essential $(A_{450})_{initial}$ is in the linear range of the standard curve.

4. Incubate the plate at 37C 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 (12.5nmole/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 $[(A_{450})_{final}\}$ 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.