USE OF EXOGENOUS GROWTH PROMOTANTS IN FINISHING CATTLE

by

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B.S., Colorado State University, 2007

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Animal Sciences and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2014

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Abstract

Exogenous growth promotants, such as the synthetic beta agonist zilpaterol hydrochloride (ZH), have been shown to increase carcass weight by repartitioning energy toward increased skeletal muscle at the expense of adipose tissue, which is associated with a decline in tenderness. More recently, essential oils such as menthol have been observed to have growth promoting properties in livestock. The objectives of this research were to determine effects of ZH on blood parameters and long chain fatty acids in plasma and adipose tissue, to determine if the decline in tenderness can be negated by temporary depletion of calcium during ZH supplementation, and to determine effects of crystalline menthol on blood parameters. Blood samples were collected in 7-d intervals during ZH administration. Zilpaterol hydrochloride decreased concentrations of plasma urea nitrogen and whole blood glucose ($P < 0.10$), but had no effects on concentrations of plasma glucose, lactate, beta-hydroxybutyrate, NEFA, or whole blood lactate ($P > 0.10$). Total long chain fatty acids of plasma and adipose tissue were unaffected ($P > 0.10$); however, ZH supplementation increased HCW, dressing percentage, and LM area ($P < 0.10$). Calcium was temporarily depleted during ZH supplementation in an attempt to increase tenderness of meat. No differences ($P > 0.10$) were observed for Warner-Bratzler shear force values, live animal performance, or carcass measurements. Addition of 0, 0.003, 0.03, 0.3% menthol (diet DM) to diets of steers resulted in a menthol × time within day interaction ($P < 0.01$) for IGF-1 concentration and BW; however, glucose, lactate, and PUN concentrations were unaffected ($P > 0.05$). Furthermore, concentrations of VFA were not different ($P > 0.05$), but production of fermentative gas was decreased ($P < 0.01$) when menthol was added at 0, 0.003, 0.03, 0.3% of substrate DM in a 24 h *in vitro* fermentation trial. Results from these studies suggest ZH improved efficiency of nutrient utilization for increased skeletal muscle growth; however, the decline in tenderness was not negated by the temporary depletion of calcium in the diet. Overall, ZH affected components related to increased skeletal muscle growth, but menthol did not affect blood parameters associated with growth.

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Acknowledgements

There are many individuals who I would like to thank that helped make this thesis possible. I would like to thank Dr. Jim Drouillard for serving as my major professor and allowing me to have the opportunity to earn my masters. I would also like to thank Dr. Larry Hollis and Dr. Terry Houser for serving on my committee. Their willingness to answer any question I may have had was invaluable.

I would also like to thank Cheryl Armendariz for her assistance in the laboratory. Without Cheryl's help to guide me through the laboratory analyses would have made obtaining my masters much more difficult. I would like to thank the managers and student employees of the Beef Cattle Research Center for their help in conducting my research. I am also very appreciative of all the help I received from my fellow graduate students, both still here at Kansas State University as well as the ones who have moved on in their careers. Completing my research would not have been possible without all of their assistance those cold early mornings. I am also very thankful for all the wonderful people I have met here at Kansas State University that have made my masters program enjoyable.

Above all, I would like to thank my husband for his continued advice, and support through everything I set out to accomplish. I would like to thank my family for their continued love and support. In particular, I would like to thank my parents John and Christy for always giving me the courage to shoot for the stars in anything I choose to do. Their love and encouragement has given me the strength I needed to complete my program. My brothers Noah, Nathaniel, Jake, and Caleb you helped to keep me grounded and to not take life so seriously. My sister Jessa you were always the light that kept shining no matter what. I would also like to thank my sister-in law Shannon, Cody, Chad, Eric, Callie, Krystal, and Christina for their continued support and friendship.

Dedication

I would like to dedicate this thesis to my Lord and Savior Jesus Christ. Without His strength I would not have been able to endure the hardships and trials encountered.

The Lord is my shepherd, I shall not be in want. He makes me lie down in green pastures, He leads me beside quiet waters, He restores my soul.

Psalm 23:1-3

Chapter 1 - Literature Review: Effects of β-Adrenergic Agonists on the Digestive, Respiratory, Endocrine, and Muscular Systems of Ruminants

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Abstract

Beta agonists have been utilized in research trials with livestock since the 1980's as repartitioning agents, increasing the deposition of lean tissue in comparison to adipose tissue. In mammalian tissue, 3 subtypes of beta-adrenergic receptors (β-AR) exist: β_1-AR, β_2-AR, and β_3-AR. Different tissues within animals vary with respect to different distributions of β-AR, and therefore respond differently with administration of β-adrenergic agonists (β-AA). For example, the heart and digestive tract contain greater proportions of β_1-AR relative to β_2-AR or β_3-AR, whereas lung and skeletal muscle contain greater proportions of β_2-AR, and adipose tissue contains primarily β_3-AR. Due to the wide range of tissues containing β-AR, different systems within the body of ruminants are affected with β-AA administration. Calves treated with different β-AA and had respiratory parameters measured. In treated calves there was an initial increase in respiration rate with oral administration of β-AA; however, this effect was diminished with chronic exposure. Lambs treated with terbutaline had higher liquid clearance from their lungs. The digestive system has not responded consistently to β-AA administration. In some instances contractile activity is unchanged, whereas in other studies smooth muscle contraction is altered. Fermentation responses within the rumen microorganisms varied between trials suggesting rumen microorganisms respond differently to different β-AA. Furthermore, carcass composition is altered with administration of β-AA. In general, an increase in lean tissue mass is observed while adipose tissue is either decreased or not affected depending on type of β-AA administered. Differences observed in the literature may be due to many factors which include: species, age of animal, weight of the animal, and/or genetics, β-AA administered, dosage of β-AA, duration of treatment. All of which make comparing β-AA, and trying to quantify physiological responses difficult.
Key words: beta-adrenergic agonists, beta-adrenergic receptors, ruminants

Introduction

Adrenergic receptors (AR) are membrane-bound receptors located on the cell surface of most cells throughout the body. Naturally occurring catecholamines (i.e. epinephrine, and

norepinephrine) and sympathomimetic drugs exert their physiological effects by binding these receptors (Badino et al., 2005). In the late 1940's, AR were characterized into two types, α- and β-adrenergic receptors, based on excitatory or inhibitory responses to catecholamines (reviewed by Badino et al., 2005). Primary responses observed during alpha adrenergic receptor activation include maintenance of vascular tone, blood pressure, glucose homeostasis, and contraction of vascular smooth muscle (Badino et al., 2005). Due to the diversity of cells in which receptors are located, every body system is affected with beta-adrenergic agonist (β-AA) administration. Three β-receptor subtypes have been identified: β_1, β_2, and β_3-AR, and distribution of subtypes varies among tissues. For example, the heart contains primarily β_1-AR, whereas skeletal muscle contains more β_2-AR, and adipose tissue contains predominantly $ß_3$-AR; therefore, the effect of each agonist varies depending on which β-AR is affected (Badino et al., 2005). Distributions of receptor subtypes also vary among species, making it difficult to extrapolate information from humans to livestock (Badino et al., 2005). Beta-adrenergic agonists administered to livestock are orally active, and general responses observed are: 1) increased ADG; 2) improved feed efficiency; 3) decreased adipose tissue; and 4) increased dressing percent (Moody et al., 2000). Although responses within the muscular system are most commonly studied, other organ systems within treated animals are affected by oral administration of β-AA. The purpose of this review is to summarize effects of β-AA on digestive, respiratory, endocrine, and muscular systems of ruminants.

Beta-Receptor classification and activation

In 1948, Ahlquist discovered 2 types of receptors on smooth muscle for which catecholamines, such as epinephrine and norepinephrine, exerted excitatory or inhibitory effects. Classification of the receptors was based on potency of different agonists in stimulating responses (Stiles et al., 1984). These receptors were classified as α- or β-receptors (Badino et al., 2005). In 1967, β-ARs were further subdivided into β_1 or β_2 subtypes based on their affinity for epinephrine or norepinephrine. Beta_1 receptors, commonly found in cardiac tissue, had equal affinities for both epinephrine and norepinephrine, whereas β_2 receptors, found in the trachea and vasculature, had greater affinity for epinephrine compared to norepinephrine. Additionally, both receptor subtypes can exist in the same organ or tissue (Badino et al., 2005). In 1989, another β-

AR was identified leading to designation of the β_3-AR subtype for which norepinephrine is more stimulatory than epinephrine (Emorine et al., 1989). Distribution of receptor subtypes varies greatly among tissue types and animal species. Beta$_2$ receptors found in the smooth muscle of lungs are responsible for relaxation, conferring an important role of β_2-AA for control of asthma in humans. Agonists primarily affecting β_1 and β_2 receptors have been used in human health for the treatment of cardiac inotropic support, asthma, and as uterine relaxants (Moody et al., 2000). Beta$_3$ receptors were found in rat adipose tissue, and most likely are involved with regulation of lipolysis and body temperature (Badino et al., 2005).

The different classifications of β-AR have been confirmed with ligand binding studies. In 1974, the first successful radioactively labeled β-antagonist was evaluated (Stiles et al., 1984). A summary of common radioligands used for β-receptor studies is found in table 1.1. Researchers use antagonist radioligands to study adrenergic receptor abundance and properties, and agonist radioligands for researching mechanisms of action of agonist attachment to adrenergic receptors (Stiles et al, 1984). Additionally, researchers have been able to study the differences in receptor subtype. Use of β_1 or β_2 specific ligands has made it possible to accurately assess relative proportions of receptor types within a given tissue.

Binding of agonists to β-AR located on cell surfaces causes a cascade of events to occur. First, binding of agonist to receptor stimulates G-proteins to activate adenylate cyclase. Adenylate cyclase then synthesizes cAMP, the intracellular messenger for receptors (Mersmann, 2002). Different mechanisms of action occur depending on tissue type after synthesis of cAMP. In general, cAMP binds to the regulatory unit of protein kinase A to release its catalytic subunit (Moody et al., 2000). Phosphorylation of protein kinase A regulates cellular enzymes, including hormone-sensitive lipases and lipogenic enzymes (Moody et al., 2000). The cAMP response binding protein (CREB) is also phosphorylated by protein kinase A and initiates transcription for a number of genes in mammalian tissues (Mersmann, 1998). Phosphorylation causes some enzymes, including the rate limiting enzyme for long-chain fatty acid biosynthesis, acetyl-CoA carboxylase, to become inactive (Mersmann, 1998). Examples of physiological effects include regulation of heart rate and blood pressure, bronchial muscle contraction or relaxation, uterine contractions or relaxation, and in the case of food animals, increased skeletal muscle accretion. Due to the wide distribution of β-AR in mammalian tissues, effects observed *in vivo* may be

primary and secondary in nature, caused by β-AA binding to receptors in one type of tissue or in combination with binding of receptors in another tissue (Mersmann, 1998). For example, direct effects of β-AA on skeletal muscle may be augmented through indirect effects on vasculature that increase blood flow to skeletal muscle, thus making more nutrients available to support muscle accretion.

Chronic exposure to β-AA causes cells to become desensitized, thus decreasing response to β-AA. Different mechanisms for desensitization can occur: functional uncoupling of receptors, physical sequestration of receptors away from the cell surface, and down regulation of the number of receptors (Badino, 2005). The time frame over which this can occur is seconds to hours after exposure; however, response to β-AA administration is not eliminated completely.

Respiratory system

Beta-agonists have been used for many years in treatment of human asthma, and effects on respiratory function have been extensively researched. $Beta_2$ receptors are the predominant receptor involved in bronchodilation, whereas cardiac effects are mediated through β_1 receptors. $Beta_2$ agonists were introduced in the 1960's as a means of controlling asthma without the common side effect of increased heart rate (Barnes, 1988). The primary benefits of treating asthma using β_2-agonists are bronchodilation (Barnes, 1997) and production of surfactant by mucous cells lining airway smooth muscle (Enhoning, 2008). In the early 1980's, synthetic β-agonists made their entry into livestock research trials as growth stimulants. Ricks et al. (1984) were among the first to administer synthetic β-AA to livestock and observe their effects on carcass composition in several meat animal species including cattle (Mersmann, 2002). The effect on body composition has been extensively researched (Ricks et al., 1984; Beermann et al., 1987; Avendaño-Reyes et al., 2006); however, effects of β-AA on the respiratory system in cattle is poorly understood.

Beta-agonist treatment caused respiration rate to increase in cattle. Blum and Flueckiger (1988) administered P-5369 and Q-2636 orally in milk replacer of calves and observed an increased respiration rate at h 5, 6.5, and 8 with addition of P-5369 ($P < 0.05$), and h 3.5, 6.5, and 8 with Q-2636 ($P < 0.05$) compared to control counterparts. Similarly, Zimmerli and Blum (1990) fed the β-AA T-3660 (group T) and P-5369 (group P) to calves and observed 50 to 60%

rise in respiration rate on d 0 and 28 for calves in group T ($P < 0.05$) compared to control calves, but calves in group P were not affected ($P > 0.05$). Bruckmaier and Blum (1992) observed increased respiration rates in calves treated with clenbuterol before during, and, after exercise on d 1, 14, and 35 ($P \leq 0.05$) when compared to calves with no β-AA. Substance P-5369 was later found to be clenbuterol, and was classified as primarily a β_2-agonist (Blum and Flueckiger, 1988). Substances Q-2636 and T-3660 had no data available for comparable characterization, but have been shown to have β_2-agonist activity as evident by their action on respiration rate.

Respiration minute volume (RMV) was affected with oral administration of β-AA. Respiratory minute volume is the amount of new air entering the lungs each minute. Substances P-5369 and Q-2636 increased RMV in treated calves h 2 thru 8 ($P < 0.05$) when compared to control calves (Blum and Flueckiger, 1988). Correspondingly, Zimmerli and Blum (1990) fed the β-AA T-3660 and P-5369 to calves for 28 d and observed an increase in RMV of treated calves compared to control calves 6 h after administration of both β-AA ($P < 0.05$); however, after d 28 of treatment this effect persisted only in calves treated with T-3660 ($P < 0.05$). These results may indicate greater β_2-receptor activity for T-3660 compared to P-5369 due to differences on d 28 in RMV. Bruckmaier and Blum (1992) performed a study assessing respiration parameters during exercising of calves that received 0 or 25 mg/kg BW clenbuterol. Respiratory minute volume was higher in clenbuterol treated calves on d 1 and 14 up to 12 min of exercise and remained higher during recovery ($P \leq 0.05$), but was elevated for only 6 min of exercise on d 35 ($P \leq 0.05$) compared to control calves. Results from these studies suggest initial exposure to β-AA cause the greatest change in RMV, but as administration persists subtle differences were observed indicating possible tachyphylaxis. Direct effects of β-AA on lung tissue could have modulated the differences observed in respiration parameters (Blum and Flueckiger, 1988).

Berthiaume et al. (1987) performed a study using anesthetized sheep. Sheep were surgically fitted with tracheosotomy tubes to assess whether or not the β_2-agonist terbutaline could increase lung liquid clearance. Addition of terbutaline mixed with a serum solution added directly to air spaces in the lungs decreased ($P < 0.05$) water accumulation in the lungs compared to lungs of sheep instilled with serum alone. Additionally, when terbutaline was administered intravenously the amount of excess liquid measured in the lungs was less than that of serum

infusion ($P < 0.05$). These results were confirmed to be due to the action of β-AR through use of propranolol (beta blocker). The authors hypothesized that the mechanism of action was through induction of sodium transport by the sodium-potassium pump located on the interstitial side of the plasma membrane. Movement of sodium out of the lumen in the lungs caused water and chloride to passively move through the cells, following the movement of sodium.

Results of these studies lead to the conclusion that addition of β-AA does have an initial effect, causing an increase in respiration rate. Depending on β-AA administered, cells located in lungs can become desensitized. In addition, ruminants that have or have had respiratory disease problems may benefit from the addition of β-AA as suggested by the results of Berthiaume et al. (1987).

Digestive system

Naturally occurring catecholamines, norepinephrine and epinephrine, affect gut motility by increasing or decreasing motility (Leek, 2001). Phenethanolamines may act in a manner similar to that of catecholamines. Miert (1968) observed α and β-AR in the ruminal wall. Strips taken from the dorsal sac of the rumen in sheep were treated with oxymetazoline hydrochloride for α-receptor stimulation, or isoprenaline hydrochloride for β-receptor stimulation (Miert, 1968). Miert (1968) concluded α- receptors caused a stimulatory response, and β-AR caused an inhibitory response in ruminal smooth muscle.

Leek (2001) suggested $beta_1$-receptors mediate inhibition of gastric centers and β_2-receptors mediate inhibition of intrinsic and extrinsic motility of the reticulorumen. Rumenoreticular motility was analyzed in Holstein calves by Guard et al. (1988). No effect was observed ($P > 0.05$) for ruminal motility with addition of clenbuterol, indicating clenbuterol does not have inhibitory actions on forestomach motility of healthy calves. On the other hand, Ricks et al. (1984) observed a decrease in rumen fermentation ($P \leq 0.01$) with addition of 500 mg/d clenbuterol to the diet of steers compared to steers without clenbuterol. The authors suggested decreased fermentation may have been caused by decreased ruminal motility. Brikas et al. (1990) infused doputamine, a β_1-AA, intravenously to sheep and observed a decrease in frequency of reticular, ruminal, and abomasal contractions, but an increased number of contractions in the duodenum ($P < 0.05$). When the authors infused ritodrine, a β_2-AA, there was no difference on

motility in forestomach or jejunum, but the frequency at which abomasal contractions occurred was reduced ($P < 0.05$) compared to control ewes. These findings may be due to the gastro-intestinal tract containing primarily β_1-receptors (Brikas, 1989). Ritodrine and clenbuterol are primarily β_2-AA, which would explain the lack of effect on gastro-intestinal motility.

Moloney et al. (1994) measured ruminal digesta kinetics (liquid volume, ammonia, liquid outflow rate, solid outflow rate, and mean retention time of liquid and solids) to measure the impact of cimaterol, primarily a β_2-agonist, on digesta passage. Steers were fed 0 or 15 mg/kg cimaterol for a 35-d feeding period. The authors observed no differences among treatments ($P > 0.05$), leading them to conclude passage rate from the rumen was not affected by cimaterol due to the lack of effect on ruminal motility.

Beta-agonists may act directly on receptors in the gastro-intestinal tract to decrease contractions thereby increasing retention time and potentially increasing digestion (Brikas, 1989). Fiems et al. (1991) observed a decrease ($P < 0.05$) in crude fiber digestibility in sheep, and a decrease ($P < 0.05$) in crude fiber, DM, and OM digestibility in bulls when both species were fed cimaterol. These findings were attributed to an increased rate of passage for bulls fed *ad libitum*, whereas sheep were fed a limited diet, therefore decreasing the rate of passage. Similarly, Chikhou et al. (1991a) observed a decrease ($P < 0.001$) in DM digestibility, with no effect observed for crude fiber, NDF, ADF, or CP digestibility ($P > 0.05$) with addition of cimaterol to the diet of steers fed a limited diet. Walker et al. (2007) performed a study that fed 200 mg/d ractopamine for 28, 29, and 30 d to steers. The authors observed an increase in DM digestibility ($P < 0.05$) with addition of ractopamine. Furthermore, nitrogen intake, fecal nitrogen output and urinary nitrogen output decreased ($P < 0.05$) when compared to control cattle, indicating increased nitrogen retention. Increased DM digestibility is in agreement with Brikas (1989), suggesting β-AA act on β-receptors in the gastrointestinal tract to decrease contractions and increase retention time (Walker et al., 2007). López-Carlos et al. (2010) performed a study in which lambs were supplemented with 0.35, 0.70, 1.05 mg/kg BW ractopamine or 0.1, 0.2, 0.3 mg/kg BW zilpaterol. The authors observed no differences in DM, CP, ADF, or NDF digestibility ($P > 0.05$) with either β-AA. They concluded addition of β-AA to the diet of sheep does not compromise digestibility. Due to differences in amount of feed offered to animals it is difficult to extrapolate information and determine if differences in effects observed between

studies are due to β-AA treatment, rate of passage, or both; however, species differences are evident.

Ruminal fermentation was reviewed in more detail due to the important role of fermentation in the digestion process of ruminant animals, and conflicting results were observed. Inclusion of clenbuterol to diets of steers had no effect ($P > 0.05$) on ruminal fermentation when samples were collected at the time of slaughter (Ricks et al., 1984). Similarly, Moloney et al. (1994) analyzed ruminal fermentation in sheep fed *ad libitum* with 0 or 10 mg/kg diet DM cimaterol for 64 d. They observed no change in concentrations of acetate, propionate, or butyrate ($P > 0.05$); however, concentration of valerate was less ($P < 0.01$) in treated lambs. The authors regarded the decrease in valerate as unimportant due to the minor role of valerate as a fermentative end product. Moloney et al. (1994) fed a pelleted ration that contained 0 or 15 mg/kg cimaterol to steers, and intake was maintained at 15.5 g DM/kg BW fed twice daily. Ruminal fluid samples were collected on d 17 and 21 at 2, 4, 8, 12, 16, and 24 h after feeding. Addition of cimaterol to the diet caused a cimaterol × time interaction for acetate, propionate, and butyrate ($P < 0.001$), but concentration of valerate was not different from control animals ($P > 0.05$). Molar proportion of acetate was higher ($P < 0.05$) 12 h after the first feeding with addition of cimaterol, but was not different at other times. The molar proportion of propionate was higher at h 8, 12, and 16 ($P < 0.01$) in animals fed cimaterol, and the molar proportion of butyrate was lower ($P < 0.05$) in cimaterol fed steers 12 and 16 h after the first feeding. The authors concluded cimaterol had a small but beneficial effect on the concentrations of ruminal VFA. Fiems et al. (1991) conducted a trial that measured VFA content in sheep fed at maintenance level with 0 or 4 mg/kg diet DM cimaterol. Ruminal fluid samples were collected 4 consecutive days at 2.5 h after feeding. They observed increased concentrations of propionic and acetic acid, and a decreased concentration of butyric acid ($P < 0.05$) in cimaterol treated sheep. The data suggest ruminal microorganisms respond to β-AA treatment; however, the response is not consistent between studies. Differences may be due to diurnal variation in rumen fermentation, amount of feed offered, or species differences.

Walker and Drouillard (2010) conducted an experiment *in vitro* to assess impact on mixed ruminal microbes with addition of ractopamine to substrate. The authors observed an increase in gas production up to 22.6 mg/L ractopamine ($P < 0.05$), but no differences in acetate, propionate, or butyrate acid concentrations ($P > 0.05$). Additionally, a ractopamine × time of

fermentation interaction was observed when analyzing IVDMD ($P < 0.01$). Cultures containing ractopamine had greater DM disappearance suggesting ractopamine stimulates fermentation by rumen microbes.

Metabolic Effects

Metabolic parameters have been measured in an effort to explain shifts in carcass composition commonly observed with β-AA administration. Common metabolites measured include glucose, NEFA, and BUN concentration. Analyzing NEFA content can be used as an indicator of fatty acid oxidation, where low concentrations are normally found in blood, but increased concentrations indicate catabolism of adipose tissue. Beermann et al. (1987) measured NEFA concentration in lambs fed 10 mg/kg cimaterol. The authors observed a 60% increase in NEFA concentration in lambs receiving cimaterol when compared to control lambs ($P < 0.01$). Similarly, Blum and Flueckiger (1988) fed P-5369 and Q-2636 to calves through milk replacer and observed an increase in NEFA concentration ($P < 0.05$) up to 4 h after administration of P-5369; followed by a return to basal levels. Administration of Q-2636 resulted in an increase in NEFA concentration ($P < 0.05$) up to 6 h compared to control calves. Additionally, Chikhou et al. (1991b) observed increased NEFA concentration d 1 and 13 ($P < 0.05$), but NEFA were not different on d 20 ($P > 0.05$) for animals with and without orally administered cimaterol. In agreement with theses findings, Zimmerli and Blum (1990) observed a postprandial increase in NEFA concentration ($P < 0.05$) on d 1 of administration of β-AA T-3660 and P-5369, but no differences at later sampling points when compared to control animals. These results suggest chronic exposure to β-AA may cause desensitization of receptors in adipose tissue. The consistent increase in NEFA concentration in plasma with addition of β-AA suggests lipolysis is occurring in treated animals, and may be in combination with decreases in re-esterification or uptake of free fatty acids from circulation (Chikhou et al., 1991b). Increased blood flow with β-AA administration may contribute to decreased free fatty acid uptake and increased beta oxidation by skeletal muscle.

Glucose is an important energy substrate for skeletal muscle development (Du et al., 2010); however, β-AA treatment has minor effects on plasma glucose concentrations. Beermann et al. (1987) measured glucose concentration in lambs fed 10 mg/kg cimaterol, and observed no change in plasma glucose concentration ($P > 0.01$). Likewise, Quirke et al. (1988) observed

blood concentrations of glucose to be unaffected by administration of cimaterol ($P > 0.05$) to steers for 85 d. These results suggest animals may be able to regulate blood glucose levels more closely when compared to BUN or plasma NEFA concentration, which may explain the lack of effect of β-AA on plasma glucose concentrations. Blum and Flueckiger (1988) fed P-5369 and Q-2636 to calves through milk replacer and observed increased concentrations of glucose up to 10 h after administration ($P < 0.05$) of either β-AA. Similarly, Chikhou et al. (1991b) observed increases in plasma glucose concentrations on d 1 ($P < 0.05$) compared to control animals, but concentrations did not differ among treatments on d 13 and 20 ($P > 0.05$). Zimmerli and Blum (1990) observed a postprandial increase in glucose ($P < 0.05$) on d 1 of administration of T-3660 and P-5369 to calves, but no further changes on following sampling days. In disagreement, Walker et al. (2007) observed a tendency ($P = 0.08$) to decrease plasma glucose with ractopamine administration to steers, while Walker et al. (2010) demonstrated ractopamine had no effect on plasma glucose concentrations in steers ($P = 0.34$). Early effects of β-AA administration on concentrations of glucose may represent the initial signal for target tissues, ultimately inducing changes in nutrient repartitioning, and body composition (Blum and Flueckiger, 1988).

Cattle fed β-AA had initial increases in lactate concentration, but effects diminished with chronic administration. Lactate is produced in cells that have high demand for glucose utilization. During glycolysis, glucose is broken down to produce pyruvate. When intracellular concentrations of pyruvate become elevated it is enzymatically converted to lactate (Kravitz, 2005). Blum and Flueckiger (1988) fed P-5369 and Q-2636 to calves through milk replacer and observed an increase in concentrations of lactate up to 10 h after administration ($P < 0.05$) of either β-AA. In agreement, Zimmerli and Blum (1990) observed a postprandial increase in lactate ($P < 0.05$) on d 1 of administration of T-3660 and P-5369 to calves, but no further changes in following sampling days. These results coincide with results of plasma glucose, with an initial rise in glucose suggesting initial mobilization of glucose caused by β-AA treatment thus inducing an initial increase in lactate. When homeostatic mechanisms return glucose to normal levels, normal concentrations of lactate also are restored.

Plasma urea nitrogen (PUN) and blood urea nitrogen (BUN) concentrations commonly are decreased in cattle fed β-AA. Urea is produced in the liver in response to excess nitrogen

intake or as a result of tissue protein catabolism, thus making PUN and BUN concentrations useful as indicators of protein turnover. Ricks et al. (1984) reported a decrease ($P < 0.05$) in BUN concentration in steers fed 500 mg/d clenbuterol compared to untreated steers. In agreement, Quirke et al. (1988) observed linear decrease in blood PUN concentration ($P < 0.001$) after steers were fed 8.25, 12.38, or 16.50 mg/kg diet DM cimaterol compared to control steers. Chikhou et al. (1991b) observed a decrease in PUN concentration on d 1 ($P < 0.05$), but concentrations on d 13 and 20 were not different than control steers with administration of 0.09 mg/kg BW cimaterol. Conversely, Eisemann et al. (1988) fed 8 mg/d clenbuterol to steers, and observed no change ($P > 0.05$) in PUN concentration. Plasma urea nitrogen was numerically lower than control steers; however, the amount of clenbuterol fed may not have been sufficient to cause a significant difference. Similarly, Walker et al. (2007) observed no change for PUN concentration ($P > 0.05$) when 200 mg/d ractopamine was fed to steers; however, Walker et al. (2010) demonstrated ractopamine had a tendency to decrease PUN concentration ($P = 0.06$). Although not all studies observed significant decreases in urea nitrogen concentrations, numerical differences have been observed, which may be indicative of decreased protein degradation and/or increased protein synthesis (Mersmann, 1998).

Endocrine System

Due to the vast abundance of β-AR located throughout the body, circulating hormones that may influence growth have been analyzed in an attempt to identify indirect mechanisms of action. Studies performed to determine indirect effects of β-AA have yielded conflicting results.

Somatotropin causes the release of somatomedin, which ultimately mediates growth. Beermann et al. (1987) observed increased concentrations of somatotropin by wk 6 of administration ($P < 0.01$), but concentrations were not different wk 12 ($P > 0.05$) when cimaterol was fed to lambs. In contrast, Quirke et al. (1988) observed blood concentrations of GH were not different ($P > 0.05$) among steers treated with 0, 8.25, 12.38, or 16.5 mg/kg cimaterol. Similarly, Dawson et al. (1993) demonstrated that addition of cimaterol to diets of steers for 23 wk caused no differences in concentration of GH ($P > 0.01$) between treatments. Zimmerli and Blum (1990) observed an initial decrease in GH on d 0 for calves treated with P-5369 or T-3660 ($P < 0.05$) compared to control calves, but no changes were observed on subsequent sampling days ($P > 0.05$). This may be indicative of differences in the β-AA primary target receptor for which

binding occurs. Chikhou et al. (1991b) observed a similar acute decrease in GH concentration for steers 8 h after administration of cimaterol; however, chronic administration of cimaterol resulted in an increase in GH concentration when compared to untreated cattle. Beta agonists can acutely inhibit release of GH, but the mechanism responsible is not understood. It has been suggested that chronic administration of β-AA may cause desensitization in the β-receptors of the hypothalamic-peptidergic neurons thereby decreasing somatostatin secretion which regulates GH secretion (Chikhou et al., 1991b).

Insulin-like growth factor 1 plays an important role in growth of developing animals due to its impact on cell growth, proliferation, and metabolism (Micke et al., 2010). Beermann et al. (1987) observed lower ($P < 0.01$) IGF-1 concentrations in lambs treated with cimaterol compared to their control counterparts. Similarly, Chikhou et al. (1991b) observed concentration of IGF-1 decreased with chronic administration of cimaterol to steers. Walker et al. (2007) observed a tendency ($P = 0.10$) to decrease serum IGF-1 concentrations with addition of ractopamine. On the contrary, Winterholler et al. (2007) observed no difference ($P > 0.05$) in IGF-1 concentration in steers fed ractopamine compared to control animals. The authors suggested ractopamine may have an effect on IGF-binding protein (IGFBP) concentrations, which may account for differences observed among studies. Walker et al. (2010) reported a response to ractopamine, in which they found a difference between steers and heifers for IGF-1 concentration. Heifers had decreased concentration of IGF-1 d 14 and 28. Steers had decreased concentrations on day 14, but by day 28 ractopamine treated steers had greater concentrations of IGF-1 than their control counterparts. The authors suggested this may be in response to ractopamine having a direct effect on the liver, or it may alter IGF-1 clearance from the blood by affecting IGFBP. Contradicting these results, Quirke et al. (1988) found blood concentrations of IGF-1 were not different ($P > 0.05$) for steers treated with 0, 8.25, 12.38, or 16.5 mg/kg cimaterol. Likewise, Zimmerli and Blum (1990) observed concentrations of IGF-1 were not different ($P > 0.05$) when calves were treated with P-5369 or T-3660 compared to control calves. Dawson et al. (1993) demonstrated that addition of cimaterol to diets of steers for 23 wk resulted in no difference in concentration of IGF-1 ($P > 0.01$) between treatments. Most of the circulating concentration of IGF-1 was thought to be released from the liver, therefore decreased concentrations of IGF-1 in steers administered β-AA may be due to the effect of β-AA on hepatic IGF-1 synthesis (Walker et al., 2007). Amount of β-AA administered may have contributed the differences among studies.

Variable responses have been observed for insulin concentrations in livestock fed β-AA, while differences in glucagon concentration were not different between treated and untreated animals. Insulin and glucagon are hormones secreted by the pancreas, and work together to maintain glucose homeostasis in blood. Insulin is secreted when blood glucose levels are too high, whereas glucagon is secreted when blood glucose is low. Beermann et al. (1987) observed that insulin concentrations were decreased wk 6 and 12 ($P < 0.01$) after cimaterol feeding to lambs. Walker et al. (2007) observed a tendency ($P = 0.10$) for decreased insulin concentrations with addition of ractopamine. Conversely, Blum and Flueckiger (1988) observed, with acute administration of Q-2636 and P-5369 to calves, increased ($P < 0.05$) insulin concentrations up to 6 h after administration. Furthermore, Zimmerli and Blum, 1990 observed increased insulin concentrations ($P < 0.05$) with addition of T-3660, but insulin concentrations were not different ($P > 0.05$) with administration of P-5369 compared to control calves, indicating potential differences in β-AR affected. Chikhou et al. (1991b) fed cimaterol to steers, and also observed increased insulin concentrations on d 1 ($P < 0.05$), but on d 13 and 20 the steers administered cimaterol had insulin concentrations similar to control steers. Lower insulin concentrations may be due to direct effects on the pancreas, as β-AA may stimulate both alpha and beta receptors (Beermann et al., 1987). The differences observed between studies for response of insulin may reflect differences in primary target receptors of the β-AA administered. Although insulin concentration was observed to have variable responses with β-AA administration, Blum and Flueckiger (1988) observed no difference for glucagon concentration in calves treated with Q-2636 or P-5369 ($P > 0.05$) compared to control calves. These results may reflect the differences in β-receptor density on the cells responsible for release of glucogon when compared to β-receptors located on cells that secrete insulin.

Triiodothyronine (T3) and thyroxine (T4) are hormones secreted by the thyroid gland affecting many processes in the body including, growth, heart rate, and cellular metabolism, and have been observed to be decreased or un-affected by β-AA treatment. Blum and Flueckiger (1988) observed concentrations of T3 were not different ($P > 0.05$) after Q-2636 or P-5369 administration compared to control calves. Similarly, Zimmerli and Blum (1990) noted that T3, and T4 concentrations were unchanged ($P > 0.05$) after treatment with T-3660 or P-5369. Chikhou et al. (1991b) also observed no effect on T3 or T4 ($P > 0.05$) when steers were fed

cimaterol. Conversely, Fiems et al. 1989 observed decreased concentrations of T3 ($P < 0.05$) when cimaterol was administered to Belgian White-blue bulls for 84 and 196 d compared to control bulls, and decreased ($P < 0.05$) T4 concentrations after 28 and 84 d administration compared to controls. The bulls in the experiment by Fiems et al. were genetically predisposed to heavy muscling, which may partially explain the difference in T3 and T4 concentration, when compared to other experiments; however, other experiments would suggest β-AA treatment does not affect thyroid gland secretions of T3 and T4.

Acute effects of β-AA on hormone concentrations are no doubt evident; however tissues may become desensitized to β-AA, and effects may be attenuated with chronic exposure. These observations support the contention that indirect effects of the endocrine system may play a lesser role in explaining the growth response observed with β-AA administration. Additionally, inconsistent results observed in response to β-AA treatment would also suggest the hormones analyzed may not constitute important mechanisms by which β-AA exert their effect (Moody et al., 2000). A study by Byrem et al. (1998), in which cimaterol was administered to steers through direct hindlimb infusion, showed an increase in muscle protein accretion, suggesting a direct response rather than indirect effect of β-AA (Moody et al., 2000).

Skeletal Muscle

A common observation with administration of β-AA is an increase in skeletal muscle mass in proportion to mass of adipose tissue. Figure 1.3 illustrates difference between loin eye sizes from the 12th rib face of treated versus non-treated steers. The process by which nutrients are shifted to the tissues with greatest priority is under homeorrhetic control (Anderson et al., 1991). The nutrient shift is accomplished by binding of β-AA with β-receptors in the arteries and veins, which increases blood flow to skeletal muscle in cattle and sheep. Increased blood flow may increase nutrient transport to skeletal muscle to accommodate increases in protein synthesis and resulting hypertrophy of muscle tissue in livestock (Mersmann, 1998). Beta adrenoceptors in skeletal muscle were reviewed by Kim and Sainz (1992), who noted that the primary receptor found in skeletal muscle is the β_2-subtype (Kim and Sainz, 1992). The mechanism by which β-AA exert their effect on skeletal muscle is less clear for livestock species (Moody et al., 2000), as studies are inconclusive. This lack of congruence among published studies may be due to

differences in animal species, β-AA used, dosage, and (or) duration of treatment. Moody et al. (2000) hypothesized that protein synthesis is enhanced and/or protein degradation is inhibited by treatment with β_2-agonists cimaterol, clenbuterol, and $L_{644,969}$, whereas the β_1-agonist ractopamine affects only protein synthesis.

Addition of β-AA to diets of ruminants has increased carcass weight, live weight, or both. Yang and McElligott (1989) described the response to β-AA in skeletal muscle as being hypertrophic rather than hyperplastic. Mice fed clenbuterol had reduced muscle wasting when fed a nutritionally poor diet compared to mice without clenbuterol, and atrophy was attenuated when denervated or suspended rat skeletal muscle was evaluated (Kim and Sainz, 1992).

Beta agonist administration also influences fiber type and size. Beermann (2002) described the increase in muscle mass as being attributable to hypertrophy of type II muscle fibers. Type II fibers (fast-contracting, mixed glycolytic-oxidative) of lambs were reported to consistently increase in cross sectional-area, whereas type I fibers (slow contracting oxidative) were less affected (Yang and McElligott, 1989). Beermann et al. (1987) performed a study feeding cimaterol to lambs. The *biceps femoris*, *semitendinosis*, and *semimembranosis* in treated lambs were heavier after 12 wk of administration ($P < 0.01$) compared to those of non-treated lambs. Beermann et al. (1987) attributed the heavier muscle weights to hypertrophy of both type I and type II fibers. Additionally, Beermann et al. (1987) observed a decrease in percentage of type I fibers throughout the 12 wk experimentation period for all 3 muscle types. Distribution of fiber type also was notably different among treatments; the largest fibers were 35% greater in cimaterol treated lambs compared to control lambs (Beermann et al., 1987). Kellermeier et al. (2009) performed a study in which zilpaterol was administered to steers to evaluate effects on yield characteristics and tenderness, and observed a 5.87% increase ($P < 0.001$) in *longissimus* muscle fiber diameter in steers treated with zilpaterol compared to control steers. Furthermore, Kim et al. (1987) performed a study feeding cimaterol to lambs, and observed a 50% increase in ($P < 0.01$) cross-sectional areas of type II fibers in the *longissimus dorsi* and *semitendinosus*, but not in any other cut of meat. Additionally, there were no differences ($P > 0.05$) in cross-sectional area of type I fibers; indicating increases in muscle mass were due to hypertrophy of type II muscle fibers. Clearly, muscle hypertrophy occurred in β-AA treated animals.

Ractopamine is one of two β-AA approved for use in beef cattle in the United States. Cull beef cows treated with ractopamine had increased ($P < 0.05$) fiber diameter and cross-sectional area of type I fibers; however, type II fiber diameter and cross-sectional area were unaffected ($P > 0.05$) by addition of ractopamine (Gonzalez et al., 2007). Ractopamine is primarily a β_1-agonist, which could have a different effect on fiber size when compared to β_2-agonists. Contradicting previous work, Gonzalez et al. (2009) fed steers ractopamine and found no differences ($P > 0.05$) in cross-section area of type I or type II fibers in 6 different muscles, but did observe a shift from type I to type II fibers ($P < 0.05$) in the *adductor*, *gracilis*, *longissimus lumborum*, *rectus femoris*, and *vastus lateralis*. Discrepancies between studies may be due to age of animals tested, as cull cows tend to have fewer type II fibers compared to young animals. Differences observed among muscle types are attributable to density of β-receptors (β_1 vs. β_2 vs. β_3) as well as distribution of type I and type II muscle fibers (Kim and Sainz, 1992). Depending on the β-AA used, type I fibers may or may not be affected by β-AA treatment.

Mersmann (1998) described the increase in skeletal muscle as being due to increases in protein synthesis and decreased protein degradation. Due to the difficulty with measuring protein degradation, protease activity was evaluated. Protease activity decreased when β-AA was administered, suggesting decreased protein degradation (Mersmann, 1998). Wheeler and Koohmaraie (1992) calculated fractional degradation rates of myofibrillar proteins to be lower ($P < 0.05$) after 3 wk and fractional accretion rates to be greater ($P < 0.05$) in steers treated with $L_{644,969}$ suggesting β-AA administration decreases protein turnover.

When carcass composition is evaluated, response to β-AA most frequently is characterized by increased carcass muscle mass and decreased carcass adipose tissue; however, different β-AA have different effects. Ricks et al. (1984) noted REA at the 12th rib face was 11 and 16% greater, and back fat decreased 35 and 42% when steers were fed 10 or 500 mg/d of clenbuterol, respectively. Additionally, clenbuterol treated steers had more carcass protein and less fat than their control counterparts. Steers also had more water content in muscle than control animals. In agreement, Quirke et al. (1988) administered 33, 49.5, 66 mg/d cimaterol to steers, and observed a linear and quadratic decrease in percent fat ($P < 0.001$) with steers treated with 66 mg/d having the lowest percent fat, and a linear and quadratic increase in percent lean tissue ($P < 0.001$) with 66 mg/d having the highest percent lean tissue. Additionally, percent moisture

and protein increased linearly in response to cimaterol (P < 0.01). $L_{644,969}$ was administered to Friesian steers at rates of 0, 0.25, 1 or 4 mg/kg BW by Moloney et al. (1990), who observed linear and quadratic increase in percent lean tissue ($P < 0.001$) with steers fed 4 mg/kg BW having the greatest amount of percent lean tissue, and control steers having the least amount of percent lean tissue. A linear and quadratic decrease in percent fat ($P < 0.001$) was observed with steers fed 4 mg/kg containing the least amount of carcass fat, and control steers had the greatest amount of carcass fat. Steers fed 4 mg/kg had the greatest lean:fat ratio ($P < 0.001$) in the *M. longissimus* compared to the other treatments. Additionally, percent protein had a quadratic effect ($P < 0.01$) with steers fed 1 mg/kg having the greatest percent carcass protein. Percent carcass ash increased linearly ($P < 0.05$), and a linear and quadratic effect was observed for percent fat with 1 mg/kg having the lowest percent carcass fat ($P < 0.01$), and percent carcass moisture was unaffected by treatment of $L_{644,969}$ compared to untreated steers (Moloney et al., 1990). In agreement, Wheeler and Koohmaraie (1992) observed a 21% increase in *semitendinosis* muscle weight ($P < 0.05$), and total carcass protein was increased 23.5% ($P < 0.05$) with addition of $L_{644,969}$ to diets of steers. In general, carcass composition is altered with an increase in amount of muscle mass, and decreased amount of carcass fat.

Caine and Mathison (1992) fed cimaterol to lambs at rates of 1.1or 2.2 × maintenance. Fat concentration (g/g tissue) was lower in the *psoas major* muscle and *semitendinosus* muscle ($P < 0.01$), and tended to be lower in the *gastrocnemius* muscle ($P = 0.09$). Contrary to the previous studies with cattle, Caine and Matheson (1992) observed no difference ($P > 0.05$) in the amount of protein in *gastrocnemius* or *semitendinosus* muscles; however, protein:fat ratio in the *gastrocnemius*, *psoas major*, and *semitendinosus* muscles increased ($P < 0.01$). The differences observed may reflect receptor density in the muscles evaluated, indicating muscle types have different β receptor densities.

Zilpaterol hydrochloride was approved for use in the United States in 2006, and also affects body composition of cattle. Avendaño-Reyes et al. (2006) performed a study in which zilpaterol was administered to steers. They observed no differences ($P > 0.05$) in percent lean, bone, or fat in treated steer carcasses compared to control animals. Conversely, Leheska et al. (2009) observed an increase in carcass muscle deposition in both steer and heifer carcasses ($P < 0.01$) with zilpaterol administration, but percent fat and moisture content were unaffected ($P > 0.05$) by treatment. Percent bone was decreased ($P = 0.02$) in heifers, but not different in steer

carcasses ($P > 0.10$), and percent carcass ash was unaffected ($P > 0.05$) by β-AA treatment. The authors concluded that effects observed in their study, compared to those reported by Avendaño-Reyes et al. (2006) may have been due to the lower dose of zilpaterol hydrochloride administered (60 mg/d) by Avendaño-Reyes et al. or differences in genetic make-up of the cattle. Results by Shook et al. (2009) are in agreement with those of Leheska et al. (2009), demonstrating an increase in percent lean tissue ($P < 0.05$), no change in percent carcass fat ($P > 0.05$) , and a decrease in percent bone ($P < 0.05$) with zilpaterol hydrochloride administration. Hilton et al. (2009) observed a decrease in estimated carcass fat ($P < 0.01$) and an increase in estimated carcass moisture and protein ($P < 0.01$) with zilpaterol hydrochloride administration; however, the decrease in carcass fat was not as pronounced as seen with cimaterol or clenbuterol administration. Table 1.2 summarizes effects of β-AA on cattle growth and carcass composition.

The calpain proteolytic system, which is partly responsible for post-mortem tenderization of meat, also is affected by β-AA. Calpains play an important role in turnover of myofibrillar proteins in live animals and in post-mortem aging (Koohmaraie et al., 1991). Administration of $L_{644,969}$ to lambs increased total extractable μ-calpain and calpastatin by 24.6 and 62.8%, respectively, at d 0 of storage, and were 89.2 and 227.2% greater than control lambs on d 7 (Koohmaraie et al., 1991). The increase in calpastatin, which inhibits calpains, supports the argument β-AA interfere with muscle protein degradation. Contrary to the findings by Koohmaraie et al. (1991), Wheeler and Koohmaraie (1992) administered $L_{644,969}$ to steers, and observed that μ-calpain and calpastatin activity decreased over a 7-d aging period for both treated and non-treated animals ($P < 0.05$). The authors also found that total activity of calpastatin in β-AA treated animals increased ($P < 0.05$) in comparison to their control counterparts indicative of reduced protein degradation in treated steers. Post-mortem proteolysis and tenderization were reduced ($P < 0.05$) in steers fed $L_{644,969}$, as shear force values did not vary from d 1 to 14 postmortem (Wheeler and Koohmaraie, 1992). The authors concluded that inhibition of calpain activity by increased calpastatin activity in β-AA fed steers was responsible for decreases in post-mortem proteolysis. Hilton et al. (2009) analyzed the calpain proteolytic system with administration of zilpaterol, and found that calpastatin, μ-, and m-calpain were unaffected ($P > 0.05$) by zilpaterol supplementation. The authors attributed the lack of effect to the possibility of zilpaterol having a greater influence on protein accretion rather than decreasing

protein degradation. Beta agonists cause different responses in the proteolytic system indicating differences in target receptor activity.

Adipose Tissue

Administration of β-AA has been noted to decrease adipose tissue content of carcasses. Yang and McElligott (1989) suggested that decreased body fat may be a result of increased fat mobilization, decreased fat synthesis, or both. They proffered that binding of β-AA to adipose tissue β-receptors results in a cascade of events that lead to activation of lipase, and resulting triacylglycerol hydrolysis (Yang and McElliogott, 1989). Lipogenic enzymes were measured *in vitro* and activities of enzymes were decreased in subcutaneous adipose tissue, but not in intramuscular or perirenal adipose tissue (Yang and McElligott, 1989). This observation may help to explain inconsistencies observed in carcass composition measurements described previously in this review. I*n vivo* measurements are commonly evaluated by measuring concentrations of NEFA or glycerol in blood (Yang and McElligott, 1989; Mersmann 2002). Administration of β-AA caused NEFA levels to rise in treated animals, which would suggest activation of the adipocyte lipolytic system (Beermann et al., 1987; Blum and Flueckiger, 1988; Chikhou et al., 1991). After the acute response to β-AA administration, responses decrease with chronic exposure (Eisemann et al., 1988; Zimmerli and Blum, 1990; Chikhou et al., 1991).

In reviewing studies regarding carcass composition, most reported decreases in amount of carcass fat; however, there are a few research trials in which carcass fat was not affected with the addition of β-AA (Avendaño-Reyes et al., 2006; Leheska et al., 2009; Shook et al., 2009). This may be explained by differences in study design. In earlier experiments the β-AA was fed to the animals or not, and then carcass composition was compared among treatments. This type of design has migrated to comparisons in which cattle fed β-AA were fed to large carcass weights, but similar fatness. Studies in which zilpaterol was administered to livestock are inconsistent with regards to its effect on adipose tissue as compared to studies in which cimaterol or clenbuterol are administered. An explanation for this observation may be that zilpaterol stimulates protein accretion more so than lipolysis, thus increasing carcass muscle as opposed to mobilizing body fat (Mersmann, 2002). Furthermore, differences in study design, such as genetic make-up of animals tested may be a cause of discrepancies between studies (Mersmann, 1998).

Chronic exposure to β-AA may cause inactivation or removal of the β-receptor from the cell surface quicker in adipose tissue when compared to skeletal muscle, thus attenuating the response in adipose tissue (Mersmann, 1998). Miller et al. (2012) performed an *in vitro* trial treating freshly obtained adipose tissue with 4 different concentrations of zilpaterol, and concluded that effects of zilpaterol on de novo fatty acid synthesis were minimal, and that changes in carcass fat are likely caused by redirection of nutrients from adipose tissue fatty acid biosynthesis to muscle accretion.

Conclusion

Although β-AA have been researched extensively in the livestock industry for their effects on carcass composition, it is clear that these compounds influence a broad range of body systems. Beta agonists can cause an initial rise in respiration rate. Inconsistent results were observed when analyzing the smooth muscle of the digestive tract and microbial fermentation. Hormonal response to β-AA treatment also is not consistent between studies. Variable responses are to be expected when comparing responses to different β-AA because a single agonist has not been tailored to all species (Mersmann, 2002). Many factors can cause inconsistent results demonstrated in this review. These factors include species of animal, age, dose of β-AA, duration of treatment, diet, genetics, and possible even animal weight; all of which make it very difficult to quantify mechanisms of action of β-AA in general.

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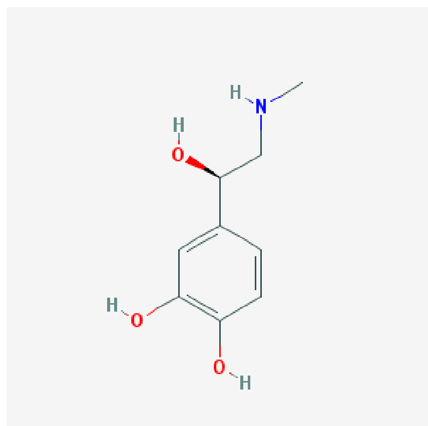
Table 1.1: Radioligands for β-adrenergic receptors[1]

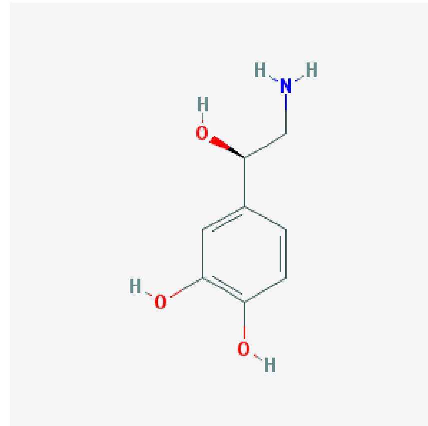
Radioligand	Specificity
Antagonist	
l-[^{3}H]dihydroalprenolol	$ß_1 = ß_2$
dl-[^{3}H]propranolol	$ß_1 = ß_2$
l-[^{3}H]propranolol	$ß_1 = ß_2$
dl-[^{3}H]carazolol	$ß_1 = ß_2$
l-[^{3}H]carazolol	$ß_1 = ß_2$
l-[^{3}H]bupranolol	$ß_1 = ß_2$
Agonists	
dl-[^{3}H]hydroxybenzylisoproterenol	$ß_1 < ß_2$
l-[^{3}H]epinephrine	$ß_1 < ß_2$
l-[^{3}H]norepinephrine	$ß_1 > ß_2$
dl-[^{3}H]isoproterenol	$ß_1 = ß_2$

[1]Antagonist radioligands are used to study adrenergic receptor abundance and properties, and agonist radioligands for researching mechanisms of action of agonist attachment to adrenergic receptors. Table adapted from Stiles et al. 1984.

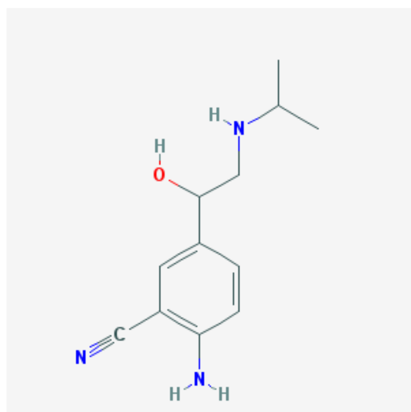
Figure 1.1: Structure of naturally occurring catecholamines and common beta agonists



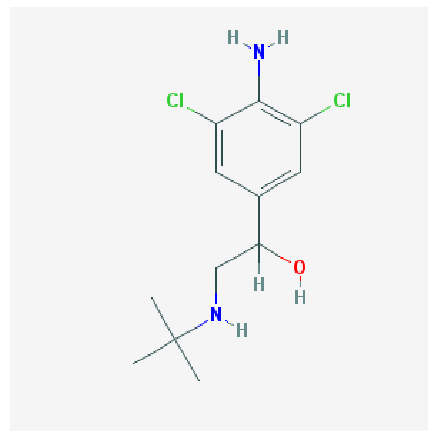
Epinephrine



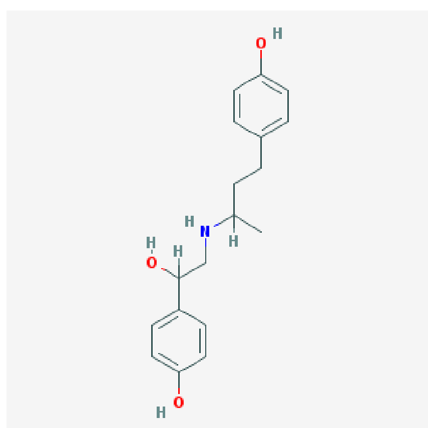
Norepinephrine



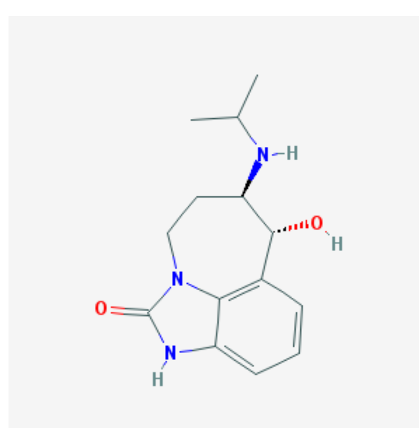
Cimaterol



Clenbuterol



Ractopamine Hydrochloride



Zilpaterol Hydrochloride

Figures from pubchem.ncbi.nlm.nih.gov

Table 1.2: Effects of beta-agonists on carcass composition of growing cattle

		Control values and proportional responses (%)				
				Carcass composition		
Treatment Dose (mg/kg)	Treatment period (d)	ADG[a] (g/d)	F:G[b]	Protein	Lipid	Reference
Clenbuterol						
0	98	1007	11.83	15.4%[c]	35.2%[c]	Ricks et al. (1984)
10		-8	-2.1	+13*	-20*	
500		-20	0	+14*	-30*	
Cimaterol						
0	91	816	11.7	203 kg[d]	75.1 kg[d]	Hanrahan et al. (1986)
3.5		+18*	-23.5*	+24.5*	-28.1*	
5.1		+30.4*	-30.6*	+30.0*	-30.4*	
7		+6.0	-8.2	+27.6*	-35.0*	
L-644,969						
0	84	780	10.2	60.4%[e]	21.4%[e]	Moloney et al. (1990)
0.25		+10	-14.0	+3	-14.5*	
1.0		+16	-19.6	+4.5	-29*	
4.0		+10	-23.4	+15.6*	-35*	
Ractopamine						
0	56	1430	5.37	13.2%[c]	42.6%[c]	Anderson et al. (1991)
20		+7.7	-8.2*	+4.5	-4.9	
40		+7.7	-8.6*	+6*	-4.7	
60		+9	-10.6*	+7.3*	-8.7*	
80		+16.8*	-17.7*	+9.8*	-9.8*	

Table adapted from Beermann (1994)

[a]Average daily live-weight gain.

[b]kg feed per kg live-weight gain.

[c]Chemical composition of 9th to 11th rib section.

[d]Total carcass muscle and adipose tissue weights based on half carcass physical dissection.

[e]Percentage of physically dissected skeletal muscle and adipose from carcass side.

*$P < 0.05$.

Figure 1.2: Twelfth rib face of steers treated with and without ractopamine.
The loin on the right came from a steer that had been treated with ractopamine for 42 d prior to harvest, and the loin on the left came from an untreated steer.

Chapter 2 - Effects of zilpaterol hydrochloride on blood metabolites in finishing steers

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Abstract

Effects of zilpaterol hydrochloride (ZH) on blood metabolites and carcass traits were evaluated in crossbred finishing steers (n=18, BW 639 kg ± 12.69) that were stratified by BW and randomly assigned, within strata (block), to receive 0 (control) or 8.33 mg/kg diet DM ZH. Cattle were fed once daily *ad libitum* in individual feeding pens (9 pens/treatment). ZH was fed 23 d and withdrawn 3 d before harvest. Blood samples and measures of BW were taken on d 0, 7, 14, and 21. Concentrations of beta-hydroxybutyrate (BHB), glucose, and lactate were determined for whole blood, and NEFA, urea nitrogen (PUN), and long-chain fatty acids (LCFA) concentrations were analyzed from plasma. Adipose tissue samples (approximately 20 g) from subcutaneous fat covering the lumbar vertebrae were collected after 48-h of refrigeration and analyzed for LCFA profiles. Feeding ZH decreased DMI by 8% ($P < 0.10$), but did not impact live BW gain or efficiency ($P > 0.10$). Addition of ZH resulted in greater HCW, dressing percentage, and LM area ($P < 0.10$), but did not influence other carcass traits ($P > 0.10$). ZH decreased glucose and PUN concentrations ($P < 0.10$). A ZH × d interaction also was observed ($P < 0.10$) for PUN concentrations. Concentration of PUN increased over time in control cattle, but decreased in cattle receiving ZH. Nonesterified fatty acids, BHB, plasma glucose, whole blood and plasma lactate concentrations were unaffected by ZH ($P > 0.10$). Zilpaterol hydrochloride increased plasma concentrations of elaidic, vaccenic, and docosapentaenoic acids ($P < 0.10$), but LCFA concentrations of adipose tissue were unaffected ($P > 0.10$), suggesting no preferential oxidation of specific fatty acids. ZH supplementation decreased plasma nitrogen concentration to accommodate increased muscle accretion at the expense of adipose tissue.
Key words; glucose, plasma urea nitrogen, zilpaterol hydrochloride

Introduction

Synthetic β-adrenergic agonists (βAA), which are similar in structure to the naturally occurring catecholamines norepinephrine and epinephrine, are known to induce accretion of skeletal muscle at the expense of adipose tissue (Mersmann, 1998). Plasma urea nitrogen (PUN) is a measurement associated with nitrogen metabolism and has been observed to decrease in plasma with clenbuterol and cimaterol administration (Ricks et al., 1984 and Byrem et al., 1996).

Additionally, blood metabolites associated with carbohydrate and lipid metabolism (i.e. lactate, NEFA) have been shown to increase in response to cimaterol and clenbuterol feeding (Eisemann et al., 1988 and Chikhou et al., 1991), while plasma glucose concentrations have been observed to either increase or decrease during administration of P 5369, Q 2636, or cimaterol (Blum and Flueckiger, 1988; Quirke et al., 1988). Zilpaterol hydrochloride (ZH), a β_2-AA merchandized under the trade name, Zilmax (Merck Animal Health, Millsboro, DE), has been reported to improve feed efficiency, ADG, dressing percentage, HCW, and LM area (Montgomery at al., 2009; Plascencia et al., 2008). Hilton et al. (2009) reported decreases in USDA yield grade and total carcass fat with ZH use. Our interest was in assessing the underlying changes in metabolism with addition of ZH to diets of finishing cattle. We hypothesized blood components related to muscle growth (i.e. glucose, and PUN) will be decreased to accommodate the increase in muscle mass, while blood components related to lipid metabolism (i.e. NEFA, and β-hydroxybutyrate, lactate) will be increased in plasma. The objectives of our study were to evaluate the effects of ZH on blood concentrations of glucose, lactate, NEFA, β-hydroxybutyrate (BHB), and plasma urea nitrogen (PUN), and to assess changes in long chain fatty acid (LCFA) profiles of plasma and adipose tissue.

Materials and Methods

Animals and sampling:

Protocols and procedures followed in this study were approved by the Kansas State University Institutional Animal Care and Use Committee.

The study was conducted at the Kansas State University Beef Cattle Research Center, located in Manhattan, KS. Eighteen crossbred steers (initial BW 614 kg ± 12.69) were stratified by initial body weight, and randomly allocated, within strata, to two treatment groups. Cattle were randomly assigned to individual, partially covered feeding pens equipped with concrete floors, fence line feed bunks, automatic water fountains (9 pens/treatment), and were fed once daily *ad libitum*. Treatments consisted of diets with or without ZH. Zilpaterol hydrochloride was fed at a rate of 8.33 mg/kg of diet DM beginning 26 d before the end of the study and withdrawn from the diet 3 d prior to harvest. Diet composition is presented in table 2.1. Body weights were measured for each steer at 7-d intervals, and again on the d of harvest. Blood was drawn from

each steer via jugular venipuncture prior to feeding on d 0, 7, 14, and 21. Blood was collected in two 6-mL EDTA-containing vacutainer tubes/animal (Becton Dickinson and Company, Franklin Lakes, NJ). The first tube was placed on ice and transported immediately (approximately 10 min) to the Pre-Harvest Food Safety Laboratory located at Kansas State University's main campus, and analyzed to determine whole blood concentrations of glucose and lactate. The second blood sample was immediately placed on ice then centrifuged within 20 min of sampling at 2,000 × g for 10 min to recover plasma. Plasma was transferred to 5-mL plastic tubes, capped, and stored at -20ºC until analyzed for concentrations of plasma urea nitrogen (PUN), NEFA, glucose, and lactate. Additionally, fatty acid profiles of plasma were measured for each steer at each time point. Finally, BHB was measured using fresh blood by allowing a small amount of blood to drop onto test strips prior to removal of the needle from the jugular vein. Concentrations of BHB were analyzed using the Precision Xtra Advanced Diabetes Management System (Abbott Diabetes Care Inc., Alameda, CA). Precision Xtra Blood β-ketone test strips (Abbott Diabetes Care Inc., Alameda, CA) were required for BHB concentration analysis. The Precision Xtra was placed in ketone mode and blood was analyzed according to kit directions. Results for BHB were provided within 10 s after initiation of the test.

Feed intakes were monitored daily, and unconsumed feed was removed from the bunk, weighed, and dried at 55ºC for 48 h at weekly intervals or as needed to determine actual feed intake. Feedstuffs were sampled weekly and analyzed for DM, starch, NDF, CP, S, and ether extract. Portions of ground samples of feedstuffs were dried in a forced air oven at 105°C overnight to determine DM (Undersander et al., 1993). Starch contents of feedstuffs were determined according to Herrera-Saldana and Huber (1989) with a Technicon Autoanalyzer III (SEAL Analytical, Mequon, WI). Determination of NDF was conducted using an ANKOM fiber analyzer (ANKOM Technology Corp., Fairport, NY) according to Van Soest et al. (1991). Heat-stable α-amylase (ANKOM Technology Corp) was added to remove residual starch from feedstuff samples. Determination of CP was accomplished by measuring N content with a LECO FP–2000 nitrogen analyzer (LECO Corp., St Joseph, MI) following AOAC (1995) official method 990.03. Ether extract analysis was performed according to AOAC (1995) official method 920.39.

Average daily gains were computed by subtracting initial shrunk live weight from shrunk final BW (gross BW × 0.96), and dividing by days on feed (DOF). Gain efficiencies were

computed by dividing ADG by DMI. Final BW (gross BW × 0.96) were determined immediately before cattle were shipped 451 km to a commercial abattoir in Holcomb, KS. Hot carcass weight and incidence and severity of liver abscesses were recorded the d of harvest. Liver abscesses were scored according to the Elanco scoring system (Liver Abscess Technical Information AI 6288, Elanco Animal Health, Greenfield, IN): 0 = no abscesses, A^- = 1 or 2 small abscesses or abscess scars, A^0 = 2 to 4 small, well-organized abscesses and A^+ = 1 or more large or active abscesses with or without adhesions. USDA Yield Grade, USDA quality grade, marbling score, 12th rib fat thickness, loin-eye area, percent KPH, and incidence and severity of dark cutting beef were collected after carcasses were refrigerated for 48 h. Finally, a 20-g sample of subcutaneous adipose tissue was removed from fat covering the lumbar vertebrae on the left side of each carcass and placed on ice. Adipose samples were transported to Kansas State University and placed into a freezer at -20ºC until analyzed for composition of fatty acids (LCFA).

Chemical Analyses

Glucose and lactate concentrations of both plasma and whole blood were analyzed in duplicate using the YSI 2300 STAT Plus Glucose and L-lactate Analyzer (YSI Inc., Yellow Springs, OH). Whole blood glucose and lactate were measured within 10 min after sampling. The HR Series NEFA-HR kit (Wako Diagnostics, Richmond, VA) was used for determination of NEFA concentrations in plasma. Samples were measured in duplicate with the Biotek PowerWave XS (Winoski, VT) using a wavelength of 550 nm. Plasma urea nitrogen concentration was determined according to procedures described by Marsh et al. (1965) using an Auto Analyzer II (Seal Analytical, Mequon, WI).

For analysis of plasma fatty acids, 500 μL of plasma were frozen and subsequently lyophilized overnight using the Labconco Freeze Dryer 5 (Labconco Corporation, Kansas City, MO). One milliliter of benzene containing internal standard 1,000 μg/mL methyl-C:13 was added to each tube. Samples were capped immediately and vortexed to dissolve the pellet. Four milliliters of BF_3-methanol reagent (Supelco B1252; Sigma-Aldrich, St. Louis, MO) were added, tubes were recapped, mixed gently by inverting the tubes, then incubated at 60°C for 60 min. Samples were allowed to cool to room temperature, and 4 mL of distilled deionized water and 1 mL hexane were then added. Tubes were recapped and vortexed. Samples were centrifuged at 1,000 × g for 5 min, and supernatant was transferred (1 to 2 mL) to gas chromatography vials.

Determination of adipose tissue LCFA concentration was performed following the procedure described by Sukhija and Palmquist (1988). Concentrations of LCFA for both plasma and adipose tissue were analyzed using a Shimadzu Model GC-17A (Columbia, MD) gas chromatograph equipped with a Supelco SP-2560 capillary column (100 m X 0.25 mm X 0.20 *u*m film). Injection and detector temperatures were set at 260°C. Initial oven temperature was 140°C, held 5 min, then increased at the rate of 4ºC/min to a final temperature of 240°C. Column flow rate was 1.1 mL/min, and nitrogen was used as the carrier gas. Supelco 37 FAME mix (Supelco # 47885-U; Sigma-Aldrich, St. Louis, MO) was used as the standard.

Statistical Analyses

Blood metabolites and plasma LCFA were analyzed as repeated measures using the MIXED procedure of SAS version 9.1 (SAS Inst. Inc., Cary, NC). Animal was the experimental unit, and the model included fixed effects of ZH, d, and ZH × d. The random effects were block and block × ZH. Likewise, live performance and carcass traits were analyzed using the MIXED procedure where ZH was the fixed effect, and block and block × ZH were the random effects. The LSMEANS statement was used for means and standard error calculations, and the PDIFF option was used for separation of means. Means separations were declared significant at an α level ≤ 0.10 to accommodate the small number of experimental units.

Results and Discussion

One animal from the control group was removed from the study due to respiratory disease. The steer began to display symptoms the second week of the study.

Live animal performance is presented in table 2.2. Zilpaterol hydrochloride supplementation did not affect final BW ($P > 0.10$). Observations of BW change have not been consistent among published studies. In agreement, Vasconcelos et al. (2008) and Holland et al. (2010) observed no difference ($P > 0.10$) in final BW with ZH administration when compared to un-treated cattle. Contradicting our results, Montgomery et al. (2009b) observed an increase ($P = 0.01$) in final BW for ZH supplemented steers compared to steers receiving no ZH in a study observing effects of ZH in combination with monensin and tylosin. Additionally, Scramlin et al. (2010) performed a study that fed either ZH or ractopamine to cattle and compared growth performance and carcass responses. The authors observed an increase in final BW ($P < 0.05$)

with ZH supplementation when compared to control cattle. Differences between trials may be attributed to genetic differences in animals utilized as genetics can affect response to βAA treatment (Mersmann, 1998).

Feeding ZH to steers decreased DMI by 8% (*P* = 0.03). Zilpaterol hydrochloride supplementation has been reported to either have no effect (*P* > 0.05) on DMI (Avendaño- Reyes et al., 2006; Plascencia et al., 2008; Parr et al., 2011), or, in agreement with our results, has been observed to cause a decrease (*P* < 0.05) in DMI (Montgomery et al., 2009b; Holland et al., 2010; Scramlin et al., 2010). Despite the decrease in DMI in the present study, neither ADG (*P* = 0.83) nor G:F (*P* = 0.56) were influenced by ZH supplementation. Contradicting our results, Elam et al. (2009) found a linear increase in ADG and G:F when comparing ZH supplementation for 0, 20, 30, or 40 d. Other studies have also reported an increase in G:F with ZH supplementation when compared to un-treated cattle (Montgomery et al., 2009a; Avendaño- Reyes et al., 2006; Scramlin et al., 2010). Parr et al. (2011) performed a study that compared responses to anabolic implants Revalor-S and Revalor-XS with and without ZH. The authors observed an improvement in feed efficiency (*P* = 0.01) as well as an increase in ADG (*P* = 0.01) with ZH administration. Performance of steers in the present study may have been compromised by the weekly handling of the steers which could be a possible explanation for the lack of an effect on ADG and feed efficiency. Furthermore, the low statistical power of this study may also be a reason no differences were observed for ADG and G:F.

The goal of this experiment was to evaluate blood components related to growth with ZH supplementation. Glucose is an important source of energy for mammalian cell function. There was a ZH × sampling d interaction (*P* = 0.06) for whole blood glucose; concentration decreased over time with addition of ZH; however, there was no ZH × sampling d interaction (*P* = 0.37), ZH (*P* = 0.52), or sampling d (*P* = 0.68) effects for plasma glucose concentration (table 2.3). The difference between whole blood and plasma glucose concentration may reflect the inaccuracy of attempting to measure whole blood glucose samples when the measuring device is not on site. The active cells in the blood samples used for whole blood analysis may affect the ability to measure glucose values when the cells are given the opportunity to continue to metabolize glucose during transportation. Additionally, Hasegawa and Townley (1983) performed a study to determine down-regulation of beta receptors in spleen and lung tissue, both of which were found to contain beta-adrenergic receptors. Zilpaterol hydrochloride supplementation may induce

secretion of stored red blood cells in the spleen through activation of beta-adrenergic receptors, thus increasing the amount of cells available to metabolize glucose. In agreement with our results, Ricks et al. (1984) performed a study in which they fed 0, 10, or 500 mg/d of clenbuterol for 98 d to steers. The authors found no difference ($P > 0.05$) in plasma glucose concentration in steers fed clenbuterol compared to steers fed a control diet without clenbuterol. Quirke et al. (1988) administered the β_2-AA cimaterol to steers at rates of 0, 33, 49.5, or 66 mg/d. No effect ($P > 0.05$) on plasma glucose concentration was observed. Additionally, Beermann et al. (1987) performed a study using lambs which received 0 or 10 mg/kg cimaterol for 7 or 12 wk. The authors also observed no difference ($P > 0.05$) in plasma glucose concentration. Contradicting these results, Eisemann et al. (1988) performed a study in which steers were fed 8 mg/d of clenbuterol daily for 9 d. Blood samples were taken from steers on d 1 and 9. The authors observed an initial increase ($P = 0.02$) in glucose concentration; however, after d 9 of feeding, glucose concentrations were not different ($P > 0.05$). The authors suggested an initial increase in glucose rate of entry due to increased liver gluconeogenesis or glycogenolysis (Eisemann et al., 1988). Results from Eisemann et al. (1988) would suggest there is an initial response in glucose metabolism due to βAA administration, but cells become quickly desensitized and the response is no longer observed. These results would explain why our data showed no difference in plasma glucose concentration as our first sampling time was 7 d after administration of ZH.

Lactate is produced in cells that have high demand for glucose utilization. During glycolysis, glucose is broken down to produce pyruvate. As intracellular pyruvate concentration increases, 2 protons are absorbed, and lactate is produced enzymatically (Kravitz, 2005). We observed no ZH × sampling d interaction (P > 0.10) or effect of ZH ($P > 0.10$) for whole blood or plasma lactate, but an effect of sampling d ($P < 0.10$) was observed. Concentrations of whole blood and plasma lactate were lower on d 7, 14, and 21 when compared to d 0 (table 2.3). Blum and Flueckiger, (1988) fed P-5369 and Q-2636 to calves through milk replacer and observed an increase ($P < 0.05$) in concentrations of plasma lactate up to 10 h after administration of both β-agonists compared to control calves. Additionally, Byrem et al. (1996) found when cimaterol was administered to steers through arterial infusion, circulating concentrations of lactate increased ($P < 0.01$) with increasing concentrations of cimaterol. Similar findings for lactate concentrations were reported for clenbuterol suggesting an increase of peripheral glycolysis (Eisemann et al., 1988). Differences between our study and those cited may be the age difference

of the animals. Steers in our study were older than those previously utilized for study. Additionally, ZH may initially affect the demand for glucose similar to other βAA by stimulating glycogenolysis (Blum and Flueckiger, 1988) and possibly glycolysis, thus indirectly increasing concentration of lactate in plasma initially.

Decreased body fat is a common effect of βAA supplementation, and may be a consequence of increased fat mobilization, decreased lipogenic activity, or a combination of both (Yang and McElligott, 1989). Plasma NEFA concentration has been utilized as a blood parameter to estimate lipolysis as it exhibits a strong correlation to NEFA turnover (Beermann et al., 1987). In this study, no effects of ZH × sampling d interaction, ZH, or sampling d ($P = 0.97$; $P = 0.25$; $P = 0.78$, respectively) were observed for plasma NEFA concentrations in steers fed diets with and without ZH (table 2.3), which is in disagreement with previous research feeding other βAA. Beermann et al. (1987) observed a 60% increase ($P < 0.01$) in NEFA concentrations in lambs fed cimaterol, but the increased NEFA concentration was attributed to lambs being fasted 1 d prior to sampling. Quirke et al. (1988) administered 0, 33, 49.5, or 66 mg/d cimaterol to steers, and reported a quadratic increase ($P < 0.05$) in NEFA concentration. Steers fed 33 mg/d had NEFA concentrations lower than animals fed 0 mg/d cimaterol, and steers receiving 66 mg/d had the largest increase in NEFA concentration. Chikhou et al. (1991) performed a study using 12 steers fed 0 or 0.09 mg/kg BW cimaterol for a period of 13 d. The authors observed an increase in plasma NEFA concentration ($P < 0.05$) on d 1 and 13 of sampling, which was attributed to increased lipolysis and reduced uptake of free fatty acids by adipose tissue in steers fed cimaterol. Byrem et al. (1996) administered cimaterol to steers through arterial infusion, and circulating concentrations of NEFA increased ($P < 0.01$). In a follow up study, Byrem et al. (1998) observed an initial increase in plasma NEFA concentration after 6 h ($P < 0.05$) of close-arterial infusion of cimaterol, but after 24 h of infusion there was no difference ($P > 0.05$) in NEFA concentration compared to basal concentrations. The differences observed between the NEFA concentrations in our study and previous work may reflect physiological differences among animals, and the βAA utilized. Variable responses have been caused by use of different βAA because there has not been an individual βAA tailored to all species (Mersmann, 2002). Absorption rate, metabolism of the agonist by organs, and rate of excretion control the concentrations maintained in plasma and target organs (Mersmann, 2002). Moreover, fatty acid

synthesis and esterification of FFA into triacylglycerols both are inhibited by βAA. The decrease in lipogenesis with βAA administration would lead to decreased adipocyte hypertrophy, thereby decreasing fat deposition (Mersmann, 2002). Although not all βAA affect fat catabolism, there is proportionately less fat in carcasses due to increased skeletal muscle growth with βAA use. ZH may have greater impact on skeletal muscle growth than lipolysis, which could explain the relative absence of significant differences in blood parameters related to lipolysis.

Beta-hydroxybutyrate is a ketone body produced from metabolism of NEFA by the liver, and usually is elevated in states of negative energy balance (Ospina et al., 2010). We observed no ZH × sampling d interaction ($P = 0.97$) for BHB concentration (table 2.3), and no effects of ZH supplementation or sampling d ($P = 0.94$; $P = 0.74$, respectively). Likewise, Eisemann et al. (1988) observed no changes ($P > 0.10$) in BHB concentration when clenbuterol was administered to cattle. Collectively, these results suggest cattle are able to metabolize BHB concentrations at rates sufficient to avoid accumulation within blood. Another explanation to the lack of effect on BHB concentration may be correlated to the lack of effect of ZH on lipolysis evident by no response in plasma NEFA concentration.

Comparing PUN concentrations between treatments, there was a ZH × sampling d interaction ($P = 0.06$); cattle in the control and ZH treatments started with similar PUN concentrations, but cattle fed ZH had lower PUN concentration ($P < 0.05$) from d 7 to d 21 (figure 2.1). An increase in nitrogen retention suggests nitrogen is being utilized more efficiently for muscle accretion. Decreases in PUN concentrations following ZH supplementation are consistent with observations in previous experiments with other β_2-AA (Ricks et al., 1984; Eisemann et al., 1988; Quirke et al., 1988), which may reflect decreased protein catabolism in skeletal muscle with βAA supplementation.

A ZH × sampling d interaction ($P < 0.10$) was observed for plasma concentrations of elaidic (C18:1n9t), vaccenic (C18:1n7), and docosapentaenoic acids (C22:5n3). Additionally, an effect of d ($P < 0.10$) was observed for 9 of the analyzed fatty acids, and a tendency for a ZH × d interaction for total plasma fatty acid concentration ($P = 0.14$; summarized in table 2.4). Heneicosanoic acid (C21:0) was the only adipose tissue fatty acid (table 2.5) affected by ZH ($P = 0.005$). We concluded ZH supplementation had a minor effect on adipose tissue LCFA concentration. Engeseth et al. (1992) performed a study feeding ractopamine to pigs harvested at

1, 2, 4, and 6 wk of feeding. The authors collected adipose tissue at each harvest date and analyzed fatty acid profiles. Animals receiving ractopamine had lower 18:0 ($P < 0.05$) and higher 18:3 ($P < 0.05$) percentages after 4 wk of feeding, and a higher percentage of 18:2 fatty acid ($P < 0.01$) after wk 6 of feeding. Similarly, Engeseth et al. (1992) concluded ractopamine had only a minor effect on fatty acid profiles of adipose tissue. The limited changes observed in LCFA profiles of plasma and adipose tissue suggests there is no preferential oxidation of any specific fatty acid with ZH supplementation. Again, the lack of an effect on lipolysis may contribute to the minimal effect on fatty acid concentration.

Zilpaterol hydrochloride supplementation increased HCW by approximately 3% ($P = 0.02$), dressed yield percent by 1.9% ($P = 0.08$), and LM area by 11.9% ($P = 0.07$; table 2.6). Marbling score and yield grade were numerically lower with ZH supplementation, but these differences were not significant ($P = 0.12$, $P = 0.47$, respectively). Animals in the present study were not affected by liver abscesses. Additionally, there were no differences ($P > 0.10$) between cattle fed diets with and without ZH with respect to USDA quality grade, KPH, or 12th rib fat thickness. Increased HCW and dressing percentage have been observed consistently with ZH supplementation in feedlot cattle (Vasconcelos et al., 2008; Elam et al., 2009; and Hilton et al., 2009). Montgomery et al. (2009a) compared response to ZH in steers and heifers for periods of 20 or 40 d, and observed decreases in marbling scores and quality grades ($P = 0.002$) for steers, and a decrease in quality grade ($P < 0.05$) for heifers; however, ZH did not affect 12th rib fat thickness or KPH ($P > 0.10$) in carcasses from either sex. Additionally, Vasconcelos et al. (2008) observed larger LM area, lower yield grade, and lower marbling scores ($P< 0.01$) when comparing ZH supplemented for final 0, 20, 30, or 40 d for finishing periods consisting of 136, 157, 177, or 198 d on feed compared to control animals. Conversely, Placencia et al. (2008) fed steers 0 or 6 mg/kg ZH (as fed basis) to steers and reported no effect of ZH on LM area, quality grade, or marbling score ($P > 0.20$) when compared to un-treated steers. Our results suggest increases in HCW are due to increased skeletal muscle mass, which is consistent with observations of increased LM area in treated steers. The lack of effect on carcass measurements associated with fat deposition may be a consequence of limited statistical power with the small population size of cattle we utilized for this study compared to previously cited studies.

In conclusion, feeding ZH decreased PUN concentrations indicating nitrogen may be utilized more efficiently to accommodate increased carcass weight and LM area in supplemented

steers. Blood constituents, and carcass measurements related to lipolysis were mostly unchanged, which is suggestive of ZH having a smaller effect on adipose tissue lipolysis compared to skeletal muscle accretion.

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Table 2.1: Diet Composition, DM basis

Ingredients	%, DM basis
Wet distiller's grain with solubles	30.00
Steam flaked corn	58.32
Wheat straw	7.00
Monensin/tylosin premix[1]	2.16
Vitamin/mineral premix[2]	0.41
Limestone	1.81
Salt	0.30
Analyzed composition, %	
CP	15.2
Ca	0.7
P	0.4
K	0.8
NDF	18.6

[1]Formulated to provide 300 mg/d monensin and 90 mg/d tylosin (Elanco Animal Health, Greenfield, IN) in a ground corn carrier.

[2]Formulated to provide; 2,200 IU/kg vitamin A; 22 IU/kg vitamin E; 10 mg/kg added Cu; 60 mg/kg added Zn; 60 mg/kg added Mn; 0.5 mg/kg added I; 0.25 mg/kg added Se, and 0.15 mg/kg Co. Zilpaterol-HCl (Merck Animal Health, Millsboro, DE), was fed 23 d at 8.33 mg/kg of diet DM followed by a 3-d withdrawal prior to harvest.

Table 2.2: Feedlot performance of steers fed 0 or 8.33 mg/kg diet DM zilpaterol hydrochloride (ZH)[1]

Item	Control	ZH	SEM	*P*-Value
No. steers	8	9	-	-
Initial BW, kg[a]	614	615	10.48	0.60
Final BW, kg[a]	658	660	13.76	0.72
DMI, kg/d	11.87	10.95	0.46	0.03
ADG, kg	1.67	1.73	0.54	0.83
G:F	0.1399	0.1535	0.0191	0.56

[1]ZH (Merck Animal Health, Millsboro, DE) was fed for 23 d followed by a 3-d withdrawal prior to harvest.

[a]Initial and final BW are calculated as gross BW $\times$ 0.96.

Table 2.3: Concentrations of blood metabolites at weekly intervals for steers fed 0 or 8.33 mg/kg diet DM zilpaterol hydrochloride (ZH)[1]

	D 0		D 7		D 14		D 21		
Item, mM	Control	ZH	Control	ZH	Control	ZH	Control	ZH	SEM[2]
Whole blood glucose[‡]	3.32[ab]	3.39[b]	3.33[ab]	3.01[a]	3.48[ab]	3.12[a]	3.48[ab]	3.09[a]	0.24
Whole blood lactate[†]	2.83	2.80	2.45	1.43	2.37	1.37	2.16	1.54	0.48
Plasma glucose	5.17	5.21	5.33	4.77	5.23	4.96	5.38	5.10	0.36
Plasma lactate[†]	4.28	4.29	4.00	2.31	3.71	2.19	3.20	2.62	0.79
NEFA	126	175	172	192	164	184	140	174	36
BHB[3]	0.06	0.07	0.09	0.08	0.07	0.08	0.09	0.08	0.02

[1]ZH (Merck Animal Health, Millsboro, DE) was fed for 23 d followed by a 3-d withdrawal before harvest.

[‡]ZH × d interaction, $P < 0.10$.

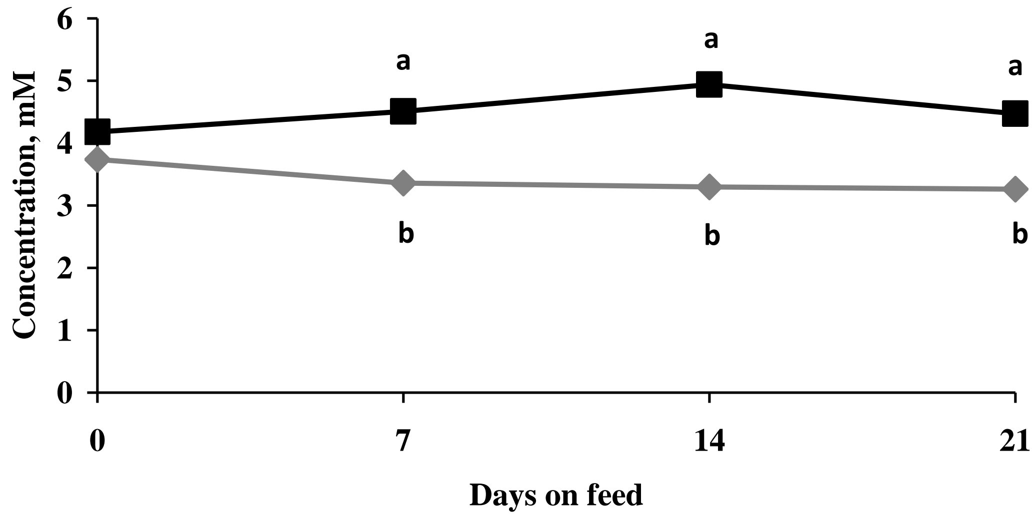
[ab]Within a row, means without a common superscript letter are different; $P < 0.10$.

[†]Effect of d, $P < 0.10$.

[2]SEM listed is greater SEM of two treatments.

[3]Beta hydroxybutyrate, BHB.

10 **Figure 2.1: Concentration of plasma urea nitrogen at weekly intervals for steers fed 0 (■)**
11 **or 8.33 (♦) mg/kg diet DM zilpaterol hydrochloride.**
12 ZH × sampling d interaction, $P = 0.06$; effect of d, $P = 0.69$; effect of ZH, $P < 0.05$; SEM = 0.24
13 [a, b] means within d with different letters are different, $P = 0.06$.



14

15 **Table 2.4: Plasma concentrations of long-chain fatty acids at weekly intervals for steers fed 0 or 8.33 mg/kg diet DM zilpaterol**
16 **hydrochloride (ZH)[1]**

	D 0		D 7		D 14		D 21		
Item (*u*g/g)[2]	Control	ZH	Control	ZH	Control	ZH	Control	ZH	SEM
C14:0	16.7	20.0	16.4	16.0	18.9	18.9	17.6	18.2	1.91
C14:1	12.7	13.6	11.5	10.8	10.6	13.6	12.6	14.0	1.16
C15:0[†]	21.4	20	20.9	23	24.9	23.9	27.1	27.9	1.64
C15:1	6.3	7.7	6.4	7	5.6	7.7	7.4	6.8	0.99
C16:0[†]	206	199	204	219	205	237	210	242	13.6
C16:1[†]	17	20	12.4	15	14.3	13.6	12.1	11.4	1.80
C17:0	24.2	26.2	25.4	26.8	28.8	30.7	25.6	28.7	2.46
C17:1	6.6	9.4	5.8	6.1	6.5	7.2	6.5	6.1	1.18
C18:0[†]	297	302	345	342	343	391	373	405	27.3
C18:1n9t[†,‡]	41.7[abc]	36.5[a]	60.3[d]	52.3[cd]	35.9[ab]	50.2[bcd]	40.1[ab]	62.6[d]	6.88
C18:1n11[†]	0	1.2	1.7	0	1.6	0.9	4.7	3	1.44
C18:1n9c[†]	121	121	108	108	108	119	111	125	8.37
C18:1n7[‡]	14.5[abc]	12.4[a]	14.9[abc]	13.7[ab]	12.7[ab]	15.1[bc]	12.7[ab]	16.1[c]	1.12
C18:2n6t	4.3	1.9	5.5	2.6	2	3.3	2.7	2.8	1.20
C18:2n6c[†]	1,201	1,142	1,224	1,286	1,246	1,380	1,270	1,478	104
C18:3n6	18.1	19.7	21	15.8	17.6	18.1	13.1	18.1	2.70
C18:3n3[†]	34.8	31.9	27	28.7	25.8	23.6	25.1	26.4	3.17
C20:3n6	33.6	41.5	26.7	35.5	31.4	30.3	28.8	38.3	5.27
C20:4n6	2.6	2.3	2.9	4.4	3.7	2.7	2.1	4.3	1.18
C20:5n3	4	2.3	4.4	2.1	3.3	2.9	3.6	5.3	1.38
C22:5n3[‡]	31.6[ab]	28.5[ab]	35.7[b]	25.7[a]	22[a]	34.3[ab]	26.5[ab]	39.8[b]	6.76
C22:6n3	33.6	25.4	41.1	27.6	27.7	39.6	30.5	42.7	10.59
Total LCFA[†]	2,149	2,082	2,221	2,268	2,194	2,463	2,262	2,623	164

17 [1]ZH (Merck Animal Health, Millsboro, DE) was fed for 23 d followed by a 3-d withdrawal prior to harvest.

18 [2]Nomenclature of fatty acids is as follows: example, C14:0 = a fatty acid with 14 carbon atoms and no double bonds; C18:1n9c = a
19 fatty acid with 18 carbon atoms and 1 double bond located at the 9th carbon in the cis configuration; “t” indicates the double bond is
20 in the trans configuration.
21 [†]Effect of d, $P < 0.10$.
22 [‡]Treatment × d interaction, $P < 0.10$.
23 [abcd]Within a row, means without a common superscript letter are different, $P < 0.10$.

24 **Table 2.5: Concentrations of long-chain fatty acids from steers fed 0 or 8.33 mg/kg diet**
25 **DM zilpaterol hydrochloride (ZH)[1] within adipose tissue collected from subcutaneous fat**
26 **covering lumbar vertebrae.**

Item, % of sample[2]	Control	ZH	SEM	*P*-Value
C14:0	2.514	2.557	0.146	0.84
C14:1	0.358	0.341	0.015	0.46
C15:0	0.012	0.011	0.001	0.59
C15:1	0.013	0.020	0.003	0.11
C16:0	18.468	18.114	0.502	0.54
C16:1	3.052	3.140	0.262	0.76
C17:0	1.122	1.028	0.052	0.18
C17:1	0.593	0.540	0.023	0.14
C18:0	11.44	11.25	0.742	0.86
C18:1n9t	2.731	2.834	0.210	0.73
C18:1n11	0.432	0.477	0.035	0.38
C18:1n9c	29.69	28.64	0.639	0.15
C18:1n7	1.284	1.264	0.051	0.78
C18:2n6t	0.159	0.176	0.010	0.21
C18:2n6c	1.547	1.580	0.121	0.85
c9, t11 CLA	0.053	0.063	0.006	0.17
t10, c12 CLA	0.011	0.015	0.005	0.60
c9, c11 CLA	0.002	0.006	0.002	0.22
t9, t11 CLA	0.203	0.235	0.021	0.20
C18:3n6	0.043	0.042	0.007	0.92
C18:3n3	0.132	0.139	0.012	0.66
C20:0	0.064	0.081	0.010	0.26
C20:1	0.180	0.189	0.019	0.76
C20:2	0.0799	0.0804	0.007	0.96
C20:3n6	0.046	0.052	0.007	0.52
C20:4n6	0.0096	0.0104	0.002	0.74
C20:5n3	0.007	0.004	0.002	0.37
C21:0	0.101	0.219	0.021	0.01
C22:0	0.018	0.014	0.005	0.45
C22:5n3	0.0049	0.0045	0.002	0.87
C22:6n3	0.006	0.009	0.002	0.30
C24:0	0.008	0.005	0.002	0.22
C24:1	0.003	0.004	0.001	0.56
Total fatty acids	74.9	73.8	1.84	0.66

27 [1]ZH (Merck Animal Health, Millsboro, DE) was fed for 23 d followed by a 3-d withdrawal
28 prior to harvest.
29

30 [2]Nomenclature of fatty acids are as follows: example, C14:0 = a fatty acid with 14 carbon atoms
31 and no double bonds; C18:1n9c = a fatty acid with 18 carbon atoms and 1 double bond located
32 at the 9th carbon and is in the cis configuration; "t" indicates the double bond is in the trans
33 configuration. c9, t11 CLA = conjugated linoleic acid with double bonds located on the 9th (cis
34 configuration) and the 11th (trans configuration) carbon atoms.
35

36

37 **Table 2.6: Carcass characteristics for steers fed 0 or 8.33 mg/kg diet DM zilpaterol**

38 **hydrochloride (ZH)[1]**

Item	Control	ZH	SEM	*P*-Value
HCW, kg	415	429	8.98	0.02
Dressed yield, %	63.1	65.0	0.68	0.08
Liver abscesses, %	0	0	-	-
LM area, cm^2	88.64	99.22	3.55	0.07
12th rib fat, cm	0.92	0.84	0.11	0.63
KPH, %	2.06	2.06	0.169	0.98
USDA yield grade	2.6	2.0	0.26	0.12
Marbling score[a]	408	378	28.5	0.47
USDA Choice, %	26.1	44.4	17.6	0.44
USDA Select, %	73.9	55.6	17.6	0.44

39 [1]ZH (Merck Animal Health, Millsboro, DE) was fed for 23 d followed by a 3-d withdrawal

40 prior to harvest.

41 [a] Marbling scores were determined by a USDA grader; Slight = 300 to 399;

42 Small = 400 to 499; Modest = 500 to 599.

43

44 # Chapter 3 - Manipulation of Dietary Calcium Concentration to
45 Potentiate Changes in Tenderness of Beef from Heifers
46 Supplemented with Zilpaterol Hydrochloride

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50

51 ## Abstract

52 Dietary calcium concentrations were manipulated during supplementation of zilpaterol
53 hydrochloride (ZH) to evaluate impact on feedlot performance, carcass characteristics, and beef
54 tenderness using 96 heifers (BW 392 kg ± 3.2). We hypothesized that temporary depletion
55 followed by repletion of dietary calcium prior to harvest would increase intracellular calcium
56 concentrations, thus stimulating postmortem activity of calcium-dependent proteases to effect
57 changes in tenderness. Heifers were stratified by initial BW and randomly assigned, within
58 strata (block), to treatments consisting of a finishing diet in which calcium was added in the
59 form of limestone (+Ca) or removed (-Ca) during ZH supplementation. Cattle were fed a
60 common diet prior to ZH supplementation, and 28 d prior to slaughter ZH was added to the diet
61 with and without supplemental calcium. Calcium content of the diets during ZH
62 supplementation was 0.74% or 0.19% (diet DM) for +Ca and -Ca, respectively. Zilpaterol
63 hydrochloride was fed for 25 d then removed from the diet 3 d prior to harvest. The final 3 d
64 before harvest all cattle were fed calcium at 0.74% of diet DM. Heifers were housed in concrete
65 surfaced pens with 8 animals/pen (6 pens/treatment). At the end of the finishing phase animals
66 were weighed and transported to an abattoir in Holcomb, Kansas. Severity of liver abscesses
67 and HCW were collected the day of harvest, and after 48-h of refrigeration USDA yield and
68 quality grades, KPH, LM area, and 12th rib subcutaneous fat thickness were determined.
69 Additionally, boneless loin sections were collected for Warner-Bratzler shear force
70 determination. Removal of calcium did not affect Warner-Bratzler shear force values ($P = 0.64$).
71 In addition, ADG, DMI, final BW and feed efficiency were unaffected by treatment ($P > 0.05$).
72 Carcass measurements also were unaffected by the temporary decrease in dietary calcium ($P >$
73 0.05). In conclusion, temporary depletion of dietary calcium did not alter beef tenderness, live
74 animal performance, or carcass measurements.
75 Key words; beef tenderness, calcium, ruminant, zilpaterol hydrochloride

76 ## Introduction

77 The addition of a zilpaterol hydrochloride (ZH) to diets of finishing cattle has
78 consistently demonstrated a decrease in meat tenderness (Leheska et al., 2009; Shook et al.,

79 2009; Garmyn et al., 2010). Tenderness is one of the most important characteristics of meat for
80 consumer acceptance (Koohmaraie, 1992). The calpain proteolytic system consists of calcium
81 dependent proteolytic enzymes m-calpain and μ-calpain, and the inhibitor calpastatin (Goll et
82 al., 1998), and is mostly responsible for post-mortem tenderization of meat. There is evidence to
83 suggest that increasing calcium ion concentrations in skeletal muscle cells results in weakening
84 of myofibrillar structures, which aids in post-mortem tenderization (Koohmaraie, 1992). To
85 potentiate the decrease in tenderness, calcium chloride has been infused in meat post-mortem
86 and observed to increase tenderness (Morgan et al., 1991).
87 Removal of calcium from diets of dairy cows prior to parturition has been used to
88 activate homeostatic mechanisms required to prevent milk fever (Thilsing-Hansen et al., 2002).
89 Decreased dietary calcium stimulates parathyroid hormone (PTH), which is necessary for
90 calcium reabsorption from glomerular filtrate and resorption of calcium stored in bone (Horst et
91 al., 1997). Activation of homeostatic mechanisms during periods of temporary depletion of
92 calcium prior to parturition in dairy cattle resulted in decreased incidences of milk fever
93 (Hillman, 1967).We hypothesized that temporary depletion of dietary calcium could be
94 exploited to increase bone resorption, and when followed by repletion of normal dietary calcium
95 concentration, elevate intracellular calcium concentration temporarily, thus stimulating
96 postmortem activity of calcium-dependent proteases. The objective of our study was to
97 temporarily deplete dietary calcium during ZH administration in an effort to overcome adverse
98 effects of ZH on meat tenderness.

99 Materials and Methods

100 *Animals and sampling*

101 Protocols and procedures followed in this study were approved by the Kansas State
102 University Institutional Animal Care and Use Committee. The study was conducted at the
103 Kansas State University Beef Cattle Research Center in Manhattan, KS.
104 Ninety six crossbred heifers (initial BW = 392 kg ± 3.2) were stratified by BW and
105 randomly allocated, within strata, to two treatment groups for a 116-d finishing trial. Heifers
106 were identified using ear tags that displayed a number unique to each study animal. Cattle were
107 allotted to feeding pens equipped with concrete floors, fence line feed bunks, and automatic

108 water fountains with 8 animals/pen (6 pens/treatment). Heifers were fed once daily to allow *ad*
109 *libitum* intake of feed. A pre-ZH diet (0.78% calcium, DM basis) was fed until the final 28 d of
110 finishing, at which time ZH was included in the diet. Zilpaterol hydrochloride was fed at a rate
111 of 8.33 mg/kg of diet DM for a period of 25 d, and then withdrawn from diets 3 d before
112 harvest. Treatments consisted of diets containing supplemental calcium (+Ca; 0.74% of DM) in
113 the form of limestone, or a diet from which limestone had been removed (-Ca; 0.19% of DM)
114 during ZH supplementation. Diets are presented in table 3.1. The final 3 d before harvest cattle
115 were fed a common diet containing 0.74% calcium (DM basis).
116 Feed deliveries to each pen were monitored daily, and unconsumed feed was removed
117 from the bunk, weighed, and dried at weekly intervals or as needed to determine actual feed
118 DMI. Feedstuffs were sampled weekly and analyzed for DM, starch, NDF, CP, S, and ether
119 extract. Portions of ground samples of feedstuffs were dried in a forced air oven at 105°C
120 overnight to determine DM (Undersander et al., 1993). Starch contents of feedstuffs were
121 determined according to Herrera-Saldana and Huber (1989) with a Technicon Autoanalyzer III
122 (SEAL Analytical, Mequon, WI). Determination of NDF was conducted using an ANKOM
123 fiber analyzer (ANKOM Technology Corp., Fairport, NY) according to Van Soest et al. (1991).
124 Heat-stable α-amylase (ANKOM Technology Corp) was added to remove residual starch from
125 feedstuff samples. Determination of CP was accomplished by measuring N content with a
126 LECO FP–2000 nitrogen analyzer (LECO Corp., St Joseph, MI) following AOAC (1995)
127 official method 990.03. Ether extract was performed according to AOAC (1995) official method
128 920.39.
129 Average daily gains were computed by subtracting pre-ZH shrunk BW from final shrunk
130 BW (gross BW * 0.96), and dividing by days on feed (DOF). Gain efficiencies were computed
131 by dividing ADG by DMI. Body weights were measured for each pen at 28-d intervals to
132 determine proper time for ZH supplementation. Body weights were measured prior to ZH
133 supplementation and shrunk final BW (gross BW * 0.96) were determined immediately before
134 cattle were shipped on the d of harvest. Heifers were harvested when they achieved an estimated
135 12th-rib subcutaneous fat thickness of 1cm. Heifers were then loaded onto a truck and
136 transported 451 km to a commercial abattoir in Holcomb, Kansas. Incidence and severity of
137 liver abscesses and HCW were recorded the day of harvest. Liver abscesses were scored
138 according to the Elanco scoring system (Liver Abscess Technical Information AI 6288, Elanco

139 Animal Health, Greenfield, IN): 0 = no abscesses; A^- = 1 or 2 small abscesses or abscess scars;
140 A^0 = 2 to 4 small well-organized abscesses; and A^+ = 1 or more large or active abscesses with or
141 without adhesions. Yield Grade, quality grade, marbling score, 12th-rib subcutaneous fat
142 thickness, LM area, percent KPH, and incidence and severity of dark cutting beef were
143 determine after 48 h of refrigeration. Finally, a loin section weighing approximately 800 g was
144 collected from one side of each carcass, placed on ice, and transported to Kansas State
145 University, Manhattan, KS.

146 ***Warner-Bratzler shear force and moisture determinations***

147 Loin sections were refrigerated overnight at 1ºC after arrival at Kansas State University.
148 The following d samples were weighed and placed into 15.2 × 30.4 cm vacuum bags (3 mil
149 standard barrier, Prime Source Vacuum Pouches; Bunzl Processor Division, Koch Supplies,
150 Kansas City, MO) that had an oxygen transmission rate of $3.5 \text{ g} \cdot 645 \text{ cm}^{-2} \cdot 24 \text{ h}^{-1}$ at 21ºC, and
151 water vapor transmission rate of $0.6 \text{ mL} \cdot 645 \text{ cm}^{-2} \cdot 24 \text{ h}^{-1}$ at 38ºC. The bags were vacuum
152 sealed and aged for 14 d post-mortem at a temperature of 1ºC. Following aging, loin sections
153 were removed from the bags, patted dry using paper towels, and weighed. Percent purge loss
154 was calculated as: [(pre-aged weight – aged weight)/pre-aged weight] × 100. Prior to cooking, a
155 single 2.54-cm steak was removed from the 13th rib face for Warner-Bratzler shear force
156 determination.

157 Steaks for Warner-Bratzler Shear (WBSF) were weighed and then cooked in a Blodgett
158 oven (model number DFG-102, The G.S. Blodgett Company, Inc. Burlington, VT) at a
159 temperature of 163°C. Thermocouple wires (thirty gauge copper and constantan; Omega
160 Engineering, Stamford, CT) were inserted into steaks and internal temperatures were monitored
161 using a Doric Minitrend 205 (VAS Engineering, San Francisco, CA). Steaks were turned when
162 internal temperature reached 40°C and removed from the oven when an internal temperature of
163 70°C was achieved. Steaks were allowed to temper for handling and then reweighed to calculate
164 percent moisture loss during cooking as: [(raw weight – cooked weight)/raw weight] × 100.
165 Steaks were then covered with plastic wrap and refrigerated at approximately 3 to 4°C for 24 h.
166 Following 24-h of refrigeration, eight 1.25-cm diameter cores were taken from each steak
167 parallel to the muscle fiber orientation and sheared perpendicular to the fiber orientation on an
168 INSTRON Model 5569 Universal Testing Machine (Canton, MA) equipped with a Warner-

169 Bratzler shear head. A 100-kg compression load cell was used with a crosshead speed of 250
170 mm/min. Shear force was determined for each core, and the mean of 8 core samples was
171 calculated.

172 ***Statistical Analyses***

173 The experiment was designed as a randomized complete block experiment with pen as
174 the experimental unit. The MIXED procedure of SAS version 9.2 (SAS Inst. Inc., Cary, NC)
175 was used to analyze performance, carcass, and shear force data. Incidence of liver abscess and
176 USDA quality grade were analyzed using the GLIMMIX procedure of SAS. Models included
177 calcium as the fixed effect and block as the random effect. For performance data, pre-treatment
178 BW, ADG, DMI and G:F were utilized as covariates for final BW, ADG, DMI, and G:F. The
179 LSMEANS statement was used for means and standard error calculations, and the PDIFF option
180 used for separation of means. Separation of means were determined to be significant at α level $\leq$
181 0.05.

182 Results and Discussion

183 One animal from the -Ca treatment was removed from the study due to a physical injury.
184 Final BW, ADG, DMI and feed efficiency (table 3.2) were not different for heifers fed
185 -Ca and +Ca ($P > 0.05$). In agreement with our results, Jackson and Hemken (1994) performed
186 a study to evaluate effects of calcium and cation-anion balance in Holstein calves. Calcium
187 concentrations were 0.42 or 0.52% of diet DM, yielding dietary anion-cation balances of -18 or
188 +13 for the low and high calcium concentrations, respectively. The authors observed no
189 differences in ADG or DMI ($P > 0.05$) between the two calcium concentrations, which
190 indicated 0.42% calcium concentration was adequate. Varner and Woods (1972) conducted 2
191 trials that used 24 steers/trial. Each trial had a length of 137 d. Steers were fed diets that
192 contained 0.20, 0.31, 0.42, or 0.53% (diet DM) calcium. The authors observed an increase in
193 ADG, DMI, and feed efficiency when steers were supplemented with 0.31 and 0.42% calcium
194 ($P < 0.05$), but found no further improvement with 0.53% calcium supplementation. Cattle in
195 the study performed by Varner and Woods (1972) were fed calcium deficient diets for a longer
196 period of time compared to heifers in our experiment possibly explaining the contrasting results.
197 The 25-d depletion of calcium in the current experiment may not have been sufficiently long to

198 observe differences in animal performance. Cattle that are beyond the primary stage of bone
199 growth can be fed calcium deficient diets for an extended period of time without developing
200 signs of calcium deficiency if previous calcium intake was adequate (NRC, 1996). Our results
201 indicate temporary depletion of calcium did not affect performance of feedlot heifers.

202 Tenderness is one of the most important characteristics of meat for consumer acceptance
203 (Koohmaraie,1992). Removing supplemental calcium from the diet had no effect on WBSF
204 values ($P > 0.05$; presented in table 3.3). Additionally, percent moisture lost as purge and during
205 cooking was not affected by calcium depletion ($P > 0.05$). Hilton et al. (2009) conducted a
206 study in which ZH was administered with and without monensin and tylosin. The authors
207 demonstrated no difference in percent weight lost during cooking ($P > 0.10$) with and without
208 ZH. Duckett et al. (2000) administered a calcium gel to steers 3 to 6 h prior to slaughter, and
209 observed steaks from steers treated with oral calcium gel had lower WBSF values at early
210 postmortem ages ($P < 0.05$), but not at later postmortem ages ($P > 0.05$) compared to steers that
211 did not receive oral calcium gel. In agreement with our study, Spears et al. (2003) observed that
212 adding calcium propionate at 4% (as fed) to diets of steers 7 d prior to slaughter did not affect
213 WBSF values ($P > 0.05$). Lack of effect on WBSF in the current study may suggest we can't
214 substantially alter intracellular calcium concentration by this method, or 3-d replenishment was
215 enough time for the heifers to reach plasma calcium homeostasis. Pickard (1975) demonstrated
216 dairy cows plasma calcium levels returned to normal 2 days after calving in all cows tested.
217 Another explanation for the lack of an effect on tenderness may be there was not a sufficient
218 amount of calcium in the diet during replenishment to increase intracellular calcium.
219 Additionally, Thilsing-Hansen et al. (2002) indicated dairy diets need to provide fewer than 20
220 g of calcium daily to release PTH. In our study, heifers fed the -Ca diet consumed
221 approximately 26 g/d, which may not have been sufficiently low to activate homeostatic
222 mechanisms.

223 Carcass characteristics in this study were unaffected by supplemental calcium ($P > 0.05$;
224 table 3.4). Varner and Woods (1972) observed no differences in carcass characteristics when
225 steers were fed diets containing 0.20, 0.31, 0.42, or 0.53% (diet DM) calcium. Additionally,
226 Duckett et al. (2000) and Spears et al. (2003) observed no differences in carcass traits when
227 steers were administered calcium gel 3 to 6 h before slaughter or calcium propionate 7 d before
228 slaughter, respectively. The lack of differences in carcass measurements may be attributed to

229 adequate storage of calcium in bone of research animals to negate calcium deficiencies
230 commonly found in young animals that have received inadequate dietary calcium (NRC, 1996).
231 In conclusion, temporarily depleting calcium from finishing diets during ZH
232 supplementation did no impact WBSF values, carcass traits, or live performance indicating we
233 did alter tenderness by temporary depletion of dietary calcium.
234

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300

301

302

303 **Table 3.1: Diet Composition, DM basis**

Ingredients, %	Pre-ZH	+Ca	-Ca
Dry-rolled corn	35.92	30.97	32.40
Wet corn gluten feed	29.96	35.00	35.00
Corn silage	9.99	10.00	10.00
Soybean hull pellets	19.97	20.00	20.00
Feed additive premix[1]	2.16	2.16	2.16
Vitamin/trace mineral premix[2]	0.12	0.12	0.12
Limestone	1.56	1.45	-
Salt	0.32	0.30	0.32
Analyzed composition, %			
CP	14.07	14.80	14.94
Ca	0.78	0.74	0.19
P	0.48	0.51	0.51
K	0.85	0.88	0.89
NDF	29.12	30.37	30.50

304 [1]Formulated to provide 300 mg/d monensin and 90 mg/d tylosin (Elanco Animal Health,
305 Greenfield, IN); and 0.5 mg MGA (Elanco Animal Health, Greenfield, IN) in a ground corn
306 carrier; Zilpaterol-HCl (Merck Animal Health, Millsboro, DE) was fed for 25 d at 8.33 mg/kg
307 of diet DM followed by a 3-d withdrawal prior to harvest.

308 [2]Formulated to provide 2,200 IU/kg vitamin A; 22 IU/kg vitamin E; 10 mg/kg added Cu; 60
309 mg/kg added Zn; 60 mg/kg added Mn; 0.5 mg/kg added I; 0.25 mg/kg added Se; and 0.15
310 mg/kg added Co.

311

312

313 **Table 3.2: Feedlot performance for heifers fed with and without supplemental calcium**

314 **during zilpaterol hydrochloride (ZH) supplementation[1]**

Item	+Ca	-Ca	SEM	*P*-value
Pre ZH BW, kg[a]	547	548	5.91	0.85
Final BW, kg[a]	580	583	2.09	0.29
ADG, kg	0.93	1.01	0.05	0.37
DMI, kg	11.93	11.03	0.31	0.11
G:F	0.0792	0.0904	0.006	0.42

315 [1]ZH (Merck Animal Health, Millsboro, DE) was supplemented at a rate of 8.33 mg/kg diet DM

316 for 25 d followed by a 3-d withdrawal prior to harvest.

317 [a]Pre ZH and final BW calculated as gross BW × 0.96.

318

319

320

321 **Table 3.3: Carcass characteristics for heifers fed with and without supplemental calcium**

322 **during zilpaterol hydrochloride (ZH) supplementation[1]**

Item	+Ca	-Ca	SEM	*P*-value
HCW, kg	372	374	3.76	0.57
Dressed yield, %	64.17	64.10	0.36	0.85
Liver abscesses, %	2.08	8.33	4.27	0.20
USDA yield grade	2.57	2.42	0.12	0.23
LM area, cm^2	94.0	96.0	1.59	0.27
12th rib fat thickness, cm	1.40	1.50	0.14	0.55
KPH, %	3.14	3.13	0.10	0.89
Marbling score[a]	496	501	14.63	0.73
USDA Prime, %	2.39	2.08	3.16	0.93
USDA Choice, %	82.74	87.50	6.37	0.49
USDA Select, %	10.71	6.25	5.77	0.47
Low grade, %	2.08	4.17	3.29	0.36
No roll, %	2.08	-	1.47	0.36

323 [1]ZH (Merck Animal Health, Millsboro, DE) was supplemented at a rate of 8.33 mg/kg diet DM

324 for 25 d followed by a 3-d withdrawal prior to harvest.

325 [a]Marbling scores were determined by a USDA grader; Slight = 300 to 399;

326 Small = 400 to 499; Modest = 500 to 599.

327

328

329 **Table 3.4: Meat tenderness and cooking attributes for heifers fed with and without**
330 **supplemental calcium during zilpaterol hydrochloride (ZH) supplementation[1]**

Item	+Ca	-Ca	SEM	*P*-value
Purge loss, %[a]	1.63	1.96	0.002	0.18
Moisture lost during cooking, %[b]	19.5	19.3	0.01	0.83
Shear force, kg	4.64	4.58	0.12	0.64

331 [1]ZH (Merck Animal Health, Millsboro, DE) was supplemented at a rate of 8.33 mg/kg diet DM
332 for 25 d followed by a 3-d withdrawal prior to harvest. Loin sections were aged 14 d post-
333 mortem.
334 [a]Purge loss refers to the amount of moisture lost during the aging process.
335 [b]Moisture lost during the cooking process when LM steaks were cooked to an internal
336 temperature of 70ºC.

337

338 # Chapter 4 - Effects of Menthol on Blood Metabolites in Cattle

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345

346 ## Abstract

347 Effects of menthol on blood metabolites were evaluated using Holstein steers (n = 52,
348 BW 573 ± 9.92 kg) that were blocked by BW and assigned within block to treatment.
349 Treatments consisted of 0%, 0.003%, 0.03%, or 0.3% crystalline menthol (DM basis) added to
350 the diet. Animals were housed in individual, partially covered pens equipped with feed bunks
351 and automatic water fountains. On d 1 of the experiment, blood samples were obtained via
352 jugular venipuncture at 0, 6, 12, 18, and 24 h after feeding. Treatment administration
353 commenced on d 2, and blood samples were again drawn at 0, 6, 12, 18, and 24 h after feeding.
354 This blood-sampling schedule was repeated on d 9, 16, 23, and 30. Plasma was analyzed for
355 plasma urea nitrogen (PUN), glucose, and lactate concentrations. Serum was used to analyze
356 IGF-1 concentration. Additionally, BW was measured on d 1, h 0 and again at each time point
357 on d 9, 16, 23, and 30. In addition to the live animal phase of the study, *in vitro* fermentations
358 were performed using ruminal fluid cultures. Measurements included VFA concentrations, and
359 fermentative gas production for cultures containing crystalline menthol at 0%, 0.003%, 0.03%,
360 or 0.3% of substrate DM. Addition of menthol to the diet of steers resulted in a menthol × time
361 within d interaction ($P < 0.01$) for concentration of IGF-1 and BW. Cattle fed 0.003% menthol
362 had greater serum concentrations of IGF-1 at the start of the study compared to other treatments
363 and maintained a greater concentration until d 23 when compared to 0.03% and 0.3% menthol
364 treated steers. A menthol × d interaction (P < 0.05) was observed for daily feed deliveries: cattle
365 fed 0.3% menthol consumed less feed d 1 thru 13. Glucose, lactate, and PUN concentrations
366 were unaffected by addition of menthol to the diet ($P > 0.05$). Furthermore, *in vitro*
367 concentrations of VFA were unaffected by menthol ($P > 0.05$); however, menthol, regardless of
368 concentration, decreased ($P < 0.01$) fermentative gas production *in vitro*. In summary, increased
369 concentrations of menthol had lower IGF-1 concentrations; however, other blood constituents
370 related to growth were unaffected by addition of menthol to the diet. Menthol decreased the
371 amount of gas produced by ruminal microorganisms, but had no effect on concentrations of
372 VFA produced.
373 Key words; IGF-1, menthol, plasma urea nitrogen, ruminant

374 ## Introduction

375 Menthol is a naturally occurring compound classified as an essential oil that gives plants
376 of the *Mentha* species their minty characteristic aroma and flavor. Menthol is utilized as a
377 cooling compound in products ranging from common cold medications to pesticides, and has
378 been found to have a wide range of biological activities on different systems within the body.
379 Menthol affects smooth muscle in the lungs and heart by interfering with movement of calcium
380 across the cell membrane (Eccles, 1994). Santos et al. (2011) discussed the ability of menthol to
381 dilate human forearm cutaneous vessels and induce vasodilation in rat arteries. Menthol also
382 affects TRP-independent channels, which can affect cellular calcium transport (Kim et al., 2008,
383 Manhieu et al., 2007, Swandulla et al., 1986). More recently, studies with livestock have
384 utilized menthol and other essential oils as potential alternatives to feed antibiotics and growth
385 promotants (Soltan, 2010).

386 Menthol has been observed to directly affect κ-opioid receptors causing antinociception
387 in mice (Galeotti et al., 2002). Kappa opioid receptors are located in the central nervous system
388 with a high density found in the hypothalamus (Meng et al., 1993). In a preliminary experiment,
389 improvements in growth and increases in circulating concentrations of IGF-1 were observed
390 with short-term oral administration of crystalline menthol (Parsons et al., Kansas State
391 University, Manhattan, KS, personal communication). It was hypothesized menthol may be
392 capable of binding to κ-opioid receptors in the hypothalamus stimulating neuropeptides
393 involved in production and release of GH, thus increasing growth. Our objective was to evaluate
394 changes in blood metabolites when menthol was incorporated into diets of steers. We
395 hypothesized that menthol would increase concentrations of IGF-1 when fed to finishing cattle.

396 ## Materials and Methods

397 ### *Animals and Sampling*

398 Protocols and procedures followed in this study were approved by the Kansas State
399 University Institutional Animal Care and Use Committee.

400 The study was conducted using 52 Holstein steers (initial BW = 573 ± 9.92 kg)
401 obtained from a single source. Steers were weighed, stratified into weight blocks, and assigned
402 randomly within block to 4 treatment groups. Steers were housed in individual feeding stalls

403 equipped with fence line mangers and automatic water fountains. The pens were partially
404 covered and grouped into 3 barns with 20 animals in 2 barns and 12 animals in the third barn.
405 Diets consisted of 93% concentrate and 7% roughage (table 4.1). Treatments consisted of
406 menthol added at 0%, 0.003%, 0.03%, or 0.3% of diet DM. Barn A was fed at approximately
407 1500 h, Barn B was fed at approximately 1600 h, and Barn C was fed at approximately 1700 h
408 daily. Menthol was provided by Ind-Swift Laboratories Limited (Dover, DE)

409 On d 1 of the experiment, blood samples were obtained via jugular venipuncture at 0, 6,
410 12, 18, and 24 h after feeding. Treatment administration commenced on d 2, and blood samples
411 were again drawn at 0, 6, 12, 18, and 24 h after feeding. This blood sampling schedule was
412 repeated on d 9, 16, 23, and 30. Body weights were measured on d 1, h 0 and then at 0, 6, 12,
413 18, and 24 h post-feeding on d 9, 16, 23, and 30. Blood samples were collected using two 6-mL
414 blood tubes containing EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) as
415 anticoagulant for analysis of glucose, lactate, and plasma urea nitrogen (PUN). A third tube of
416 blood was collected using 10-mL serum sampling tubes (Vacutainer, Becton Dickinson,
417 Franklin Lakes, NJ) to obtain serum for IGF-1 analysis. Blood samples in tubes containing
418 EDTA were immediately centrifuged at 2,494 × g for 15 min at room temperature to recover
419 plasma. Plasma was transferred to 5-mL plastic tubes, capped, and frozen at -20ºC for
420 subsequent analysis of PUN, glucose, and lactate. Serum samples were allowed to coagulate for
421 1 h at 4ºC, then samples were centrifuged, and serum collected and stored as described for
422 plasma. In addition, 0.5 mL of plasma from tubes containing EDTA were transferred into 96
423 well vials and stored at -20ºC until shipment to Pfizer Animal Health (Florham Park, NJ) for
424 analysis of menthol and metabolites of menthol, which include p-menthane-3,8-diol (II), p-
425 menthane-3,9-diol (III), 3,8-oxy-p-menthane-7-carboxylic acid (IV), and 3,8-dihyroxy-p-
426 menthane-7-carboxylic acid (V).

427 Feed intakes were monitored daily, and unconsumed feed was removed from the bunk,
428 weighed, and dried at weekly intervals or as needed to determine actual DMI.

429 ***Chemical Analyses***

430 Glucose and lactate concentrations in plasma were analyzed using the YSI 2300 STAT
431 Plus Glucose and L-lactate Analyzer (YSI Inc., Yellow Springs, OH). Concentrations of PUN
432 were determined according to procedure described by Marsh et al. (1965) using the Auto

433 Analyzer II (Seal Analytical, Mequon, WI). Insulin-like glucose factor 1 was prepared using the
434 IGF-1 enzyme-linked immunosorbent assay kit (Immunodiagnostic Systems Inc., Fountain
435 Hills, AZ), and was measured using the Wallac Victor 2 1420 Multilabel Counter (PerkinElmer,
436 Waltham, MA).

437 Measurements of menthol and metabolites of menthol in plasma were determined as
438 follows: the reference standard solution was prepared by weighing 2 mg of menthol and
439 transferring it into a vial. One milliliter of acetonitrile was added and mixed. Serial dilutions
440 were made to obtain concentrations of 2,000; 1,000; 200; 100; and 20 µg/mL in acetonitrile.
441 Aliquots of the solutions were injected to obtain a standard curve for quantification. Lab
442 standard solutions were prepared by transferring 250 µL of 20 µg/mL menthol in acetonitrile
443 solution into a 5-mL volumetric flask containing approximately 4 mL plasma; then diluted to
444 volume with plasma and mixed. This provided a theoretical concentration of 1,000 µg/mL
445 menthol in plasma. Further dilutions of the 1,000 µg/mL stock solution were made to obtain 20
446 µg/mL and 200 µg/mL solutions of menthol in plasma for subsequent runs. The 200 µg/mL
447 solution was prepared by transferring 100 µL of the 1,000 µg/mL stock solution into a vial
448 containing 400 µL of bovine plasma and mixed. The 20 µg/mL solution was prepared by
449 transferring 100 µL of the 200 µg/mL stock solution into a vial containing 900 µL of bovine
450 plasma and mixed. The lab standard solutions were stored in the refrigerator at 4ºC until
451 analysis. Sample preparation occurred as follows: 50 µL of plasma was transferred into a well
452 containing 200 µL of acetonitrile and mixed 1 min. The sample was centrifuged at 1,700 × g for
453 5 min at room temperature. The supernatant was transferred into a microvial (e.g. 300 µL
454 capacity) for analysis.

455 Metabolites of menthol were analyzed by gas chromatography/mass spectrophotometry.
456 Two microliters of sample were injected into the Agilent 7890A GC (Agilent Technologies,
457 Inc., Santa Clara, CA) with an injection port temperature of 100ºC and equipped with a 30 m ×
458 0.250 mm × 0.25 µm film, HP-5 MS column (Agilent Technologies, Inc., Santa Clara, CA).
459 Initial oven temperature was 50ºC, held for 1 min, and then increased at a rate of 40ºC/min to a
460 final oven temperature of 250ºC. Helium was used as the carrier gas held constant at 13.8 psi.
461 The Agilent mass spectrometer 5975C, EI source (Agilent Technologies, Inc., Santa Clara, CA)
462 was used as the detector, and had source and quad temperatures of 230ºC of 150ºC,
463 respectively.

464 ***In Vitro***

465 Ruminal fluid was collected from a ruminally fistulated Holstein steer at 0600 h
466 (approximately 2 h prior to feeding). The diet of the donor steer consisted of 7% ground brome
467 hay, 8% corn steep liquor, 80.2% steam-flaked corn, 2.16% feed additive premix, and 2.64%
468 vitamin/mineral premix. Ruminal fluid was placed directly into pre-warmed insulated water
469 jugs to maintain temperature, and transported 2 km to the Pre Harvest Food Safety Laboratory
470 located on the Kansas State University campus. Ruminal fluid was strained with 4 layers of
471 cheese cloth, placed into separatory funnels, and incubated at 38ºC for approximately 1 h to
472 allow ruminal fluid to stratify. The bottom sediment was removed, and the bacteria-rich middle
473 layer of strained ruminal fluid was mixed with McDougal's buffer (150 mL of McDougal's
474 buffer was combined with 50 mL ruminal fluid/flask), and added to each Ankom fermentation
475 flask (Ankom Technology, Macedon, NY). Substrate used for each fermentation flask consisted
476 of 85% corn, 5% soybean meal, and 10% alfalfa, and total substrate in each flask was 1 g. The
477 study was conducted as a randomized complete block design with treatments consisting of 0,
478 0.3, 0.03, or 0.003% menthol. Crystalline menthol was mixed with ethanol to obtain a 3%
479 menthol solution, and then the 3% menthol solution was diluted with ethanol to equal 0.3, 0.03,
480 or 0.003% menthol (substrate DM). Ethanol was used for control treatments to maintain
481 constant volume between treatments. Menthol mixtures were added at a rate of 0.1 mL/flask,
482 with five flasks/treatment. Three bottles containing no substrate served as blanks. Once all the
483 components were added to fermentation flasks, bottles were capped with the Ankom pressure
484 module (Ankom Gas Production System, Ankom Technology, Macedon, NY) and placed into a
485 Precision reciprocal shaking bath (Thermo Electron Corporation, Maretta, OH) maintained at
486 39ºC for 24 h. Gas pressure was measured every 5 min. Once 24 h was complete, flasks were
487 removed from the shaker and final pH was determined using a Thermo Orion benchtop pH
488 meter (model 230 A, Thermo Fisher Scientific Inc., Waltham, MA). Finally, 4 mL of the flask
489 fluid layer were mixed with 1 mL metaphosphoric acid and frozen overnight (-20ºC). After
490 thawing, 2 mL of each mixture were placed into micro centrifuge tubes and centrifuged at
491 17,000 × g for 10 min. Supernatant was removed, placed into gas chromatography vials, and
492 stored in a freezer at -20ºC until VFA analysis. Remaining samples in fermentation flasks were
493 placed into 19.0 × 12.7 cm aluminum pans and dried for 24 h at 105ºC to determine IVDMD.

494 Calculations of IVDMD were as follows: substrate weight – (dry pan weight – initial pan
495 weight – average residue weight of blanks)

496 ***Volatile Fatty Acid Analysis***

497 One milliliter of supernatant was pipetted into 12 × 75 tubes, 50 µm of 6N sodium
498 hydroxide were added, and tubes were then vortexed. Pivalic internal standard was added at a
499 rate of 3 mL/tube and mixed (final pivalic acid internal standard concentration was 3 mM). One
500 milliliter of the mix was transferred to a gas chromatography vial and refrigerated at 4ºC until
501 analysis. Standard was prepared by weighing 19.2 mg Li-Lactate into a 25-mL volumetric flask.
502 The flask was filled with pivalic acid internal standard (0.2 g pivalic acid, 2.52 g oxalic acid,
503 and 6.13 g formic acid mixed in 500 mL volumetric flask filled to volume with dionized water
504 and mixed). The analysis was carried out using a HP 5890 GC (Agilent Technologies, Santa
505 Clara, CA) equipped with a flame ionization detector and a 2 m × 2 mm Carbopak B-DA
506 column (Supelco, Inc., Bellefonte, PA) using nitrogen gas as the carrier gas. The injection and
507 detector temperature was 200ºC, oven temperature was 175ºC and with a flow rate of 24 mL per
508 min.

509 ***Statistical Analyses***

510 Feedlot performance was analyzed using the MIXED procedure of SAS version 9.2
511 (SAS Inst. Inc., Cary, NC), using menthol as the fixed effect, and weight block as the random
512 effect. Animal was the experimental unit. Blood measurements and BW were analyzed as
513 repeated measures using the MIXED procedure of SAS. The repeated measures statement
514 included time nested within d and the subject as animal nested within treatment. Menthol, time
515 within d, and menthol × time within d were included in the model statement as fixed effects.
516 The random effect was weight block, and animal was the experimental unit. The model
517 statement for feed fed daily (DM basis) included menthol, d, and menthol × d as fixed effects
518 with d as the repeated measure, and weight block as the random effect. Menthol and metabolites
519 of menthol were analyzed as repeated measures using the MIXED procedure of SAS. Menthol,
520 h, and menthol × h were the fixed effects, and block was the random effect. The repeated
521 measures statement included h. Gas production for *in vitro* fermentations was analyzed using
522 repeated measures with the MIXED procedure of SAS. The model statement included menthol,

523 time and menthol × time. The random effect was replication and the repeated statement
524 included time. VFA, pH, and IVDMD were analyzed using the MIXED procedure of SAS. The
525 model statement included menthol as the fixed effect and replicate as the random effect. The
526 LSMEANS statement was used for means and standard error calculations, and the PDIFF option
527 used for separation of means. Separations of means were determined to be significant at α level
528 ≤ 0.05.
529 .

530 **Results and Discussion**

531 Two animals were removed from the study. One was removed from the 0.003% menthol
532 group due to a displaced abomasum, and the other was removed from the 0% menthol group
533 due to a physical injury that required treatment using steroidal anti-inflammatory medications.

534 ***Growth Performance***

535 Limited information is available on the effects of menthol supplementation in growing
536 animals, and no studies have been published with respect to its effects on finishing cattle. We
537 observed a menthol × time within d interaction ($P < 0.01$) for BW. The treatment containing
538 0.3% menthol had the lowest BW d 1 to 22 during menthol administration; however, BW
539 measurements on d 22 and 30 were comparable to the other treatments (figure 4.1).
540 Additionally, BW increased 6 h after feeding for all treatments, indicating a greater amount of
541 gut fill from steers eating the majority of their feed after delivery. Soltan (2009) observed a
542 decrease in live BW ($P < 0.05$) in dairy calves the first month post weaning when an essential
543 oil mixture containing menthol was administered at 15.6, 31.2, or 46.8 mg/L in drinking water
544 compared to calves that did not receive the essential oil mixture. The authors also observed an
545 increase in BW the second and third month post-weaning ($P < 0.05$) for calves receiving 15.6
546 mg/1L drinking water when compared to control calves, but BW of calves receiving 31.2 and
547 46.8 mg/L of essential oil were not different ($P > 0.05$) than control calves. Toghyani et al.
548 (2010) reported an increase in BW of chicks at 28 d of age ($P < 0.05$) but no difference at 42 d
549 of age ($P > 0.05$), when dried peppermint was added to broilers diets at 4 or 8 g/kg. Similarly,
550 Al-Kassie (2010) supplemented chicks with 0.25%, 0.5%, 1.0%, or 1.5% dried peppermint and
551 observed an increase in BW ($P < 0.05$) for all peppermint supplemented broilers when

552 compared to control broilers. Soltan et al. (2010) administered an essential oil mixture
553 containing menthol to dairy cows at rates of 0, 16, 32, and 48 mg/L mixed into drinking water.
554 The authors observed an increase in BW ($P < 0.05$) for the cows receiving 16 and 32 mg/L
555 essential oil when compared to either the control or cows receiving 48 mg/L essential oil.
556 Differences in BW in the current study may be partly attributed to the fact that steers fed 0.3%
557 menthol consumed less feed initially as indicated in figure 4.2. Feed deliveries to cattle fed
558 0.3% menthol had the greatest decrease ($P < 0.05$) compared to the three other treatments until
559 d 13.

560 Average DMI over the entire 30 d feeding period were not different ($P = 0.25$) among
561 treatments (table 4.2); however, a menthol × d interaction was observed ($P < 0.01$) for amount
562 of feed fed daily. Steers fed 0.3% menthol consumed the least d 2 to d 13, at which point feed
563 delivered to cattle fed 0.3% menthol was similar to that of the other three treatments (figure
564 4.2). The decreased daily feed deliveries in cattle fed 0.3% menthol was attributed to the
565 stronger taste and aroma of menthol delivered at 0.3% of the diet DM compared to that of the
566 other treatments, evident by the dramatic decrease in feed delivered at the initiation of the trial.
567 Steers fed 0.3% menthol appeared to adapt to the increased aroma and taste of menthol and
568 began to consume amounts equivalent to 0, 0.003, and 0.03% menthol treatments d 13 of the
569 experiment. Soltan et al. (2010) observed a decrease in DMI ($P < 0.05$) when an essential oil
570 mixture was added to drinking water of dairy cattle when compared to control cattle. Soltan
571 (2009) also reported a decrease ($P < 0.05$) in intake for weaned dairy calves receiving an
572 essential oil mixture the first 2 wk post-weaning, which concurs with our findings for animals
573 fed 0.3% menthol. Studies performed using broiler chicks reported either no differences in feed
574 intake with addition of dried peppermint to the diet (Ocak et al., 2008; Toghyani et al., 2010), or
575 a decrease with addition of a 8 g/kg of dried peppermint to diets of broilers (Al-Kassie, 2010).
576 Feed intakes in the current trial may have been affected by frequent handling of the animals as
577 steers never consuming amounts equal to pre-trial DMI.

578 Average daily gain and feed efficiency were unaffected ($P > 0.05$; table 4.2) by addition
579 of menthol to diets of steers, which is in agreement with results reported by Ocak et al. (2008)
580 and Taghyani et al. (2010) for broilers. Additionally, Soltan (2009) observed no effect on ADG
581 or feed efficiency ($P > 0.05$) in dairy calves when a menthol mixture was added to drinking
582 water. Contradicting our results, Maenner et al. (2011) fed 300 mg/kg DM of essential oil

583 mixture containing anise, clove, and peppermint oils or anise, clove, and cinnamon oils to
584 piglets post-weaning. Live performance and ileal digestibly were recorded. The authors
585 observed no improvement in ADG, but G:F was greater ($P < 0.01$) for piglets supplemented
586 with peppermint oil mixture, and they attributed improvements in G:F to increased ileal
587 digestibility of CP and AA. Overall, the lack of differences in animal performance in the current
588 study may potentially be explained by the ability of menthol to affect growth in younger
589 animals by increasing digestion, but as animals age the effect is no longer observed (Toghyani
590 et al., 2010). Steers in our trial were approximately one year of age and may have responded
591 differently than younger animals. Additionally, we did not examine digestibility of feed with
592 menthol supplementation and therefore can't conclude whether or not menthol affects digestion
593 for cattle fed a finishing diet. Our results in the *in vitro* portion of the experiment showed no
594 differences in VFA profiles of ruminal microorganisms from ruminal fluid of a donor steer fed
595 93% concentrate, suggesting rumen fermentation was not affected by menthol supplementation.

596 ***Blood Parameters***

597 We observed a menthol × time within d interaction ($P < 0.01$; figure 4.3) with addition
598 of menthol to the diets of steers. Insulin-like growth factor 1 plays an important role in growth
599 of developing animals due to its impact on cell growth, proliferation, and metabolism (Micke et
600 al., 2010). Animals fed 0.003% menthol started out with a higher IGF-1 concentration
601 compared to the other three treatments. At d 9 and 16, steers fed 0.003% menthol had
602 comparable IGF-1 concentrations to steers fed 0% menthol until d 23. After d 23,
603 concentrations in steers fed 0 or 0.003% menthol began to decrease to similar concentrations as
604 the other two treatments (figure 4.3). Treatments containing 0.03 or 0.3% menthol started out
605 the same as steers fed 0% menthol, but on d 9 and 16 the concentrations were lower than steers
606 fed 0 or 0.003% menthol. To the authors knowledge IGF-1 concentrations with menthol
607 supplementation have not been previously reported in other published experiments making it
608 difficult to extrapolate and compare information. We concluded steers fed 0.003% menthol had
609 no effect on IGF-1 concentration due to IGF-1 concentrations being similar to steers fed 0%
610 menthol, but steers fed 0.03, or 0.3% menthol had a negative effect in IGF-1 concentrations
611 until d 23.

612 Plasma urea nitrogen (PUN) concentration can be used as an indicator of muscle growth
613 or protein degradation, and was observed to not be affected by menthol supplementation to
614 steers. No menthol × time within d interaction or effect of menthol ($P > 0.05$; figure 4.4) were
615 observed for plasma PUN concentrations, but an effect of time within d ($P < 0.01$) was observed
616 with PUN measurements being different at each time point within d. To the authors knowledge
617 there is no data published reporting effects of menthol on PUN concentrations. The lack of
618 differences observed in PUN concentrations suggests protein degradation is not being affected
619 with addition of menthol to diets of cattle.

620 We observed no menthol × time within d interaction for plasma glucose or lactate
621 concentrations ($P > 0.05$; figures 4.5 and 4.6, respectively) between treatments. Time of d had
622 an effect ($P < 0.01$) on glucose and lactate concentrations with concentrations varying at each
623 sampling time. Glucose and lactate concentrations are associated with carbohydrate and lipid
624 metabolism, and are important components of cellular metabolism. There is limited data
625 available to compare blood metabolite measurements that include glucose and lactate,
626 concentrations; however, Soltan (2009) measured serum glucose of calves administered an
627 essential oil mixture through their milk replacer. The author observed no difference ($P > 0.05$)
628 in glucose concentration for calves receiving 94 or 187 mg/d of essential oils compared to the
629 control, but for the calves receiving 281 mg/d serum glucose concentration was increased ($P <$
630 0.05) by 26.4% when compared to control calves. The differences observed between our study
631 and that of Soltan (2009) may be due to age differences of animals or route of administration.
632 Menthol administered as a liquid may be absorbed more quickly than if it were fed in a ration
633 potentially explaining why calves in the Soltan study receiving 281 mg/d had increased glucose
634 values, whereas steers in our study fed 0.3% menthol did not.

635 We concluded menthol does not have an effect on PUN, glucose, or lactate
636 concentrations; however, increased concentrations of menthol may reduce the amount of
637 circulating IGF-1 concentration.

638 Menthol is a lipid-soluble substance that is transported to the liver after ingestion, or can
639 be absorbed through skin or respiratory epithelium. Menthol is hydroxylated in the liver,
640 conjugated with glucuronide, and then circulated to the kidneys for excretion in urine (Eccles,
641 1994). Metabolites of menthol are the products of menthol after it is hydroxylated and
642 conjugated. Some metabolites of menthol found in urine of rats after ingestion include: p-

643 menthane-3,8-diol (II), p-menthane-3,9-diol (III), 3,8-oxy-p-menthane-7-carboxylic acid (IV),
644 and 3,8-dihyroxy-p-menthane-7-carboxylic acid (V; Madyastha and Srivatsan, 1988), and may
645 potentially be present in cattle. Metabolites of menthol in plasma were measured, and we
646 observed no menthol × h interaction ($P = 0.71$; figure 4.7), but an effect of menthol ($P = 0.05$)
647 and an effect of time ($P < 0.01$) were observed with concentrations of menthol being different at
648 different time points. At some of the sampling time points at least one steer fed 0% menthol
649 contained menthol metabolites in plasma. The reason for this is unknown, but it is conceivable
650 that some menthol was inhaled by control steers due to the volatility of menthol, and steers
651 being housed in close proximity to each other. Menthol has been utilized as a stimulant of cold
652 receptors in the upper airway of human patients in respiratory discomfort (Nishino et al., 1997).
653 It may be possible for metabolites of menthol to appear in the blood stream through passive
654 diffusion once inhaled into the lungs. Additionally, at h 702 the high menthol treatment
655 demonstrated a large increase in metabolites when compared to the other treatments due to one
656 animal having a high concentration of metabolites in the blood. Wu et al. (2010) observed the
657 half life of hard capsules containing peppermint oil to be 1.91 h and for a soft capsule
658 containing peppermint oil to be 1.4 h in beagle dogs. The half life of menthol metabolites in
659 bovine plasma is not known and it may be metabolized and excreted rather quickly which
660 would explain why some sampling times cattle receiving menthol had no metabolites of
661 menthol in plasma.

662 ***In Vitro***

663 Menthol is an essential oil that has been observed to have antibacterial activity and may
664 affect fermentation products of ruminal microorganisms (Eccles, 1994). Menthol had no effect
665 on *in vitro* concentrations of acetate, propionate, butyrate, isobutyrate, 2- and 3-methyl
666 isovalerate, valerate, or lactate ($P > 0.10$; figures 4.8 and 4.9) indicating menthol had no effect
667 on rumen fermentation. Hosoda et al. (2005) conducted an *in vivo* experiment in which rumen
668 fermentation of dairy cattle were evaluated after addition of an essential oil mixture containing
669 menthol. Cows were assigned randomly to either a control diet or a diet containing 5% of
670 peppermint (DM basis) for 8 d. In agreement with our observations, the authors reported no
671 difference for total VFA concentration, and molar rate of acetate, propionate, and butyrate ($P >$
672 0.05) between treatments. In contrast to our results, Agarwal et al. (2009) reported a linear

673 increase in the molar proportion of acetate ($P < 0.05$) and a linear decrease in the molar
674 proportion of propionate ($P < 0.001$) when cultures of mixed ruminal microbes were
675 supplemented with 0, 0.33, 1 or 2 mM peppermint oil. A quadratic effect for molar proportion
676 of butyrate ($P < 0.01$) was observed with cultures containing 1 mM peppermint oil having the
677 highest butyrate concentration and containing 0 or 2 mM having the lowest concentration. The
678 authors speculated that differences in VFA concentrations were due to accumulation of
679 molecular hydrogen as a result of decreased methanogenesis. Ando et al. (2003) observed a
680 decrease ($P < 0.01$) in propionate concentration in ruminal fluid from cannulated Holstein steers
681 that received 200 g dried peppermint daily compared to no menthol supplementation. The
682 authors postulated that antimicrobial effects of menthol may have had an adverse effect on
683 ruminal fermentation, thereby decreasing VFA production. Lack of congruence among studies
684 may be due to differences in the initial diet of the donor animal, potentially resulting in different
685 microfloral populations in ruminal fluid.
686 Some bacterial, protozoal, or fungal species may be more susceptible to menthol then
687 others. Zmora et al. (2012) conducted an *in vitro* batch fermentation trial with 5 doses of dried
688 peppermint leaves (0, 2.3, 8.17, 16.34, and 23.35 mg). The authors observed a quadratic effect
689 ($P < 0.005$) with 16.34 and 23.35 mg peppermint decreasing the methanogen population.
690 Additionally, linear and quadratic effects ($P < 0.05$) were observed in bacterial and protozoal
691 populations with 8.17 mg peppermint having the greatest decrease in bacteria, and 16.34 mg
692 inducing the greatest decrease in protozoa. These results confirm the antimicrobial properties of
693 menthol; however, the diet of the donor steer in our trial was receiving 93% concentrate. The
694 high concentrate diet may have changed the microbial population to favor microbes that were
695 less susceptible to menthol. This also may possibly explain the lack of an effect on VFA
696 concentrations in our study.
697 Terminal pH and IVDMD were not different between treatments ($P > 0.10$; figures 4.10
698 and 4.11, respectively). In agreement, Zmora et al. (2012) observed no differences between
699 treatments for pH and IVDMD ($P > 0.05$) when *mentha piperita* L. was added at rates of 2.33,
700 8.17, 16.34, or 23.35 mg to batch fermentation incubation vessels. Contradicting our results,
701 Agarwal et al. (2009) observed a quadratic decrease ($P < 0.05$) in *in vitro* true digestibility
702 (IVTD) of feed with addition of 0.33, 1.0, or 2.0 µL/mL peppermint oil to buffalo rumen liquor
703 with 1.0 µL/mL peppermint having the greatest decrease in IVTD. Additionally, Ando et al.

704 (2003) observed a decrease in rumen pH with addition of 200 mg dried peppermint to the diet of
705 cannulated steers. Differences observed between studies may reflect the differences in source of
706 menthol, time of sampling, or diet of donor animals.

707 Menthol has been administered to cattle in the form of peppermint and has consistently
708 demonstrated decreases in methane production (Argwal et al., 2009; Zmora et al., 2012). We
709 observed no menthol × time interaction ($P > 0.10$) for fermentative gas production; however, an
710 effect of menthol ($P < 0.01$) was observed. Addition of menthol decreased the amount of
711 fermentative gas produced at all menthol inclusion rates (figure 4.12). Agarwal et al. (2009)
712 observed an increase ($P < 0.01$) in total gas production with addition of 0.33 and 1.0 μL/mL
713 peppermint oil *in vitro* whereas 2.0 μL/mL peppermint resulted in no difference ($P > 0.05$) in
714 total gas produced by ruminal microflora. The concentrations of menthol used in the trial by
715 Agarwal et al. (2009) were higher than that used in our experiment, which may explain
716 differences between experiments. Jani et al. (2010) performed a study in which gas production
717 was measured *in vitro* using rumen fluid from 2 cannulated sheep. Peppermint oil was added at
718 14 or 40 μL/g DM to ruminal inoculum and gas measurements were recorded. The authors
719 observed a decrease in volume of gas produced ($P < 0.05$), which is in agreement with our
720 results. The decrease in total gas production in the current trial may be caused by antimicrobial
721 activity of some microbial species, as evidenced by the decrease in methanogenesis noted by
722 Argwal et al. 2009.

723 In conclusion, addition of menthol to diets of cattle had little or no effect on growth
724 parameters measured in this trial; however, *in vitro* fermentative gases were reduced suggesting
725 menthol may alter ruminal bacterial populations without having a negative effect on VFA
726 production.

727 ***Acknowledgments***

728 The authors would like to thank Steven Hu from Pfizer Animal Health for his assistance
729 in quantifying menthol and it derivatives in plasma.

730

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792

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794

795 **Table 4.1: Diet Composition, DM basis**

	Menthol, % diet DM			
Ingredients, % DM basis	0%	0.003%	0.03%	0.3%
Steam flaked corn	50.39	50.39	50.34	49.89
Wet corn gluten feed	33.640	33.637	33.660	33.840
Corn silage	12.00	12.00	12.00	12.00
Feed additive premix[1]	2.16	2.16	2.16	2.16
Vitamin/mineral premix[2]	0.07	0.07	0.07	0.07
Limestone	1.44	1.44	1.44	1.44
Salt	0.30	0.30	0.30	0.30
Menthol[3]	-	0.003	0.03	0.3
Calculated composition,%				
CP	14.00	14.00	14.00	14.00
Ca	0.62	0.62	0.62	0.62
P	0.51	0.51	0.51	0.51
K	0.70	0.70	0.70	0.70
NDF	20.08	20.08	20.08	20.10

796 [1]Formulated to provide 300 mg/d monensin and 90 mg/d tylosin (Elanco Animal Health,

797 Greenfield, IN) in a ground corn carrier.

798 [2]Formulated to provide 2,200 IU/kg vitamin A; 22 IU/kg vitamin E; 10 mg/kg added Cu; 60

799 mg/kg added Zn; 60 mg/kg added Mn; 0.5 mg/kg added I; 0.25 mg/kg added Se; and 0.15

800 mg/kg added Co.

801 [3]Menthol provided by Ind-Swift Laboratories Limited (Dover, DE).

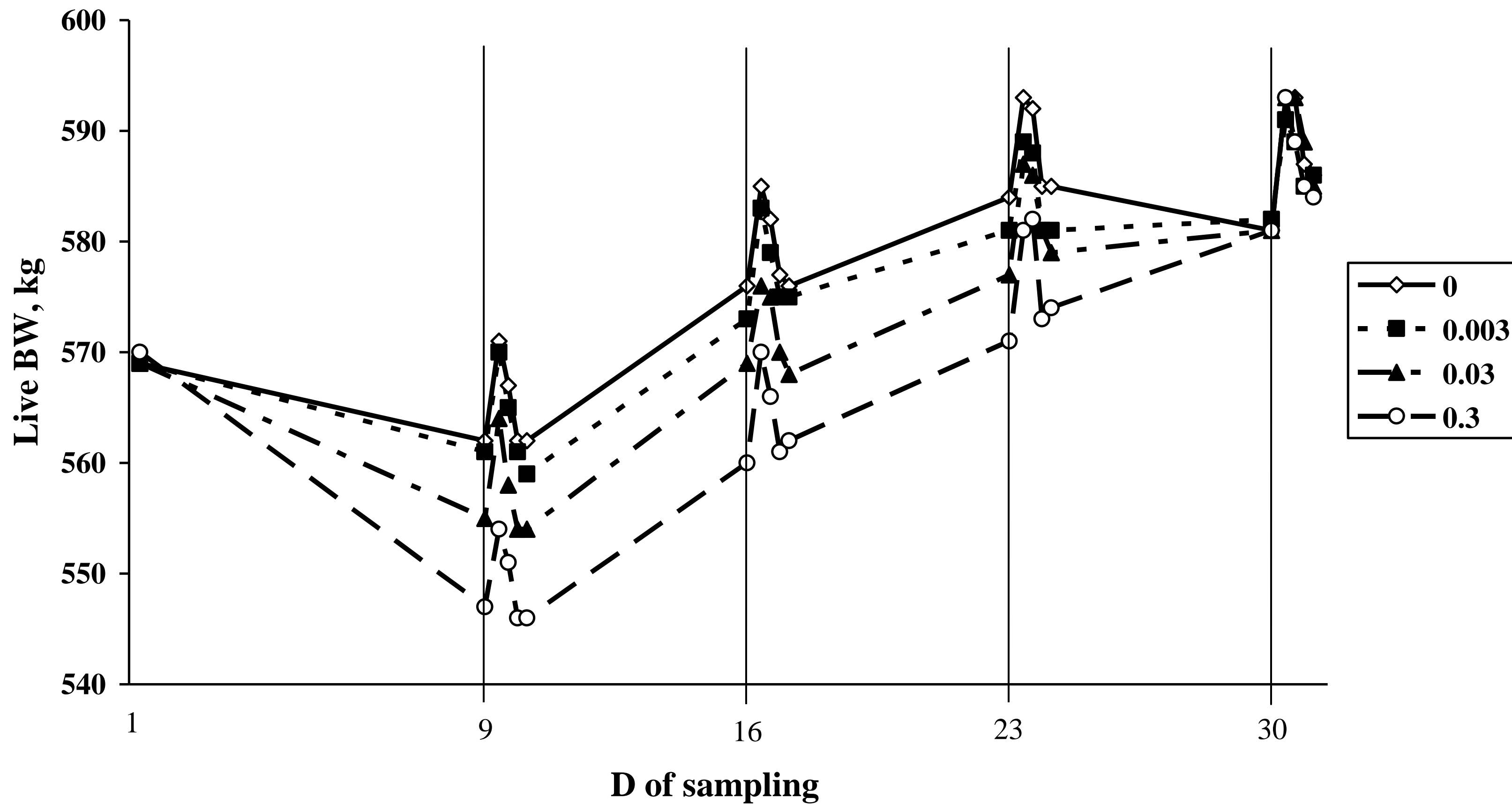
802

803 **Table 4.2: Effect of menthol on feedlot performance**

	Menthol, % of diet DM					
Item	0%	0.003%	0.03%	0.3%	SEM	*P*-value
ADG, kg	0.55	0.54	0.51	0.48	0.17	0.98
DMI, kg	7.51	7.50	7.80	7.02	0.36	0.25
G:F	0.067	0.056	0.059	0.065	0.02	0.97

804

805 **Figure 4.1: Effect of menthol on BW obtained from Holstein steers on days 1, 9, 16, 23, and 30, and at hour 0 on d 1 then h 0,**
806 **6, 12, 18, and 24 on d 9, 16, 23, and 30.**
807 Treatments consisted of 0, 0.003, 0.03, and 0.3% diet DM menthol.
808 Effect of menthol × time within d interaction $P < 0.01$; d 9, 16, 23 at h 0, 18 and 24, steers receiving 0.3% menthol had lower BW
809 than other treatments; effect of menthol $P = 0.15$; effect of time within d $P < 0.01$; SEM = 10.53.



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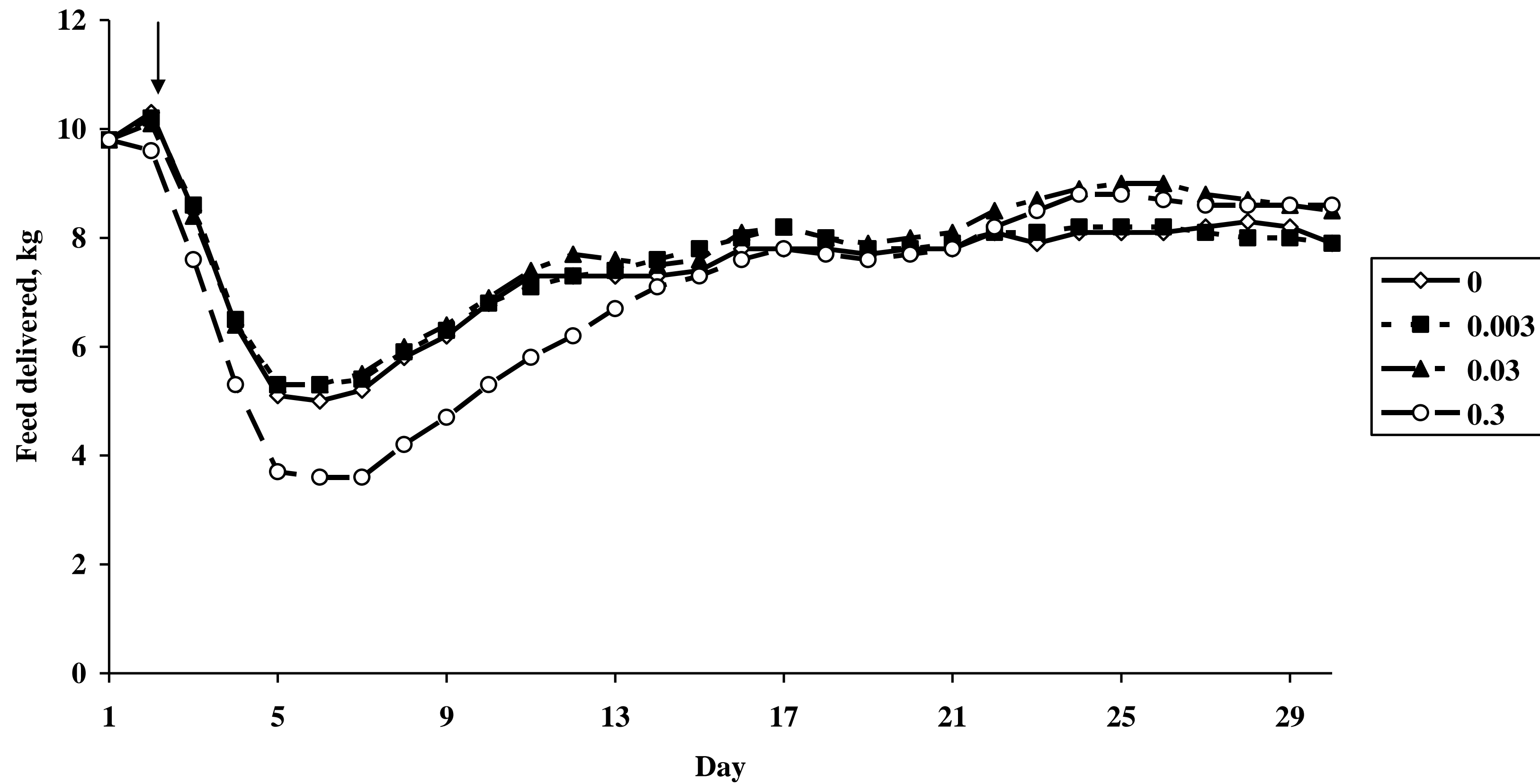
812 **Figure 4.2: Daily feed delivered to Holstein steers (DM basis).**

813 Diets were formulated to contain 0, 0.003, 0.03, and 0.3% diet DM menthol. Arrow indicates beginning of treatment administration.

814 Menthol × d interaction, $P < 0.01$, steers receiving 0.3% menthol had lower feed deliveries d 2 thru 13; effect of menthol, $P = 0.31$;

815 effect of d, $P < 0.01$. SEM = 0.78.



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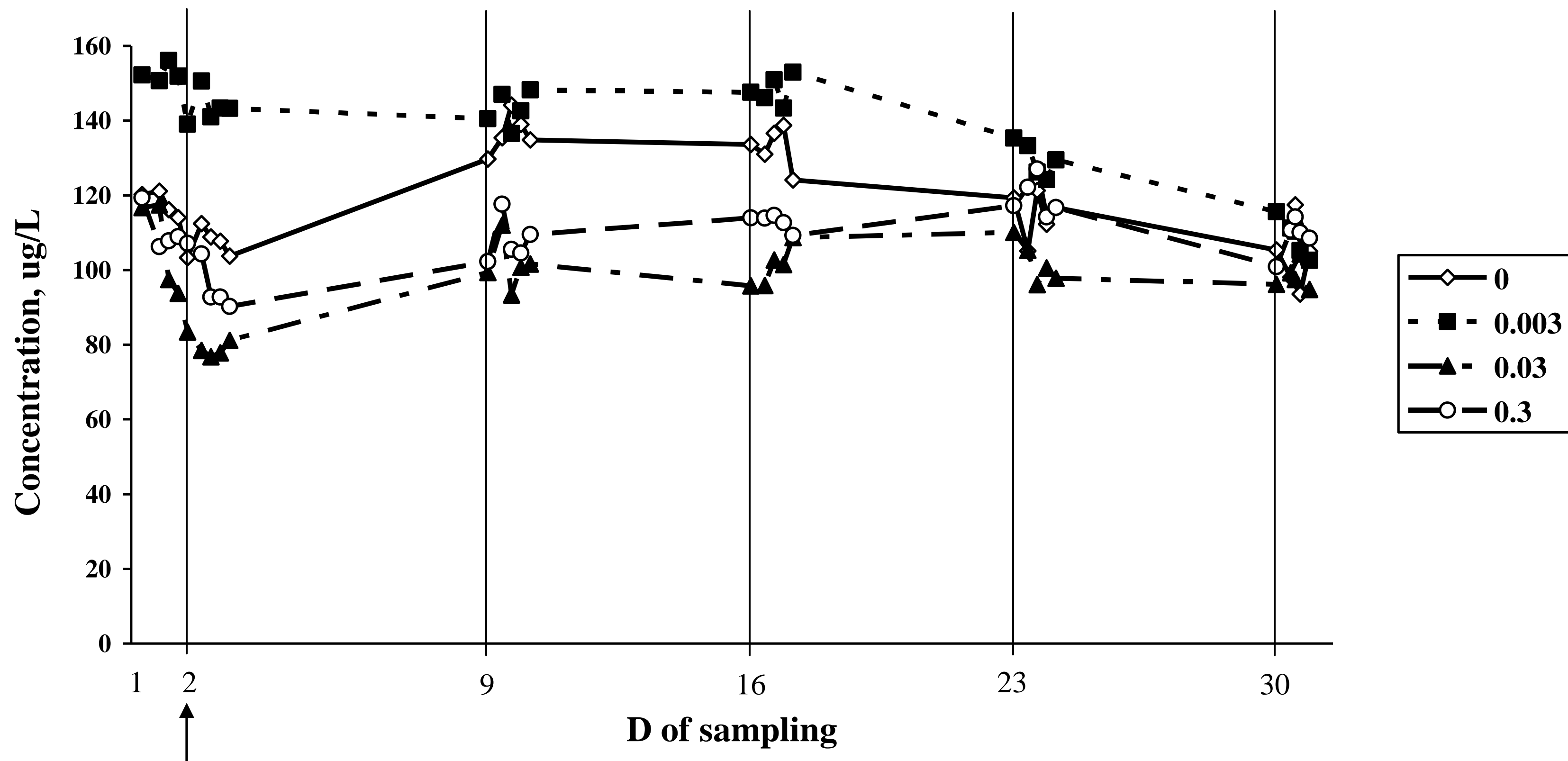
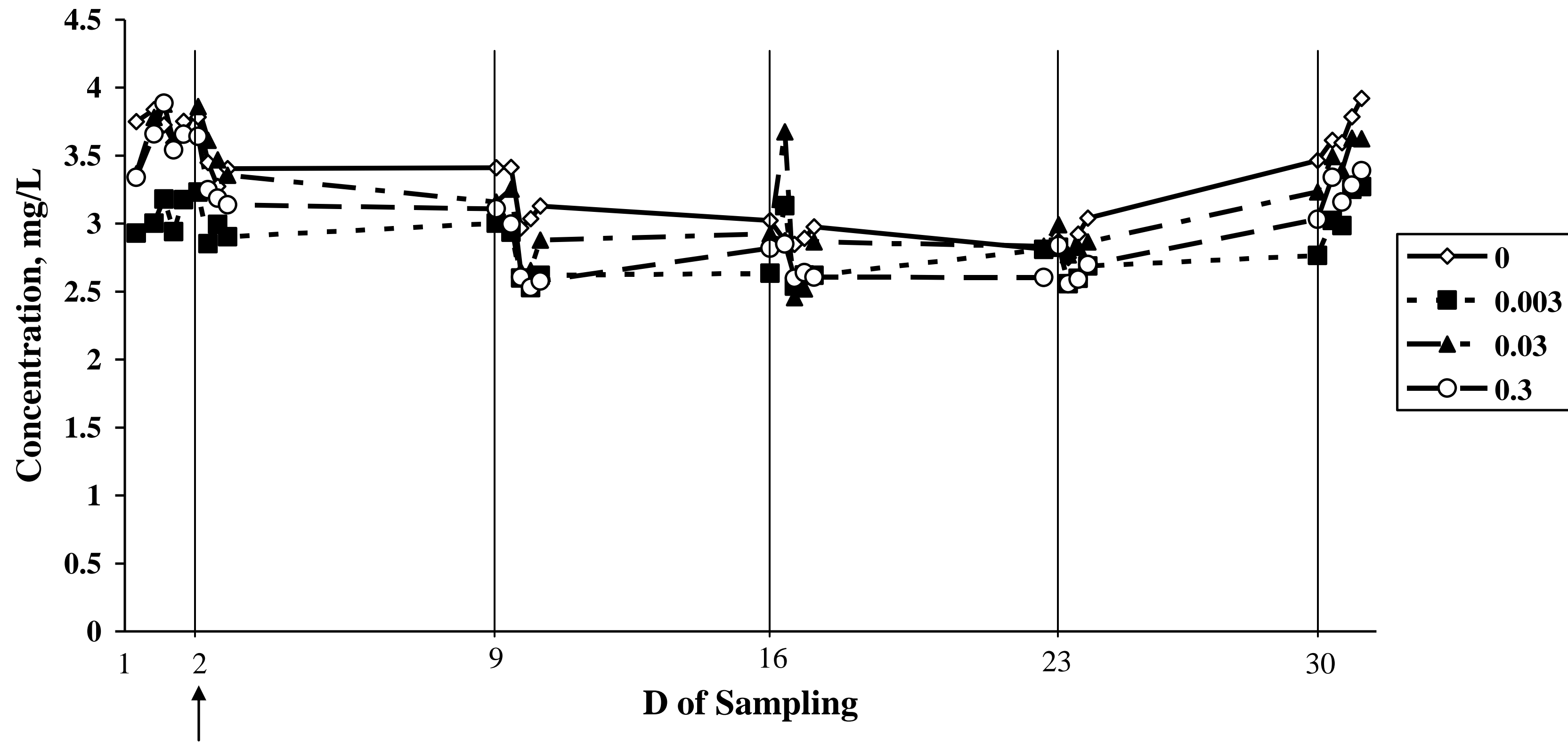
819 **Figure 4.3: Effects of menthol on IGF-1 concentration from Holstein steers on days 1, 2, 9, 16, 23, and 30, and at hours 0, 6, 12,**
820 **18, and 24.**

821 Treatments consisted of 0, 0.003, 0.03, and 0.3% diet DM menthol. Arrow indicates beginning of treatment administration.
822 Menthol × time within d interaction P < 0.01; steers receiving 0.003% menthol had higher IGF-1 concentrations d 1 and 2; d 9 and 16,
823 0% and 0.003% menthol had higher IGF-1 concentrations compared to 0.03 and 0.3% menthol; effect of menthol P = 0.26; effect of
824 time within d P < 0.01; SEM = 16.2.



825

826 **Figure 4.4: Effects of menthol on plasma urea nitrogen concentration from Holstein steers on days 1, 2, 9, 16, 23, and 30, and**
827 **at hours 0, 6, 12, 18, and 24.**
828 Treatments consisted of 0, 0.003, 0.03, and 0.3% diet DM menthol. Arrow indicates beginning of treatment administration.
829 Menthol × time within d interaction $P = 0.11$; effect of menthol $P = 0.65$; effect of time within d $P < 0.01$; SEM = 0.30.



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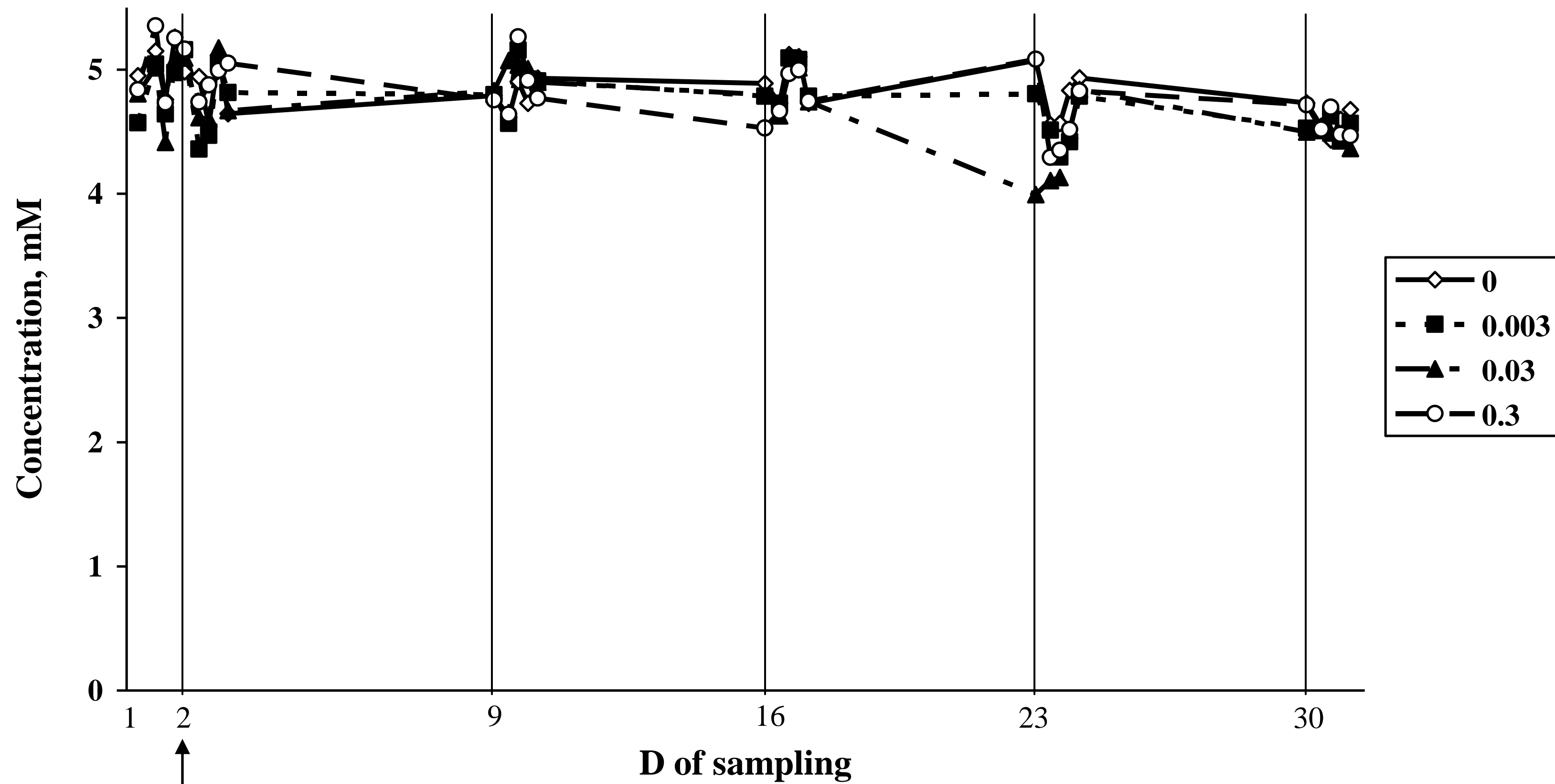
832 **Figure 4.5: Effects of menthol on plasma glucose concentration.**

833 Blood samples were obtained from 50 Holstein steers on days 1, 2, 9, 16, 23, and 30, and at hours 0, 6, 12, 18, and 24. Treatments

834 consisted of 0, 0.003, 0.03, and 0.3% diet DM menthol. Arrow indicates beginning of treatment administration.

835 Menthol × time within d interaction $P = 0.12$; effect of menthol $P = 0.46$; effect of time within d $P < 0.01$; SEM = 0.17.



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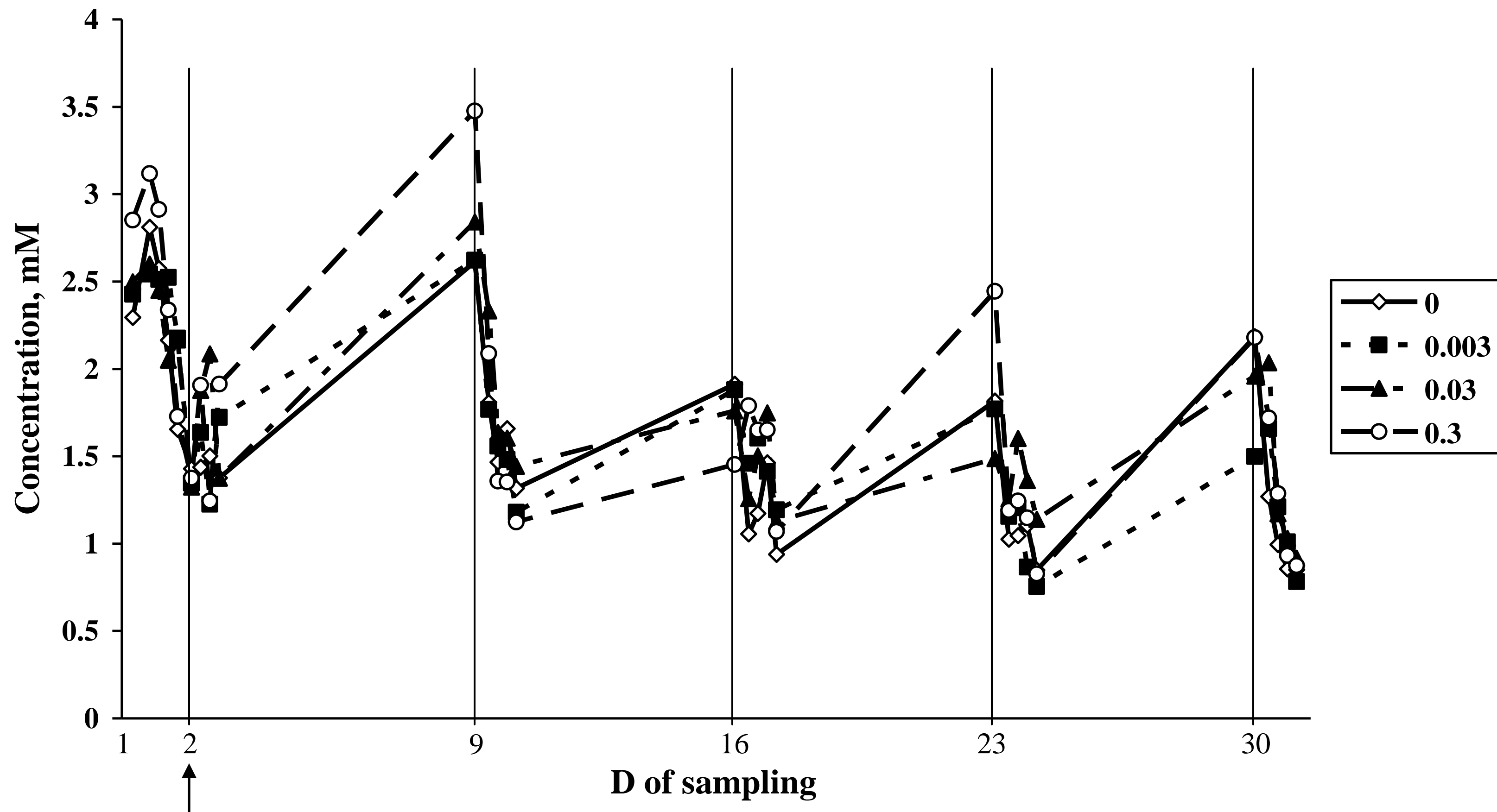
837 **Figure 4.6: Effects of menthol on plasma lactate concentration for Holstein steers on days 1, 2, 9, 16, 23, and 30, and at hours**
838 **0, 6, 12, 18, and 24.**
839 Treatments consisted of 0, 0.003, 0.03, and 0.3% diet DM menthol. Arrow indicates beginning of treatment administration.
840 Menthol × time within d interaction $P = 0.87$; effect of menthol $P = 0.50$; effect of time within d $P < 0.01$; SEM = 0.28.



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843 **Figure 4.7: Concentration of menthol metabolites in plasma obtained from Holstein steers.**

844 Diets were formulated to contain 0 (◊), 0.003 (■), 0.03 (▲), and 0.3% (○) diet DM menthol (Ind-

845 Swift Laboratories Limited, Dover, DE). Diets were delivered to pens once daily according to

846 the following feed schedule: barn A was fed at approximately 1500 h, barn B was fed at

847 approximately 1600 h, and barn C was fed at approximately 1700 h daily, arrow indicates

848 feeding relative to time of blood collection. Day 1; h 12, 18, 24, and d 2; h 6 (plate 1), d 9; h 0, 6,

849 12, and 18 (plate 2), d 16; h 6, 12, 18, 24 (plate 3), d 23; h 0, 6, 12, 18 (plate 4), d 30; h 0, 6

850 (plate 5)

851 Menthol × h interaction, $P = 0.71$; effect of menthol, $P = 0.05$; effect of h, $P < 0.01$. SEM

852 = 17.29.

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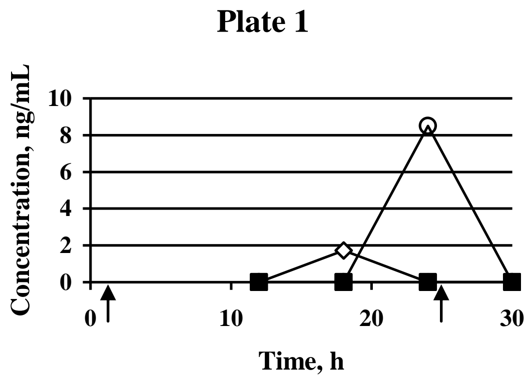

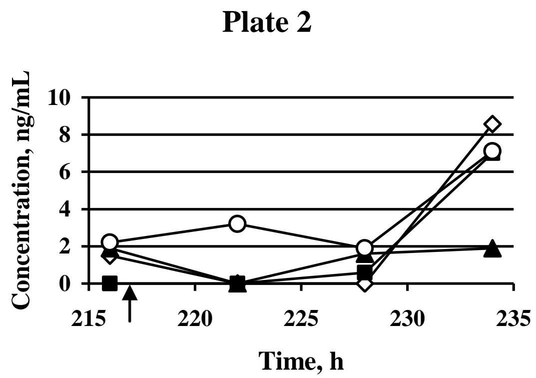

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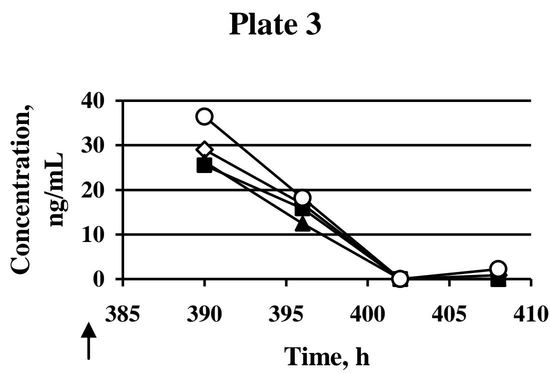

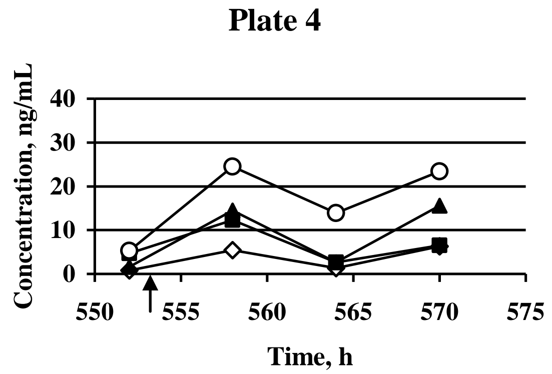

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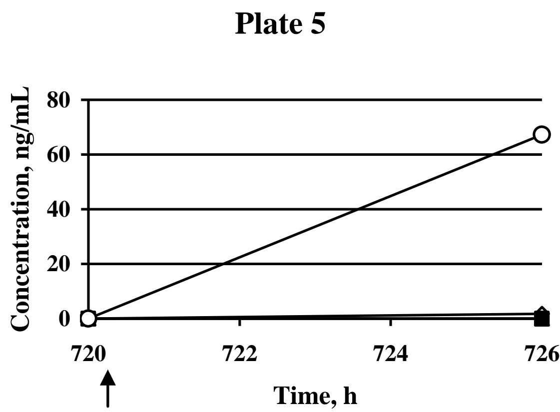

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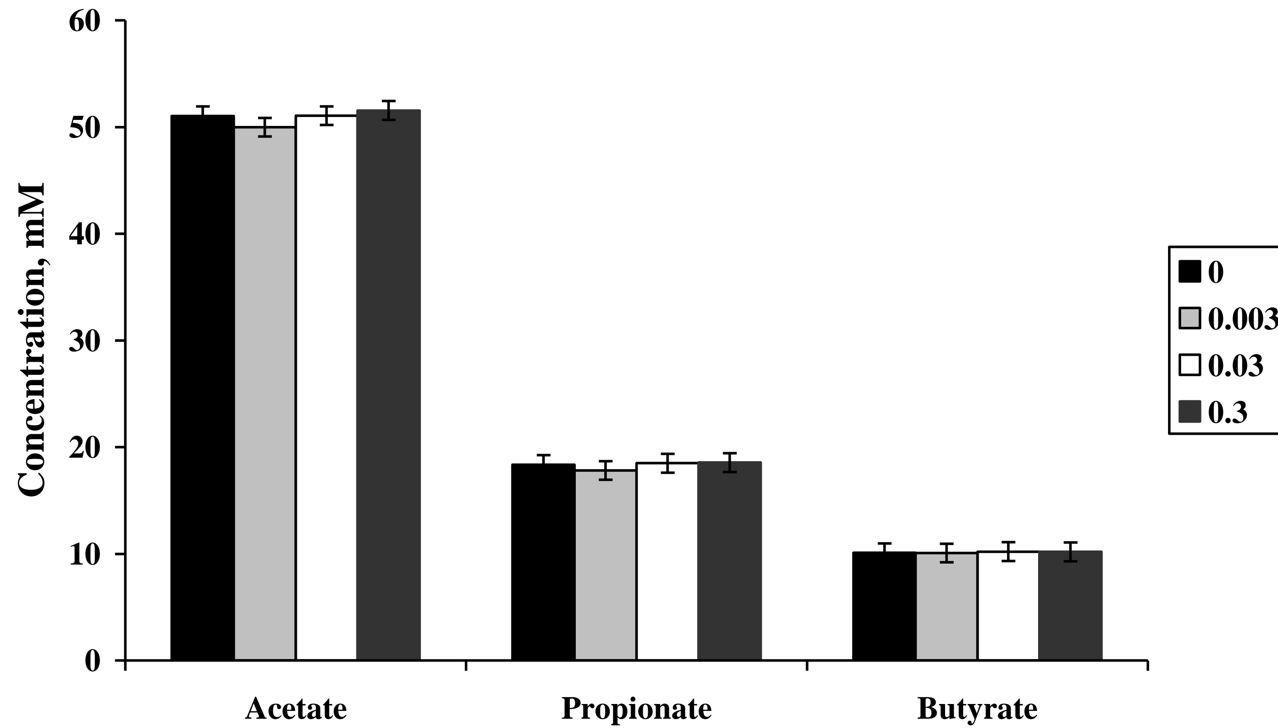
859 **Figure 4.8:** ***In vitro*** **production of major VFA by ruminal microflora with addition of 0, 0.003, 0.03, 0.3% (DM) menthol.**

860 Effect of menthol, $P > 0.10$.



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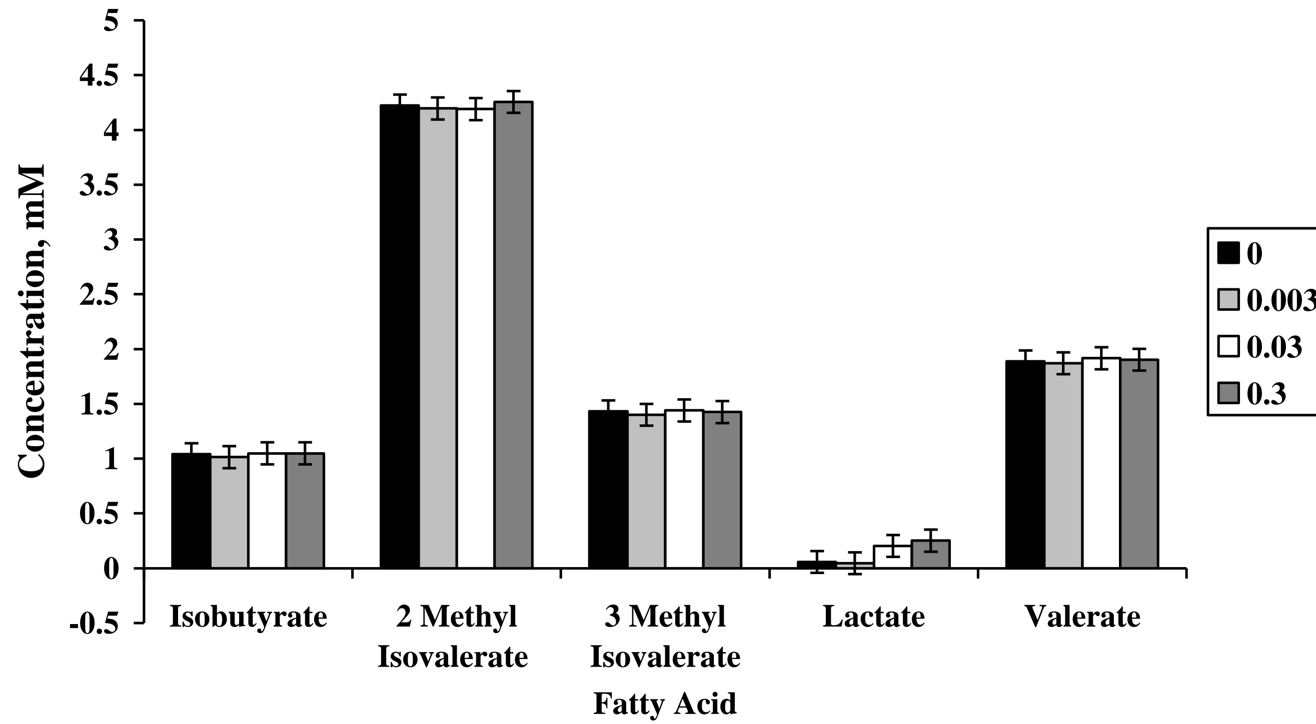
863 **Figure 4.9:** ***In vitro*** **fatty acid production by ruminal microflora with addition of 0, 0.003, 0.03, 0.3% (DM) menthol.**

864 Effect of menthol, $P > 0.10$.



865

Figure 4.10: Effect of 0, 0.003, 0.03, 0.3% menthol (substrate DM) on 24 h *in vitro* fermentation terminal pH.

Effect of menthol, $P = 0.21$; SEM = 0.025

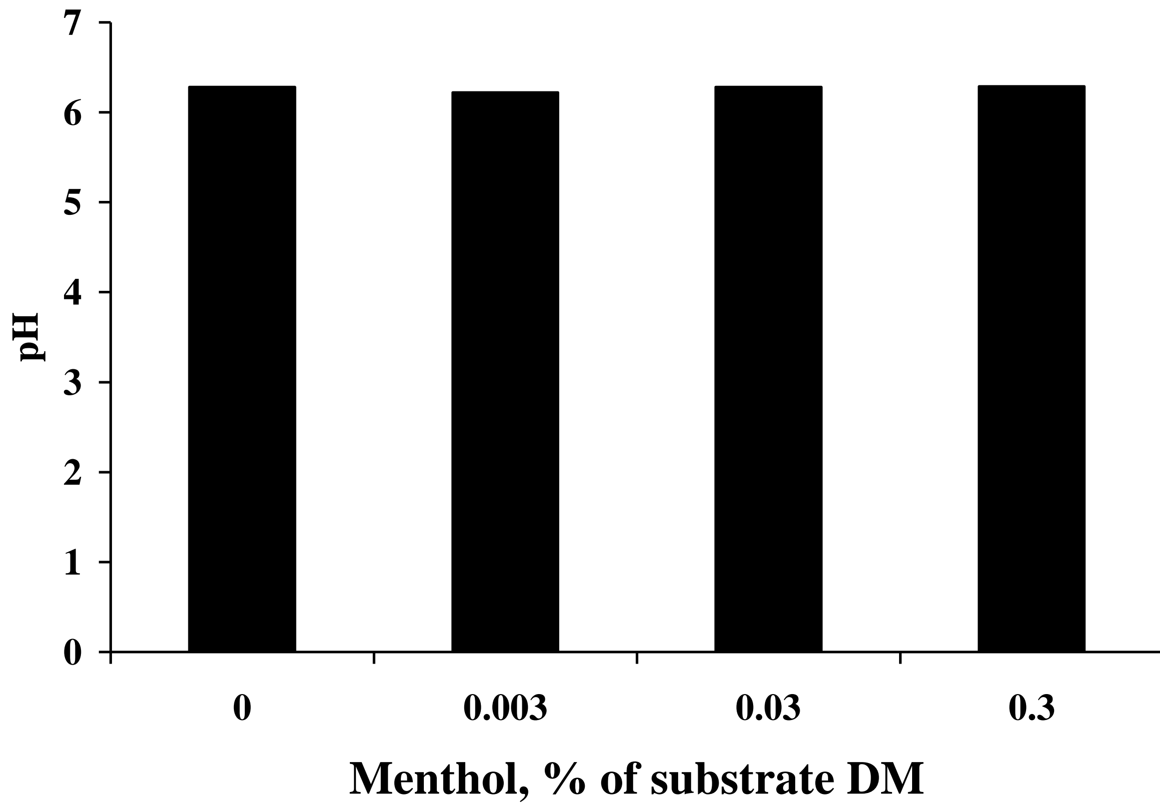


Figure 4.11: Effect of 0, 0.003, 0.03, 0.3% menthol (substrate DM) on IVDMD.

Effect of menthol, $P = 0.77$; SEM = 0.023.

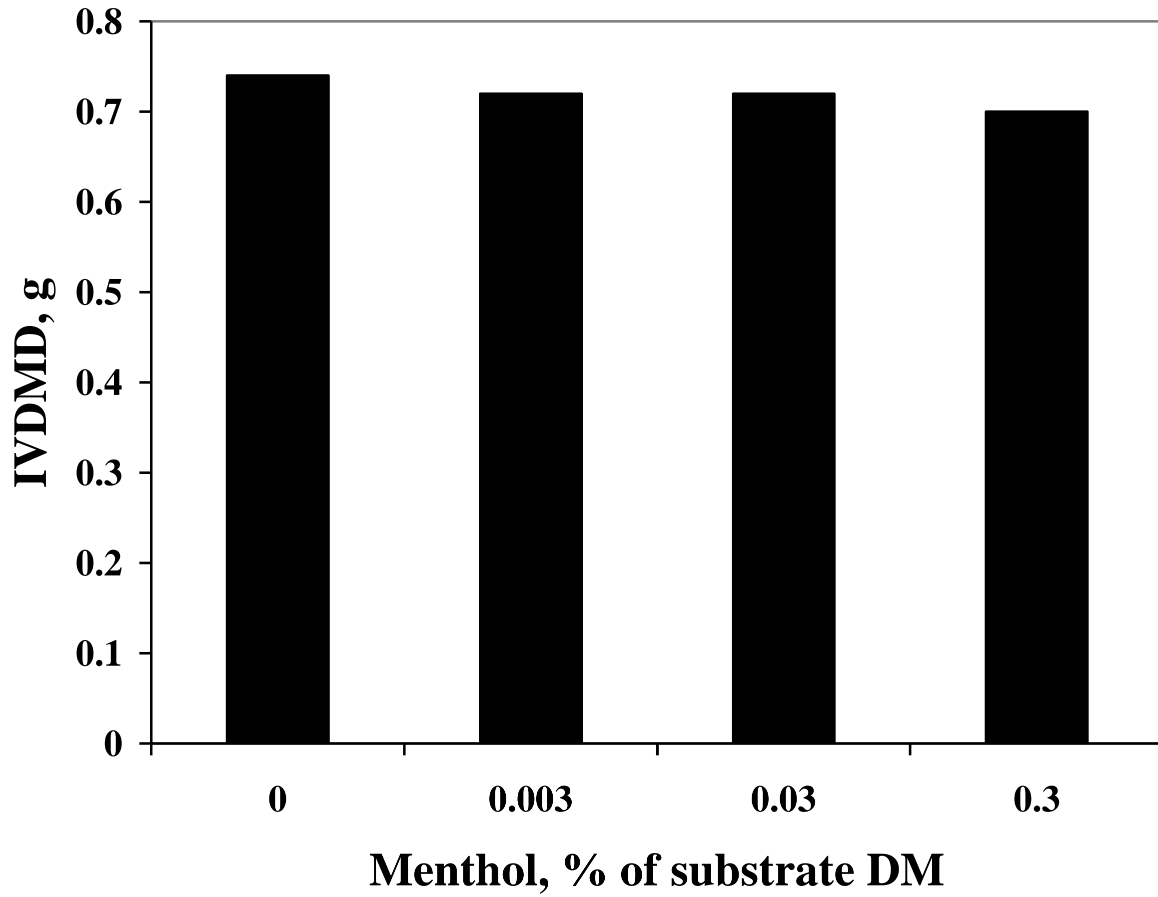


Figure 4.12: Effect of 0, 0.003, 0.03, 0.3% menthol of substrate DM on *in vitro* fermentative gas production.

Menthol × time interaction, $P > 0.05$; effect of menthol, $P < 0.001$; effect of time, $P < 0.01$; SEM = 29.32