

EFFECTS OF RACTOPAMINE HYDROCHLORIDE ON NUTRIENT DIGESTIBILITY,
ENVIRONMENTAL N EXCRETION, REGULATION OF SKELETAL MUSCLE GROWTH,
AND BETA-RECEPTOR SUBTYPES IN FINISHING BEEF STEERS

BY

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DISSERTATION

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ABSTRACT

Beta-adrenergic agonists such as ractopamine hydrochloride (Actogain, Zoetis, Parsippany, NJ) are growth-promoting technologies that when fed at the end of the finishing period in cattle act as repartitioning agents, redirecting energy toward protein accretion and away from lipid deposition. Despite nearly fifty years of research, several questions still exist surrounding beta-adrenergic receptors and the agonists to which they bind. The primary objective of this research was to evaluate these areas where relatively little literature exists. Targeted toward two independent areas of focus, this research was divided into two distinct studies. The objective of the first study was to evaluate the effects of ractopamine hydrochloride (Actogain) on nitrogen excretion and nutrient digestibility. The objective of the second study was to compare protein abundance of beta-adrenergic receptor (**β -AR**) subtypes between bovine muscle, organ, and adipose tissues through western blotting procedures. In a randomized complete block design, twelve Simmental \times Angus steers were assigned dietary treatments including a control (without Actogain; **CON**) or 400 mg·steer⁻¹·d⁻¹ ractopamine hydrochloride (**RAC**) for 35 d before slaughter. Two 5 d sampling periods were conducted on each block for total collection of feed, orts, feces and urine. No interaction ($P > 0.10$) between treatment and collection period was observed for any parameter evaluated. Dietary treatment had no effect ($P = 0.38$) on DMI, but RAC-fed steers had decreased fecal DM output (2.1 vs. 2.5 kg DM/d; $P = 0.04$) compared with CON-fed steers. Steers fed RAC had greater apparent total tract DM digestibility (72.8 vs. 68.9%; $P = 0.02$), NDF digestibility (59.2 vs. 53.3%; $P < 0.01$), and ADF digestibility (53.8 vs. 47.9%; $P = 0.05$) than CON-fed steers. Although dietary treatment did not affect nitrogen intake ($P = 0.51$) or nitrogen digestibility ($P = 0.14$), RAC-fed steers excreted less total nitrogen (120.8 vs. 138.2 g/d; $P = 0.02$) than CON-fed steers due to a tendency for

decreased fecal nitrogen output (61.5 vs. 71.8 g/d; $P = 0.09$) in RAC-fed steers compared with CON-fed steers. However, dietary treatment had no effect ($P = 0.31$) on urinary nitrogen output or percentage of nitrogen excreted as urea. An in vitro experiment was conducted to validate the effects of RAC on nutrient digestibility using a contemporary group of heifers ($N = 19$). Rumen fluid was collected by stomach tube from CON- and RAC-fed heifers to inoculate tubes containing a CON or RAC substrate in a split-plot design. No interaction between rumen fluid source and in vitro substrate was observed ($P = 0.44$). Greater IVDMD (64.0 vs. 58.6%; $P = 0.01$) was observed in tubes containing rumen fluid from RAC-fed heifers compared with rumen fluid from CON-fed heifers. Inclusion of RAC in the in vitro substrate increased IVDMD (63.1 vs. 59.5%; $P < 0.01$). Results suggest feeding RAC may impact microbial digestion of the finishing diet to increase total tract digestion and reduce nitrogen excretion in the 35 d period prior to slaughter. In the second study evaluating β -AR protein abundance, muscle (longissimus lumborum and psoas major), organ (bronchial, atrium, ventricle, and liver), and adipose tissues (visceral, subcutaneous, and intramuscular) from CON-fed steers ($N = 9$) were collected at slaughter and flash frozen in liquid nitrogen. Tissue samples were normalized to equal protein concentrations and protein abundance was determined by measuring protein band density relative to a standard sample present on every gel. Beta 1-AR protein was more highly enriched in muscle tissue than all other tissue types ($P < 0.001$). Beta 2-AR protein was most prevalent in bovine heart and lung tissue. No differences in β 2-AR protein were observed between muscle tissues ($P = 0.25$) and adipose tissues ($P \geq 0.17$). However, a tendency ($P = 0.06$) was observed for greater β 2-AR protein in subcutaneous fat than longissimus tissue. Although differences in β 3-AR protein abundance were observed between tissues ($P = 0.03$), β 3-AR was not especially enriched in any tissue evaluated. Overall, data verify the presence of all three β -AR subtypes in

the tissues evaluated and provide a first look at β -AR subtype protein abundance between different tissues. Determining the relative abundance of β -AR subtypes between different tissues may provide critical information to aid in the discovery and development of future β -AR-targeting small molecule drug compounds for beef cattle.

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Chapter 1

REVIEW OF LITERATURE

INTRODUCTION

Growth-promoting technologies are a proven method for increasing carcass yields and have helped to improve beef's overall price competitiveness compared with other proteins while reducing resource inputs. Beta-adrenergic agonists (β -AA) are one such category of growth-promoting technologies. When fed at the end of the finishing period in beef, β -AA act as repartitioning agents, redirecting energy toward protein accretion and away from lipid deposition. Early research evaluating the effects and properties of β -AA quickly identified the potential for use in marketed livestock species. Recognized as much for their value to human medicine, β -AA and the receptors they bind to have been intensely studied since the late 1970s. Given both the economic and sustainability benefits of β -AA, it comes as no surprise that a survey of feedlot consulting nutritionists, servicing over 14,000,000 cattle annually, reported 85% of their clients used a β -AA during the finishing phase (Samuelson et al., 2016).

Currently, there are two β -AA approved by the U.S. Food and Drug Administration for cattle: ractopamine hydrochloride (Optaflex, Elanco Animal Health, Greenfield, IN.; Actogain, Zoetis Inc., Parsippany, NJ) and zilpaterol hydrochloride (Zilmax, Merck Animal Health). Ractopamine hydrochloride, indicated for use as a complete feed or as a top-dress on feed, is approved for use at a rate of 70-430 mg·animal⁻¹·d⁻¹ (8.2 – 24.6 g/ton) for the last 28 - 42 d prior to slaughter. Ractopamine has an effective zero-day withdrawal (< 24 h). Zilpaterol hydrochloride is approved to be fed at a rate of 8.3 mg/kg on a DM basis in a complete feed (6.8 g/ton) for the last 20 - 40 d prior to slaughter with a mandatory 3 d withdrawal period to allow

clearance of drug residues. However in 2013, Zilmax was voluntarily pulled from the U.S. market by drug sponsor Merck over potential mobility issues in fed cattle. Although both compounds are labeled for the increase in rate of BW gain, feed efficiency, and carcass leanness when fed as a complete medicated feed, ractopamine is solely indicated for increase in rate of BW gain and feed efficiency when fed as a top-dressed medicated feed.

MODE OF ACTION

Beta-adrenergic Receptors

Adrenergic receptors (**AR**) belong to the 7-transmembrane domain superfamily of proteins (Mills, 2002), within the subset of G-protein coupled receptors (**GPCR**; Strosberg, 1996). With over 1,000 GPCR identified, there is substantial variation in the types of ligands bound by members of the GPCR family (Strosberg, 1996). As such, GPCR such as the AR family comprise a substantial proportion of the druggable proteome with more than 30% of pharmaceutical products targeting this protein family (Liu et al., 2012). Investigation of the physiological effects of the endogenous catecholamines norepinephrine and epinephrine in the 1940s led to the discovery of the adrenergic receptors responsible for ligand binding and the classification of these receptors into two primary categorizations, the alpha-adrenergic receptors (**α AR**) and beta-adrenergic receptors (**β AR**; Mersmann, 1998). Further classification over the years has resulted in nine identified AR subtypes including three alpha-1 AR subtypes (1A, 1B, and 1C), three alpha-2 AR subtypes (2A, 2B, and 2C), and the three beta AR subtypes (β 1, β 2, and β 3; Bylund et al., 1994). Existence of the β 1- and β 2-AR was first demonstrated by Lands et al. (1969) with identification and acceptance of the β 3-AR occurring several years later (Emorine et al., 1989). Genomic sequencing technologies developed in the mid-90s verified the presence

of individual β -AR subtypes with unique chromosomal locations, RNA transcript lengths, protein sizes, and amino acid sequences (Mersmann, 1995).

Classification of these AR subtypes has historically been a complex process accounting for differences in ligand binding affinity and selectivity, primary mechanisms for signal transduction, molecular biology, and physiological effects (Bylund et al., 1994; Mersmann, 1998). Embedded in the plasma membrane of almost all mammalian cell types, β AR contain more than 400 amino acids configured in a continuous chain layout (Mersmann, 1998). All three β -AR subtypes consist of seven transmembrane domains (helices) connected by four extracellular and intracellular loops. The ligand binding site is located on the extracellular side of plasma membrane within a ligand binding pocket embedded in the plasma membrane (Strosberg, 1996; Mersmann, 1998). Ligand binding sites of β -AR contain three structural features common to most monoamine receptors. These structural features include 1) an acidic side chain situated in the third helix, involved in the formation of a salt bridge with the binding ligand; 2) a serine or threonine side chain positioned in the fourth and fifth helices able to form hydrogen bonds with ligand hydroxyls; and 3) aromatic residues located in the sixth and seventh helices believed to be responsible for hydrophobic interaction with the aromatic group of the ligand (Strader et al., 1989; Strosberg, 1996).

The β -AR signaling pathway occurs through the activation of protein kinase B (**Akt**) and its downstream effectors through one of three mechanisms (Berdeaux and Stewart, 2012). The primary means of signal transduction is through activation of the trimeric G_s proteins bound to the intracellular loops of the β -AR (Mersmann, 1998), causing subsequent production of cyclic adenosine monophosphate (**cAMP**) and activation of protein kinase A (**PKA**). Comprised of three subunits (α , β , and γ), the G_s protein α -subunit dissociates from the γ - and β -subunits allowing

for GTP binding and adenylyl cyclase activation. Adenylyl cyclase is then responsible for the production of cAMP. Other mechanisms by which β -AR signaling are thought to occur are the activation of Akt by β -arrestin and phosphatidylinositol 3-kinase (**PI3K**) as well as the activation of cAMP response element binding protein (**CREB**; Berdeaux and Stewart, 2012). The development of nuclear magnetic resonance technology illuminated differences between degree of signaling and effector pathways activated as the result of conformational and G-protein binding changes in the intracellular region of β -AR (Liu et al., 2012).

In the presence of continuous or repeated exposure to β -AA, downstream signaling of β -AR diminishes over time, a major limitation to the clinical efficacy of many drug candidates (Spurlock et al. 1994). Given the physiological need for both short- and long-term desensitization of β -AR, multiple receptor desensitization mechanisms exist including receptor phosphorylation, sequestration, and receptor degradation (Hausdorff et al., 1990; Spurlock et al., 1994). Short-term desensitization, or the stoppage of effect that occurs minutes after isolated exposure, is thought to occur through the phosphorylation of sites located on the third intracellular loop and carboxyl tail of the receptor by beta adrenergic receptor kinase (**β ARK**) or PKA. This secondary-messenger dependent phosphorylation is thought to change receptor conformation, preventing G-protein coupling (Freedman and Lefkowitz, 1996). In comparison, the loss of responsiveness observed from β -AA exposure over a period of days is likely the result of receptor internalization through β -arrestin-initiated endocytosis (Pierce and Lefkowitz, 2001). An even more important consideration for the development of β -AA compounds, there are differences in phosphorylation and desensitization potential between the three β -AR subtypes. Whereas the β 1- and β 2-AR appear to have a similar number of sites for phosphorylation by PKA and β ARK, both subtypes have a greater number of sites for phosphorylation than the β 3-

AR (Hausdorff et al., 1990). This is reflected in several studies reporting little to no desensitization effect with compounds targeting β 3-AR (Nantel et al., 1993; Langin et al., 1995).

For all the similarities in signaling pathways, there are considerable differences in the properties of the three β -AR subtypes. Coded for by the ADRB genes, in cattle, *ADRB1* (β 1-AR gene) and *ADRB2* (β 2-AR gene) are intronless genes encoding for 467- and 418-amino acid proteins, respectively; whereas the *ADRB3* (β 3-AR gene) contains 2 introns needed for a 405-amino acid protein (Gene, 2004). As expected given the unique transcript lengths of each, there is less sequence homology between the three receptor subtypes. Work conducted by McNeel and Mersmann (1999) showed nucleotide homology $\leq 65\%$ between β -AR subtypes in porcine tissue. Nonetheless, individual β -AR subtypes are highly homologous across species with bovine β -AR sharing $\geq 80\%$ homology with β -AR of rat and human (Gene, 2004).

β -AR Subtype Tissue Distribution

Despite similarities in β -AR subtype sequence homology across species, differences in subtype distribution exist across both tissues and species. Liang and Mills (2002) and McNeel and Mersmann (1999) quantified β -AR subtypes in porcine tissue using radioligand binding and mRNA transcript presence, respectively. Studies evaluating β -AR distribution have reported β 1: β 2-AR ratios of approximately 80:20 in human (Bristow et al., 1986) and rat heart tissue (Minneman et al., 1979) and 15:85 in rat and 30:70 in human lung tissue (Minneman et al., 1979). Liang and Mills (2002) reported β -AR subtype mRNA transcript abundance in porcine tissue β 1: β 2-AR ratios of 81:19 in subcutaneous adipose tissue, 59:41 in skeletal muscle, 72:28 in heart tissue, 58:42 in lung tissue, and 50:50 in liver tissue. Beta 3-AR abundance has been demonstrated in human, mouse, and rat adipose tissue and have been implicated in the regulation of thermogenesis in BAT and lipolysis of white adipose tissue (WAT; Emorine et al., 1989). Of

adult human tissues, greater β 3-AR abundance was found in perirenal and omental adipose tissue than subcutaneous (Krief et al., 1993). Beta 3-AR presence has been found in rat brain tissue including cerebral cortex, hippocampus, hypothalamus, and amygdala (Summers et al., 1995), human urogenital tissue including prostate and bladder (Berkowitz et al., 1995; Yamaguchi et al., 2007), and human gastrointestinal tissue including gallbladder, colon, stomach, and small intestine (Krief et al., 1993; Berkowitz et al., 1995).

Despite characterization of β -AR subtype distribution in human (Krief et al., 1993), porcine (Liang and Mills, 2002; McNeel and Mersmann (1999), and guinea-pig tissue (Tanaka et al., 2005), relatively little information is available regarding β -AR subtype distribution in bovine tissue. Sillence and Matthews (1994) evaluated β -AR subtype proportions in cattle reporting > 99% β 2-AR in skeletal muscle and > 90% β 2-AR in adipose tissue, although it is important to note authors recognized the potential for inaccuracy associated with identification via radioligand binding. The likelihood for possible inaccuracies in these proportions is even greater considering the increase in carcass protein accretion observed in skeletal muscle of cattle fed ractopamine (a β 1-AR selective agonist). Evaluations of mRNA abundance in bovine skeletal muscle demonstrated the presence of β 1-AR mRNA in semimembranosus, however β 2-AR expression was almost 1,000 times greater than β 1-AR, indicating a strong likelihood β 2-ARs are the predominant subtype in skeletal muscle (Baxa et al., 2010).

Studies evaluating differences in β -AR subtype presence and distribution on the same tissue between different species give even greater credence to the need for β -AR information specific to cattle, especially given the fact common physiological responses may be mediated by different β -AR subtypes in different species (Ursino, 2009). Beta-AR-induced detrusor muscle relaxation is one such example of these differences. Beta-AR are implicated in relaxation of the

detrusor muscle, responsible for storage and release of urine (Ursino et al., 2009). Nonetheless, differences in detrusor muscle β -AR subtype presence between humans, mice, rats, and dogs (Yamaguchi et al., 2007; Ursino et al., 2009) indicate β -AR-induced detrusor muscle relaxation is mediated by targeting different receptors in different species. With physiological responses mediated by different receptors in different species, comparing β -AR protein abundance between tissues and relative β -AR subtype proportions present in various tissue in beef cattle would aid in the development of highly specific drug candidates.

β -AR Subtype Selectivity

Despite differences in receptor nucleotide and protein sequences, the binding pocket of the β -AR is highly conserved amongst the GPCR subfamilies (Conn et al., 2009). As a result, the development of highly potent, subtype-selective β -AA has proven difficult in the pharmaceutical industry. Nonetheless, β -AR subtype binding affinities have been observed for both the β_1 and β_2 receptors. Of the endogenous catecholamines, norepinephrine displays the greatest affinity for β_1 -AR with β_2 -AR intermediate and β_3 -AR the least, whereas epinephrine exhibits similar affinity for both β_1 - and β_2 -AR binding (Mersmann, 1998; Mills, 2002). Differences in the specificity of synthetic β -AA for receptor subtypes demonstrate a spectrum of effects from no effect to full agonist (Johnson et al., 2014). Whereas the halogenated β -AA clenbuterol is considered highly specific to the β_2 -AR, other β -AA compounds exhibit specificity for more than one receptor subtype. For example, although ractopamine exhibits primary specificity for β_1 -AR, it also acts as a partial β_2 -AA. Levy et al. (1993) demonstrated that β_1 -AR relative activity (evaluated as G protein/AC stimulation) at full stimulation, was only 80% of β_2 -AR relative activity at full stimulation. This was confirmed by Schroeder et al. (2003); feeding steers 200 mg·steer⁻¹·d⁻¹ ractopamine had no effect on objective tenderness of steaks, whereas steers

receiving 300 mg·steer⁻¹·d⁻¹ showed decreased tenderness, a response more commonly associated with β₂-AR use.

Beta-Adrenergic Agonists

Beta-adrenergic agonists are classified as sympathomimetic compounds used to elicit a physiological response through binding to β-AR. Much like the endogenous catecholamines norepinephrine and epinephrine, all βAA conform to similar structural principles with a six-membered aromatic ring, β carbon bound hydroxyl group, a positively charged N in the ethylamine side chain, and a bulky R group adjacent to the aliphatic N necessary for biological activity (Smith, 1998). Although these basic principles apply to the majority of studied βAAs, differences in aromatic substitution and R group both confer specificity and serve for categorization of the compounds.

Whereas, the catecholamines contain hydroxyl groups at the third and fourth position of the aromatic ring, ractopamine hydrochloride is classified as a phenethanolamine because of the hydrogen present on the third carbon of its aromatic ring. This aromatic substitution associated with phenethanolamines has a substantial effect on route of metabolism and effectiveness compared with several catecholamines. Many catecholamines are readily deactivated after oral administration by methylation of the 3-hydroxyl group. Therefore, this substitution in the aromatic ring of phenethanolamines prevents rapid deactivation (Smith, 1998). Stereochemistry of βAA compounds also plays a large role in binding affinity, with most beta-adrenergic receptors having much greater affinity for the R(-) configuration (Ruffolo, 1991; Smith, 1998). This finding was validated in ractopamine stereoisomers by Ricke et al. (1999) reporting the RR isomer demonstrated the greatest receptor affinity for all β-AR subtypes using cloned beta-adrenergic receptors in Chinese hamster ovary cells.

Absorption of β -AA usually occurs rapidly after oral administration. Ungemach (2004) reported peak plasma concentration for ractopamine at 0.5 – 2 hr after dosing and elimination half-life at 6 – 7 hr after initial dosing. The extent of absorption and short half-life of ractopamine is partially due to metabolism by conjugation primarily through glucuronidation (Smith, 1998). Despite the extensive research on both rate and extent of absorption of β -AA in livestock species (Dalidowicz et al., 1992; Smith and Paulson, 1997), there is relatively little published information available regarding the site of absorption. Although this is the case, it is generally believed the small intestine is the primary site of absorption through passive diffusion. Due to the more neutral pH of the small intestine, β -AA molecules would be less likely to form cations at the phenethanolamine nitrogen aiding in greater passive absorption across intestinal mucosa (Smith, 1998). Of the few studies that have evaluated site of absorption, Yuge et al. (1984) showed extensive absorption of the halogenated compound mabuterol in the small intestine of rats with little absorption in stomach and Borgstrom et al. (1990) reported terbutaline absorption in the duodenum of humans. Very little, if any, published information exists on the primary site of absorption in ruminant species.

B-AA Effects on Protein Accretion

The process of protein turnover is well recognized as an incredibly inefficient, albeit necessary, process responsible for 15 to 22% of total energy expenditure in growing livestock species (Reeds et al., 1985). Total protein accretion, composed of both catabolic and anabolic processes, is far outweighed by the individual rates of protein synthesis and protein degradation. Beta-AA have been implicated in both processes through mechanistically distinct pathways. Activation of Akt by β -AR signaling, as described previously, has important functions in the activation of a number of downstream targets affecting protein synthesis. One such target is the

activation of mammalian target of rapamycin (**mTOR**) increasing protein synthesis through activation of the ribosomal protein s6 kinase (p70^{s6k}) associated with encouragement of translation elongation and indirect activation of eIF4E involved in assembly of the initiation complex for protein translation (Norton and Layman, 2006). Akt also inhibits protein breakdown by phosphorylating and inactivating forkhead box O, a transcription factor for E3 ubiquitin ligases (Sandri, 2004; Stitt, 2004).

Effects of β -AA on these classical signaling pathways have been demonstrated through measured increases in the activity of proteins associated with degradation through the calpain system. Outside of the Akt signaling pathway, CREB activation increases calpastatin production inhibiting the calpain proteases, also reducing protein degradation (Cong et al., 1998). Both increased calpastatin and reduced calpain activity were reported by Pringle et al. (1993) in lambs fed a β -AA. Similar findings were shown in steers fed ractopamine, with even greater effect observed when steers were treated with more potent β -AA such as zilpaterol and clenbuterol (Strydom et al., 2009). Transcript presence for both skeletal muscle proteins (Helferich et al., 1990; Koohmaraie et al., 1991) and calpastatin were also shown greater in β -AA fed steers (Killefer and Koohmaraie, 1994). This in vivo effect on protein degradation validates postmortem effects on objective tenderness with a wide range of effects documented.

Despite the effects of β -AA signaling on downstream target abundance and activity, the primary mode of protein accretion appears to be through increased muscle hypertrophy without concomitant increases in myonuclei. Although evidence of increased satellite cell proliferation has been reported in vitro, fusion of satellite cells was not observed (Grant et al., 1990; Cook et al., 2005). Additionally, stimulation of skeletal muscle growth may be attenuated by indirect modulation of β -AA effect by insulin-like growth factor 1 (**IGF-I**; Johnson et al., 2014). A

number of studies have shown decreased regional and circulating IGF-I concentrations after several weeks feeding β -AA (Beerman et al., 1987; Walker et al., 2010).

B-AA Effects on Lipid Metabolism

Beta-adrenergic agonists also exhibit lipolytic and anti-lipogenic properties responsible, in part, for their characteristic repartitioning effect (Ricks et al., 1984). Increased production of PKA results in the phosphorylation of numerous downstream targets, one of which is hormone sensitive lipase. Active when phosphorylated, hormone sensitive lipase partially hydrolyzes triglycerides (TG). This response has been documented through increased circulating free fatty acids in pigs (Hu et al., 1988) and cattle (Stoffel and Meyer et al., 1993) receiving clenbuterol both via diet and intraperitoneal infusion. A second mechanism by which β -AA decrease fat deposition is the phosphorylation and inactivation of acetyl-coA carboxylase, necessary for the de novo synthesis of fatty acids (Mersmann, 1998). This phosphorylation has also been shown to reduce lipogenic gene expression (Mills and Mersmann, 1995). Despite these effects, it is important to note that, in general, changes in carcass fatness associated with β -AA are generally considered more transient than changes in protein accretion. This is likely a result of greater receptor desensitization observed in adipose tissue than skeletal muscle (Spurlock et al., 1994).

LIVE PERFORMANCE AND NUTRIENT DIGESTIBILITY

Early β -AA research quickly identified the potential for use in livestock species to enhance efficiency of live gain as well as increase carcass leanness. Given the repartitioning effects of β -AAs on protein and lipid metabolism are largely dependent on treated species, dose, duration, and potency of β -AA compounds, a wide range of performance and carcass effects have been observed in β -AA trials.

Growth Performance

The effects of β -AA on growth performance traits in finishing cattle have been well documented, and include increased average daily gain, increased feed efficiency, and decreased dry matter intake. A meta-analysis of the effects of β -AA ractopamine hydrochloride and zilpaterol hydrochloride reported average mean effects of the β -AA on growth performance traits (Lean et al., 2014). A statistical summarization of at least 26 zilpaterol studies reported an increase of 8.15 kg in final BW, 0.15 kg/d increase in ADG, and 0.02 kg/kg ratio increase in G:F of feedlot cattle fed zilpaterol compared with control-fed cattle (Lean et al., 2014). With a larger body of available research, summarization of 41 ractopamine studies revealed an average, albeit smaller, improvement in final body weight (7.57 kg), ADG (0.19 kg/d), and G:F (0.018 kg/kg ratio) of ractopamine-fed feedlot cattle compared with control-fed cattle (Lean et al., 2014). Improvements in ADG and G:F were concomitant with an average decrease in DMI of 0.12 kg/d in studies feeding zilpaterol, however no average effect on DMI (-0.003 kg/d) was reported in studies feeding ractopamine. Effects of β -AA on DMI are likely potency dependent as studies with more potent compounds such as clenbuterol demonstrated significant depression of DMI compared with control- and ractopamine-fed steers and numerically lesser DMI compared with zilpaterol-fed steers while still improving carcass-adjusted ADG compared with both control-fed and ractopamine-fed steers (Strydom et al., 2009). Nonetheless, unadjusted ADG of clenbuterol-fed steers was more similar to that of control-fed steers, likely a result of the physiological adaptation period associated with highly potent β -AA. Brockway et al. (1987) reported appetite suppression effects for the first 5 d of feeding clenbuterol to sheep.

Nutrient Digestibility

Given β -AA are small molecule drug compounds absorbed via passive diffusion, they are believed to exert effects on growth performance that are the primary result of downstream target activation occurring post-absorption. As such, relatively little research has evaluated the pre-absorptive effects of β -AA on diet component digestibility. Of the little data available, most of the ruminant metabolism studies have been conducted in sheep. Despite the well-recognized effects of β -AA on ADG and G:F, most of the available studies report feeding a β -AA had no effect on DM or protein digestibility (Kim et al., 1989; Rikhardsson et al., 1991; López-Carlos et al., 2010) compared with control-fed lambs. Similarly, a pair of studies evaluating energy metabolism, performance, and carcass characteristics in steers reported no effect of ractopamine on DM digestibility or N digestibility (Koontz et al., 2009; Strydom et al., 2009). Nonetheless, despite a lack of statistical difference, López-Carlos et al. (2010) reported feedlot lambs fed 1.05 mg·kg⁻¹ of BW·d⁻¹ ractopamine demonstrated a 2% unit increase in DM digestibility, 2% unit increase in crude protein digestibility, as well as a 4% unit and 3% unit increase in NDF and ADF digestibility, respectively, compared with control-fed lambs. In contrast, Fiems et al. (1991) reported an almost 3% unit reduction in DM digestibility and a 6% unit reduction in crude fiber digestibility of cimaterol-fed bulls. However, it is important to note bulls in this study were fed a β -AA for well longer than what is considered the normal feeding duration (225 d).

CARCASS CHARACTERISTICS AND MEAT QUALITY

Given the labeled carcass leanness effects, carcass characteristics and meat quality attributes of β -AA-fed cattle have been intensely studied. In addition to live performance effects, the Lean et al. (2014) meta-analysis also reviewed effects of ractopamine and zilpaterol on carcass characteristics. With more than 45 studies included, Lean et al. (2014) reported feeding

ractopamine resulted in an average increase of 6.18 kg in HCW, 1.84 cm² increase in LM area, and a 0.23% unit increase in DP compared with carcass characteristics of control-fed cattle. No average effect (-0.003 cm) of ractopamine usage on 12th-rib fat thickness was reported (Lean et al., 2014). Greater magnitude of response for zilpaterol on carcass characteristics has been historically observed given its function as a β_2 specific agonist. Lean et al. (2014) determined average effects of 35 zilpaterol studies reporting greater HCW (15.12 kg), greater LM area (8.01 cm²), decreased 12th-rib fat thickness (0.11 cm) and greater DP (1.71% units) than carcasses from control-fed cattle.

As expected given the improvements in HCW, LM area, and DP, β -AA have been shown to increase carcass cutability and saleable yield (Boler et al., 2009; Hilton et al., 2009; Arp et al., 2014). Zilpaterol has been reported to exert greatest effect on subprimal yields, expressed as a percentage of cold carcass weight, of cuts from the round and to a lesser extent, the loin (Boler et al., 2009). The potential for improvements in carcass yield of ractopamine-fed steers is primarily dependent on dosage. Arp et al. (2014) reported no difference in round subprimal cut yield or whole muscle yield of carcasses from control-fed and 200 mg·steer⁻¹·d⁻¹ ractopamine-fed steers, whereas steers receiving 400 mg·steer⁻¹·d⁻¹ ractopamine exhibited greater round subprimal yield, strip loin yield, and whole muscle yield.

The use of β -AA have relatively little effect on color or pH. The Lean et al. (2014) meta-analysis reported no average effect of zilpaterol or ractopamine usage on ultimate pH of LM. However, effects of β -AA on lean color of LM steak differ with compound used. Both Avendaño-Reyes et al. (2006) and Martin et al. (2014) reported lesser objective redness (a^* values) and less saturation of the primary color hue (chroma) in steaks from steers fed zilpaterol for 33 d. However, no differences in a^* value or chroma were observed between steaks from

control- and RAC-fed cattle (Quinn et al., 2008; Martin et al., 2014). Objective redness findings from 18 zilpaterol studies were also evaluated by Lean et al. (2014), reporting no average effect on objective redness.

As noted previously, use of more potent β -AA may result in reduced objective tenderness. Studies in which steers and heifers were fed a β -AA with partial agonist activity, at a low-dosage, or for shorter feeding periods demonstrate modest effects on shear force values when compared with control cattle, whereas in studies where cattle were fed more potent agonists, greater doses, or for longer periods prior to slaughter show marked increases in shear values. The Lean et al. (2014) meta-analysis reported steaks from zilpaterol-fed cattle demonstrated an average increase in WBSF of 0.84 kg, whereas steaks from ractopamine-fed cattle demonstrated only a 0.20 kg increase WBSF. Postmortem aging strategies have been proven effective in reducing detrimental effects of β -AA on objective tenderness (Boler et al., 2012; Martin et al., 2014) although improvements in objective tenderness did not change the proportion of steaks from cattle fed zilpaterol or ractopamine classified as “tender” (WBSF < 4.4 kg; SSF < 15.3 kg) according to ASTM guidelines (Martin et al., 2014).

Similarly, β -AA potency, dose, and length of usage all have been shown to affect marbling score. Historically, β AA usage has been associated with decreased marbling scores with β 2-selective agonists having a more pronounced effect on marbling. The Lean et al. (2014) meta-analysis reported a 22 unit decrease in USDA marbling score of steaks from cattle fed zilpaterol compared with those from control-fed cattle, whereas steaks from ractopamine-fed cattle exhibited only a 2 unit decrease. Montgomery et al. (2009) reported a decrease in the percentage of carcasses grading USDA Choice as feeding duration of zilpaterol increased from 20 to 40 d. Lean et al. (2014) also reported an effect of experimental unit evaluated on marbling

score. In studies where cattle were individually fed, no effect of β -AA use on marbling score was observed whereas in studies where cattle were pen fed, substantial decreases in marbling score were observed. Authors suggest slight stress responses associated with competitive feeding behaviors may further exacerbate responsiveness to adrenergic stimulation (Lean et al., 2014).

Sex is another important factor when considering β -AA responsiveness. Quinn et al. (2008) reported feeding heifers 200 mg·heifer⁻¹·d⁻¹ ractopamine for 28 d prior to slaughter had no effect on carcass weight, LM area, 12th-rib fat thickness, yield- or quality grade. Furthermore, a second experiment evaluating the effectiveness of greater feeding durations or higher dosage showed little change in carcass characteristics when heifers were fed for 0, 28, or 42 d prior to slaughter or at a dosage of 0, 200, or 300 mg·heifer⁻¹·d⁻¹ (Quinn et al., 2008). Despite the lack of carcass effects, ractopamine did have a modest effect on feed efficiency in heifers with greater response observed in heifers fed ractopamine for 42 d prior to slaughter (Quinn et al., 2008). Similar response in heifer performance and carcass characteristics was observed by Walker et al., (2006). The interaction between sex and β -AA supplementation is, however, largely compound dependent. Montgomery et al. (2009) reported feeding 8.3 mg/kg DM of zilpaterol to heifers increased HCW, and LM area compared with control-fed heifers. Decreased yield grade, marbling score, and quality grade of zilpaterol-fed heifers was also observed compared with control-fed heifers (Montgomery et al., 2009). Nonetheless, feeding zilpaterol still resulted in a smaller growth and carcass performance response in heifers than observed in steers at the same dosage and duration (Montgomery et al., 2009).

Another consideration is the effect of β -AA addition in *Bos taurus*- vs. *Bos indicus*-type cattle. Gruber et al. (2008) reported an interaction between ractopamine supplementation and biological breed type for objective tenderness such that 200 mg·steer⁻¹·d⁻¹ ractopamine inclusion

had a more detrimental effect on shear force values of steaks from Brahman-cross steers than British-breed steers, with Continental-cross steers intermediate.

ENVIRONMENTAL SUSTAINABILITY

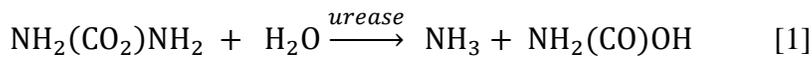
Basic calorimetry proves that at least some portion of the nutrients consumed by all animals are lost to energetic inefficiencies and excretion, whether through gaseous emissions, heat production, urine, or feces. Although early studies evaluating greenhouse gas (**GHG**) emissions, nitrogen, and phosphorus (P) excretions were primarily focused on reducing the production and energy inefficiencies associated with feeding ruminant animals, more recent studies have focused on emissions as they relate to mitigating and minimizing the environmental effects of beef production. Recent consumer and policy-maker interest in environmental sustainability has spurred new interest in developing methods to decrease the environmental footprint of multiple industries, including beef production.

Nitrogen Excretion

Recent advances in precision feeding strategies have helped to reduce nutrient loss (Klopfenstein and Erickson, 2002; Cole 2003). Nonetheless, with feed costs averaging 60-70% of production costs for beef cattle operations (Becker, 2008), producers are primarily motivated by total cost of gain. Such that in instances where available feedstuffs such as distillers grains are cheap enough to be used as an energy source, overfeeding of protein nitrogen is still likely to occur. Excess nutrient excretion not only results in diminished profits through forfeiture of feed costs, but also create environmental challenges as well. Although nitrogen is a critical nutrient required for survival of both plants and animals, excess environmental nitrogen is considered an air, soil, and water pollutant contributing to increased aerosol formation, soil acidity, and eutrophication (Hristov et al., 2010). Formation and volatilization of the nitrogenous molecules

ammonia (**NH₃**) and nitrous oxide (**N₂O**) are primary contributors to these negative environmental impacts in both terrestrial and aquatic ecology.

Fed cattle production systems are considered a major source of excess nitrogen excretion in the environment (Schroder et al., 2003). Cole and Todd (2009) reported 30-50% of fed nitrogen was excreted in feces and 40-70% excreted in urine, with only 10 – 20% of consumed nitrogen retained in animal tissue. This is an environmental concern given 50 – 75% of total excreted nitrogen is lost to volatilization (Bierman et al., 1999). In a study of feedlot cattle, nitrogen volatilization losses averaged 79% of total urinary nitrogen excretion (Cole and Todd (2009). Although fecal nitrogen can be volatilized, it represents a considerably smaller proportion with approximately 1 – 13% of total fecal N excretion volatilized as NH₃ (Bussink and Oenma, 1998). Urea, the main nitrogenous constituent excreted by ruminant animals, represents 60 – 90% of all urinary nitrogen excreted (Bristow et al., 1992) and is considered the primary compound of NH₃ volatilization from cattle manure (Bussink and Oenma, 1998). Although urea itself is not a volatile compound, urine urea hydrolyzes rapidly into NH₃ and CO₂ on contact with feces and soil, according to the reaction shown in equation 1, due to the large number of ureolytic microbes and high urease activity present (Hristov et al., 2010).



Volatilization of NH₃ from fecal sources is a result of degradation of excreted proteins.

Extracellular enzymes break down proteins into amino acids and other nitrogen containing monomers before intracellular enzymes further hydrolyze amino acids into ammonium (**NH₄**; Hristov et al., 2010). Similar to urea, NH₄ is not volatile but is susceptible to ionization into NH₃.

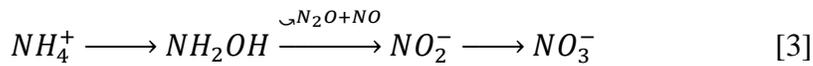
The degree to which $\text{NH}_3/\text{NH}_4^+$ equilibrium shifts is dependent on both pH and temperature (Equation 2) such that a lower pH favors NH_4^+ and reduces the potential for NH_3 volatilization.



Temperature also influences $\text{NH}_4^+/\text{NH}_3$ equilibrium such that an increase in temperature decreases the pH needed for NH_3 formation (Loehr, 1974). As a result, nitrogen volatilizations stemming from feedlot operations and other agricultural sources are greater during summer months and in warmer climates. Further accelerating the rate of nitrogen volatilization, continuous mixing of manure from cattle moving throughout the pen causes greater dissolved CO_2 release increasing both pH of the manure surface exposed to air and rate of NH_3 formation (Montes et al., 2006).

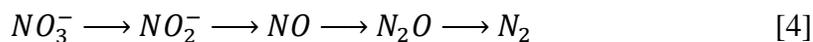
With a boiling point of -33.3°C , NH_3 volatilizes spontaneously on formation. After volatilization, NH_3 can be taken up into the atmosphere where it may bind to gaseous H_2O or react with atmospheric sulfuric or nitric acids to form small particulate matter such as ammonium sulfate, ammonium bisulfate, or ammonium nitrate. This particulate matter known as “fine particles” contribute to air pollution impairing visibility and human health (USEPA, 2004). Whereas NH_3 -bound to water vapor has a relatively short atmospheric lifespan and is deposited near its point of origination, NH_3 that has formed fine particulate matter has a longer atmospheric lifespan and can be deposited large distances from where it originated.

Manure applied to fields as a fertilizer provides an excellent source of additional nitrogen needed for plant growth. Nitrification, the process of converting NH_4 to nitrate, increases the availability of nitrogen for plants while also producing the substrate for denitrification. In the presence of oxygen, NH_4 is oxidized to nitrite (NO_2) and nitrate (NO_3) by ammonia oxidizing bacteria as shown in equation 3 (Anderson, 1964).



Increased nitrogen excretion may have detrimental effects on aquatic ecosystems as well. Dissolved oxygen consumed in the process of nitrification can create hypoxic conditions in streams, lakes, and ponds. Increased available nitrogen concentrations may give rise to algal bloom growth, some of which may produce toxins harmful to aquatic species and humans (Ward, 2013).

Nitrous oxide, released as a byproduct of the conversion of hydroxylamine to NO_2 in nitrification, can have negative environmental consequences as well. Representing a loss to the bioavailable nitrogen pool, N_2O is a potent atmospheric greenhouse gas with global warming potential 300 times that of CO_2 (Ward, 2013). Nitrous oxide is also created as a byproduct of the denitrification process by which nitrates are ultimately reduced to nitrogen gas (N_2 ; equation 4).



Given the increased social interest and goal to improve sustainability of beef production, determining baseline nitrogen excretion and NH_3 emissions data has become increasingly important. In most cases, relatively little data is available regarding these baseline emissions and nutrient excretion of farmed livestock species to set realistic standards for improvements. Furthermore, environmental attributes such as location climatic patterns, cattle bedding type, waste storage methods, and estimation techniques make baseline evaluations largely specific to the conditions under which they were evaluated with less application to other production systems.

Although growth-promoting technologies, such as steroidal implants, ionophores, and β -AA, have long been recognized for their ability to improve efficiency of BW gain and carcass leanness, more recently these technologies have entered the environmental sustainability

discussion for their ability to decrease feed resource inputs and environmental outputs per kg of beef produced. Although these secondary improvements in environmental sustainability have been documented, there is less information available regarding the direct effects of growth-promoting technologies on nutrient excretion. If β -AA are able to increase carcass protein accretion, then a greater amount of fed nitrogen would be retained in the carcass thereby reducing nitrogen excretion into the environment.

Gaseous Emissions through Eructation

Greenhouse gas emissions such as methane (CH_4) have been identified as a potential contributor to global warming and climate change (Tyler, 1991) with emissions stemming from cattle production accounting for an estimated 2% of the total global warming change in the next 50 years (Johnson and Johnson, 1995). On average, cattle lose 2-12% of GE intake in the form of eructated methane losses (Johnson et al., 2000), but this range of methane emissions in beef cattle is largely dependent on animal size, dietary composition and feed intake (Johnson and Johnson, 1995; Beuchemin and McGinn, 2006). Feeding high concentrate diets results in less methane production per unit of substrate, a result of the faster fermentation rate of non-structural carbohydrates than structural carbohydrates such as cellulose and hemicellulose. Lower ruminal pH, associated with higher concentrate diets, also inhibits activity of methanogenic bacterial species reducing total ruminal methanogenesis (Demeyer and Henderickx, 1967; Lana et al., 1998). Further, feed intake above maintenance has been shown to reduce methane production. Where limit-fed diets resulted in greater CH_4 production, the same diets fed for ad libitum intake actually decreased CH_4 emissions (Blaxter and Clapperton, 1965; Johnson et al., 1993). As such, it is accepted that feedlot cattle receiving high concentrate diets have reduced methane emissions

compared with other beef cattle, losing on average only 3.5% GE intake as CH₄ (Houghton et al., 1996).

Growth-promoting technologies have also been evaluated for their ability to decrease GHG emissions per kg of beef. In a study evaluating the effects of growth-promoting technologies on feedlot GHG emissions and profitability, Coopriider et al. (2010) reported no difference in total GHG output of steers receiving conventional production technologies (anabolic steroids, ionophores, antimicrobial drugs, β -AA) and those receiving none. However, when put on a basis of GHG emissions per unit of BW gain or saleable product, steers raised using these production technologies demonstrated a 31% decrease in emissions per finished steer compared with steers raised without (Coopriider et al., 2010). As a small molecule drug compound, little data is available regarding the pre-absorption effects of β -AA and even less on the direct effects of β -AA on GHG emissions. Small improvements in nutrient digestibility (López-Carlos et al., 2010; Walker and Drouillard et al., 2010) and changes to microbial populations (Edrington et al., 2006; Walker, 2009) have been observed in cattle fed β -AA. As a result, it is reasonable to expect altered ruminal fermentation of these cattle may decrease fermentation-associated methane production.

LITERATURE CITED

- Anderson, J. 1964. The metabolism of hydroxylamine to nitrite by *Nitrosomonas*. *Biochem. J.* 91: 8-17.
- AMSA. 2012. Meat color measurement guidelines. 2nd ed. Am. Meat Sci. Assoc., Champaign, IL.
- Arp, T. S., S. T. Howard, D. R. Woerner, J. A. Scanga, D. R. McKenna, W. H. Kolath, P. L. Chapman, J. D. Tatum, and K. E. Belk. 2014. Effects of dietary ractopamine hydrochloride and zilpaterol hydrochloride on performance, carcass traits, and carcass cutability in beef steers. *J. Anim. Sci.* 92: 836-843. doi: 10.2527/jas2013-7122
- Avendaño-Reyes, L., V. Torres-Rodríguez, F. J. Meraz-Murillo, C. Pérez-Linares, F. Figueroa-Saavedra, and P. H. Robinson. Effects of two β -adrenergic agonists on finishing performance, carcass characteristics, and meat quality of feedlot steers. *J. Anim. Sci.* 84: 3259-3265. doi: 10.2527/jas.2006-173
- Beauchemin, K. A., and S. M. McGinn. 2005. Methane emissions from feedlot cattle fed barley or corn diets. *J. Anim. Sci.* 83: 653-661. doi:10.2527/2005.833653x
- Becker, G. S. 2008. Livestock Feed Costs: Concerns and Options. Congressional Research Service – Library of Congress. <http://nationalaglawcenter.org/wp-content/uploads/assets/crs/RS22908.pdf> Accessed: 16 May 2018.
- Beerman, D. H., W. R. Butler, D. E. Hogue, V. K. Fishell, R. H. Dalrymple, C. A. Ricks, and C. G. Scanes. 1987. Cimaterol-induced muscle hypertrophy and altered endocrine status in lambs. *J. Anim. Sci.* 65: 1514-1524.

- Bierman, S., G. E. Erickson, T. J. Klopfenstein, R. A. Stock, D. H. Shain. 1999. Evaluation of nitrogen and organic matter balance in the feedlot as affected by level and source of dietary fiber. *J. Anim. Sci.* 77: 1645-1653. doi:10.2527/1999.7771645x
- Berdeaux, R. and R. Stewart. 2012. cAMP signaling in skeletal muscle adaptation: hypertrophy, metabolism, and regeneration. *Am. J. Physiol. Endocrinol. Metab.* 303: E1-E17. doi:10.1152/ajpendo.00555.2011
- Blaxter, K. L., and J. L. Clapperton. 1965. Prediction of the amount of methane produced by ruminants. *Br. J. Nutr.* 19: 511-522.
- Boler, D. D., S. F. Homer, F. K. McKeith, J. Killefer, D. L. VanOverbeke, G. G. Hilton, R. J. Delmore, J. L. Beckett, J. C. Brooks, R. K. Miller, D. B. Griffin, J. W. Savell, T. E. Lawrence, N. A. Elam, M. N. Streeter, W. T. Nichols, J. P. Hutcheson, D. A. Yates, and D. M. Allen. 2009. Effects of feeding zilpaterol hydrochloride for twenty to forty days on carcass cutability and subprimal yield of calf-fed Holstein steers. *J. Anim. Sci.* 87: 3722-3729. doi:10.2527/jas.2009-1830.
- Boler, D. D., A. L. Shreck, D. B. Faulkner, J. Killefer, F. K. McKeith, J. W. Himm, and J.A. Scanga. 2012. Effect of ractopamine hydrochloride (Optaflexx) dose on live animal performance, carcass characteristics, and tenderness in early weaned beef steers. *Meat Sci.* 92: 458-463. doi: 10.1016/j.meatsci.2012.05.011
- Borgstrom, L., B. M. Kennedy, B. Nilsson, and B. Angelin. 1990. Relative absorption of two enantiomers of terabutaline after duodenal administration. *Eur. J. Clin. Pharmacol.* 38: 621-623.

- Bristow, A. W., D. C. Whitehead, and J. E. Cockburn. 1992. Nitrogenous constituents in the urine of cattle, sheep, and goats. *J. Sci. Food Agric.* 59: 387-394.
doi:10.1002/jsfa.2740590316
- Brockway, J. W., J. C. MacRae, and P. E. Williams. 1987. Side effects of clenbuterol as a repartitioning agent. *Vet. Rec.* 120: 381-383.
- Bussink, D. W. and O. Oenema. 1998. Ammonia volatilization from dairy farming systems in temperate areas: a review. *Nutr. Cycl. Agroecosyst.* 51: 19-33.
doi:10.1023/A:1009747109538
- Bylund, D. B., D. C. Eikenberg, J. P. Hieble, S. Z. Langer, R. J. Lefkowitz, K. P. Minnean, P. B. Molinoff, R. R. Ruffolo, Jr., and U. 1994. Trendelenburg. International union of pharmacology nomenclature of adrenoceptors. *Pharmacol. Rev.* 46:121-135
- Cole, N. A. 2003. Precision feeding: Opportunities and limitations. TAES publication AREC 03-13, 1-19 in *Proc. Plains Nutr. Council.* Texas A&M Res. Ext. Center, Amarillo, TX.
- Cole, N. A., and R. W. Todd. 2009. Nitrogen and phosphorous balance of beef cattle feedyards. In: *Proceedings of the Texas animal manure management issues conference.* Bushland, TX: USDA-ARS-CPRL. 17 – 24.
<https://www.ars.usda.gov/research/publications/publication/?seqNo115=243602>
Accessed: 10 May 2018.
- Cong, M., V. F. Thompson, D. E. Goll, and P. B. Antin. 1998. The bovine calpastatin gene promoter and a new N-terminal region of the protein are targets for cAMP-dependent protein kinase activity. *J. Biol. Chem.* 273: 660-666. doi:10.1074/jbc.273.1.660

- Conn, P. J., A. Christopoulos, and C. W. Lindsley. 2009. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nature Rev. Drug Discov.* 8: 41-54. doi:10.1038/nrd2760
- Cook, D. R., M. R. Doumit, and R. A. Merkel. 1995. Ractopamine, cAMP, and forskolin enhance the mitogenic activity of platelet-derived growth factors-BB on cultured porcine satellite cells. *Basic Appl. Myol.* 1: 57-64.
- Coopriider, K. L., F. M. Mitloehner, T. R. Famula, E. Kebreab, Y. Zhao, and A. L. Van Eenenaam. 2010. Feedlot efficiency implications on greenhouse gas emissions and sustainability. *J. Anim. Sci.* 89: 2643-2656. doi: 10.2527/jas.2010-3539
- Dalidowicz, J. E., T. D. Thomson, and G. E. Babbitt. 1992. Ractopamine hydrochloride, a phenethanolamine repartitioning agent: Metabolism and residues. In: D. H. Hutson, D. R. Hawkins, G. D. Paulson, and C. B. Struble. *Xenobiotics and Food Producing Animals*. Pg. 234-243. American Chemical Society, Washington, DC.
- Demeyer, D. I., and H. K. Henderickx. 1967. Methane production in vitro by mixed rumen bacteria. *Biochem. J.* 105: 271-277.
- Edrington, T. S., T. R. Callaway, S. E. Ives, M. J. Engler, T. H. Welsh, D. M. Hallford, K. J. Genovese, R. C. Anderson, and D. J. Nisbet. 2006. Effect of ractopamine HCl supplementation on *Escherichia coli* O157:H7 and *Salmonella* in feedlot cattle. *Current Micro.* 53: 340-345. doi: 10.1007/s00284-006-0200-9
- Emorine, L. J., S. Marullo, M. M. Briend-Sutren, G. Patey, K. Tate, C. Delavier-Klutchko, and A. D. Strosberg. 1989. Molecular characterization of the human β 3-adrenergic receptor. *Science.* 245: 1118-1121.

- Fiems, L. C. V. Boucqué, B. G. Cottyn, and G. Van de Voorde. 1991. Effects of dietary cimaterol on performance and carcass traits in bulls and on aspects of digestion in cattle and sheep. *Ann. Zootech.* 40: 191-200.
- Freedman, N. J. and R. J. Lefkowitz. 1996. Desensitization of G protein-coupled receptors. *Recent Prog. Horm. Res.* 51: 319-351
- Gene [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004 – [cited 2017 September 27]. Available from: <https://www.ncbi.nlm.nih.gov/gene/>
- Grant, A. L., W. G. Helderich, R. A. Merkel, and W. G. Bergen. 1990. Effects of phenethanolamines and propranolol on the proliferation of cultured chick breast muscle satellite cells. *J. Anim. Sci.* 68: 652-658.
- Gruber, S. L., J. D. Tatum, T. E. Engle, K. J. Prusa, S. B. Laudert, A. L. Schroeder, and W. J. Platter. 2008. Effects of ractopamine supplementation and postmortem aging on longissimus muscle palatability of beef steers differing in biological type. *J. Anim. Sci.* 86: 205-210. doi: 10.2527/jas.2007-0201.
- Hausdorff, W. P., M. G. Caron, and R. J. Lefkowitz. 1990. Turning off the signal: desensitization of β -adrenergic receptor function. *FASEB J.* 4: 2881-2889.
- Helderich, W. G., D. B. Jump, D. B. Anderson, D. M. Skjaerlund, R. A. Merkel, and W. G. Bergen. 1990. Skeletal muscle α -actin synthesis is increased pretranslationally in pigs fed the phenethanolamine ractopamine. *Endocrin.* 126: 3096-3100.
- Hilton, G. G., J. L. Montgomery, C. R. Krehbiel, D. A. Yates, J. P. Hutcheson, W. T. Nichols, M. N. Streeter, J. R. Blanton Jr., and M. F. Miller. 2009. *J. Anim. Sci.* 87:1394-1406. doi: 10.2527/jas.2008-1170

- Houghton, J. T., L. G. Meira Foilho, B. Lim, K. Tréanton, I. Mamaty, Y. Bonduki, D. J. Griggs, and B. A. Callander. 1996. Greenhouse gas inventory reference manual. Revised 1996 IPCC Guidelines for National Gas Inventories. Vol. 3. Agricultural, Intergovernmental Panel on Climate Change, Geneva, Switzerland.
- Hristov, A. N., M. Hanigan, A. Cole, R. Todd, T. A. McAllister, P. M. Ndegwa, and A. Rotz. 2010. Review: Ammonia emissions from dairy farms and beef feedlots. *Can. J. Anim. Sci.* 91: 1-35. doi: 10.1139/CJAS10034
- Hu, C. Y., J. Novakofski, and H. J. Mersmann. 1988. Clenbuterol stimulated increase of plasma free fatty acids in reserpinized pigs. *Comp. Biochem. Physiol.* 91: 619-622.
- Johnson, D. E., T. M. Hill, B. R. Carmean, M. E. Branine, D. W. Lodman, and G. M. Ward. 1993. Principle factors varying methane emissions from ruminants and other animals. In: M. A. K. Khalil (Ed.) *Atmospheric Methane: Sources, Sinks, and Role in Global Change*. NATO ADI Series Vol 113, Springer-Verlag, Berlin, Germany.
- Johnson, K. A., and D. E. Johnson. 1995. Methane emissions from cattle. *J. Anim. Sci.* 73: 2483-2492.
- Johnson, D. E., K. A. Johnson, G. M. Ward, and M. E. Branine. 2000. Ruminants and other animals. In: *Atmospheric Methane: Its Role in the Global Environment*. M. A. K. Khalil (Ed.) *Atmospheric Methane: Sources, Sinks, and Role in Global Change*. NATO ADI Series Vol 113, Springer-Verlag, Berlin, Germany.
- Johnson, B. J., S. B. Smith, and K. Y. Chung. 2014. Historical overview of the effects of β -adrenergic agonists on beef cattle production. *Asian Australas. J. Anim. Sci.* 5: 757-766. doi: 10.5713/ajas.2012.12524

- Killefer, J., and M. Koohmaraie. 1994. Bovine skeletal muscle calpastatin: Cloning, sequence analysis, and steady-state mRNA expression. *J. Anim. Sci.* 72: 606-614. doi: 10.2527/1994.723606x
- Kim, Y. S., Y. B. Lee, W. N. Garrett, and R. H. Dalrymple. 1989. Effects of cimaterol on nitrogen retention and energy utilization in lambs. *J. Anim. Sci.* 67: 674-681.
- Klopfenstein, T. J., and G. E. Erickson. 2002. Effects of manipulating protein and phosphorus nutrition of feedlot cattle on nutrient management and the environment. 80(Suppl. 2): E106-E114
- Koohmaraie, M., S. D. Shackelford, N. E. Muggli-Cockett, and R. T. Stone. 1991. Effect of the β -adrenergic agonist L_{644,969} on muscle growth, endogenous proteinase activities, and postmortem proteolysis in wether lambs. *J. Anim. Sci.* 69: 4823-4835. doi: 10.2527/1991.69124823x
- Koontz, A. F., S. W. ElKadi, D. L. Harmon, E. S. Vanzant, J. C. Matthews, J. A. Boling, and K. R. McLeod. 2009. Effect of ractopamine on whole body and splanchnic energy metabolism in Holstein steers. *Can. J. Anim. Sci.* 90: 77-85. doi: 10.4141/CJAS09078
- Krief, S., F. Lönnqvist, S. Raimbault, B. Baude, A. Van Spronsen, P. Arner, A. D. Strosberg, D. Ricquier, and L. J. Emorine. 1993. Tissue distribution of β 3-adrenergic receptor mRNA in man. *J. Clin. Invest.* 91: 344-349.
- Lana, R. P., J. B. Russell, and M. E. Van Amburgh. 1998. The role of pH in regulation of ruminal methane and ammonia production. *J. Anim. Sci.* 76: 2190-2196. doi: 10.2527/1998.7682190x

- Lands, A. M., A. Arnolds, J. P. McAuliff, F. P. Luduena, and T. G. Brown, Jr. 1967. Differentiation of receptor systems activated by sympathomimetic amines. *Nature*. 214: 597-598.
- Langin, D., G. Tavernier, and M. Lafontan. 1995. Regulation of beta₃-adrenoceptor expression in white fat cells. *Fund. Clin. Pharm.* 9: 97-106. doi: 10.1111/j.1472-8206.1995.tb00268.x
- Liang, W., and S. E. Mills. 2002. Quantitative analysis of β -adrenergic receptor subtypes in pig tissues. *J. Anim. Sci.* 80: 963-970. doi: 10.2527/2002.804963x
- Lean, I. J., J. M. Thompson, and F. R. Dunshea. 2014. A meta-analysis of zilpaterol and ractopamine effects on feedlot performance, carcass traits and shear strength of meat in cattle. *PLOS ONE*. 9: e115904. doi: 10.1371/journal.pone.0115904
- Levy, F. O., X. Zhu, A. J. Kaumann, and L. Birnbaumer. 1993. Efficacy of β_1 -adrenergic receptors is lower than that of β_2 -adrenergic receptors. *Proc. Natl. Acad. Sci.* 90: 10798-10802.
- Liu, J. J., R. Horst, V. Katritch, R. C. Stevens, and K. Wuthrich. 2012. Biased signaling pathways in β_2 -adrenergic receptor characterized by ¹⁹F-NMR. *Science*. 335: 1106-1110. doi: 10.1126/science.1215802
- Loehr, R. C. 1974. *Agricultural waste management: Problems, processes, and approaches*. Academic Press Inc., New York, NY. pg. 576
- Lopez-Carlos, M. A., R. G. Ramírez, J. I. Aguilera-Soto, C. F. Aréchiga, F. Méndez-Llorente, H. Rodríguez, and J. M. Silva. 2010. Effect of ractopamine hydrochloride and zilpaterol hydrochloride on growth, diet digestibility, intake, and carcass characteristics of feedlot lambs. *Liv. Sci.* 131: 23-30 doi: 10.1016/j.livsci.2010.02.018

- Martin, J. N., A. J. Garmyn. M. F. Miller, J. M. Hodgen, K. D. Pfeiffer, C. L. Thomas, R. J. Rathmann, D. A. Yates, J. P. Hutcheson, and J. C. Brooks. 2014. Comparative effects of beta-adrenergic agonist supplementation on the yield and quality attributes of selected subprimals from calf-fed Holstein steers. *J. Anim. Sci.* 92: 4204-4216. doi: 10.2527/jas2014-7881
- McNeel, R. L., and H. J. Mersmann. 1999. Distribution and quantification of beta₁-, beta₂-, and beta₃-adrenergic receptor subtype transcripts in porcine tissues. *J. Anim. Sci.* 77: 611-621. doi: 10.2527/1999.773611x
- Mersmann, H. J. 1995. Species variation in mechanisms for modulation of growth by beta-adrenergic receptors. *J. Nutr.* 125(Suppl. 6): 1777S-1782S.
- Mersmann, H. J. 1998. Overview of the effects of β -adrenergic receptor agonists on animal growth including mechanisms of action. *J. Anim. Sci.* 76: 160-172.
- Mills, S. E., and Mersmann, H. J. 1995. Beta-adrenergic agonists, their receptors, and growth: Special reference to the peculiarities in pigs. In: S. B. Smith and D. R. Smith (Ed.) *Biology of Fat in Meat Animals: Current Advances*. Pg. 65-96. CAB International, Wallington, Oxon, U.K.
- Mills, S. E. 2002. Biological basis of ractopamine response. *J. Anim. Sci.* 80(E Suppl. 2): E28-E32.
- Montes, F., C. A. Rotz, and H. Chaoui. 2009. Process modeling of ammonia volatilization from ammonium solution and manure surfaces. *Trans. ASABE.* 52: 1707-1719. doi:10.13031/2013.24600
- Montgomery, J. L., C. R. Krehbiel, J. J. Cranston, D. A. Yates, J. P. Hutcheson, M. N. Streeter, D. T. Bechtol, E. Johnson. T. TerHune, and T. H. Montgomery. 2009. Dietary zilpaterol

- hydrochloride. I. Feedlot performance and carcass traits of steers and heifers. *J. Anim. Sci.* 87: 1374-1383. doi: 10.2527/jas.2008-1162
- Nantel, F., H. Bonin, L. J. Emorine, V. Zilberfarb, A. D. Strosberg, C. Bouvier, and S. Marullo. 1993. The human β_3 -adrenergic receptor is resistant to short term agonist-promoted desensitization. *Mol. Pharmacol.* 43: 548-555.
- Norton, L. E., and D. K. Layman. 2006. Leucine regulates translation initiation of protein synthesis in skeletal muscle after exercise. *J. Nutr.* 136: 5335-5375.
- Pierce, K. L., and R. J. Lefkowitz. 2001. Classical and new roles of β -arrestins in the regulation of g-protein-coupled receptors. *Nature Rev. Neuro. Sci.* 2: 727-733.
- Prados, L. F., M. L. Chizzotti, S. C. V. Filho, F. H. M. Chizzotti, P. P. Rotta, L. F. C. Silva. 2016. Environmental management and prediction of nitrogen and phosphorus excretion by beef cattle. *Nutrient Requirements of Zebu and Crossbred Cattle (BR-CORTE)*. Third Edition. S. C. V. Filho and L. F. C. Silva. (Ed.) Suprema Gráfica Ltda. Brazil.
- Pringle, T. D., C. R. Calkins, M. Koohmaraie, and S. J. Jones. 1993. Effects over time of feeding a β -adrenergic agonist to wether lambs on animal performance, muscle growth, endogenous muscle proteinase activities, and meat tenderness. *J. Anim. Sci.* 71: 636-644. doi: 10.2527/1993.713636x
- Quinn, M. J., C. D. Reinhardt, E. R. Loe, B. E. Depenbusch, M. E. Corrigan, M. L. May, and J. S. Drouillard. 2008. The effects of ractopamine-hydrogen chloride (Optaflexx) on performance, carcass characteristics, and meat quality of finishing feedlot heifers. *J. Anim. Sci.* 86: 902-908. doi: 10.2527/jas.2007-0117.

- Reeds, P. J., M. F. Fuller, and B. A. Nicholson. 1985. Metabolic basis of energy expenditure with particular reference to protein synthesis. In: J. Garrow and D. Halliday (Ed.) Substrate and Energy Metabolism in Man. Pg. 46-57. John Libbey, London.
- Ricke, E. A., G. L. Larson, D. J. Smith, V. J. Feil, and J. S. Caton. 1996. Influence of intraperitoneal administration of ractopamine HCl stereoisomers on growth, protein retention, and carcass composition in rats fed commercial diets. *J. Anim. Sci.* 74(Suppl. 1): 210.
- Ricks, C. A., R. H. Dalrymple, P. K. Baker, and D. L. Ingle. 1984. Use of a β -agonist to alter fat and muscle deposition in steers. *J. Anim. Sci.* doi: 10.2527/jas1984.5951247x
- Rikhardsson, G., K. A. Johnson, and D. E. Johnson. 1991. Effects of cimaterol on energetics and carcass characteristics of Suffolk ewe lambs. *J. Anim. Sci.* 69: 396-404 doi: 10.2527/1991.691396x
- Ruffolo, R. R. 1991. Chirality in α - and β -adrenoceptor agonists and antagonists. *Tetrahedron* 47: 9953-9980.
- Sandri, M., C. Sandri, A. Gilbert, C. Skurk, E. Calabrai, A. Picard, K. Walsh, S. Schiaffino, S. H. Lecker, and A. L. Goldberg. 2004. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell.* 117: 399-412. doi: 10.1016/S0092-8674(04)00400-3
- Samuelson, K. L., M. E. Hubbert, M. L. Galyean, and C. A. Löest. 2016. Nutritional recommendations of feedlot consulting nutritionists: The 2015 New Mexico State and Texas Tech University survey. *J. Anim. Sci.* 94: 2648-2663. doi: 10.2527/jas2016-0282

- Schroeder, A. L., D. M. Polser, S. B. Laudert, and G. J. Vogel. 2003. Effect of Optaflexx on growth performance and carcass traits of steers. Optaflexx Exchange No. 1. Elanco Animal Health, Greenfield IN.
- Schröder, J. J., H. F. M. Aarts, H. F. M. ten Berge, H. van Keulen, and J. J. Neeteson. 2003. An evaluation of whole-farm nitrogen balances and related indices for efficient nitrogen use. *Eur. J. Agron.* 20: 33-44. doi: 10.1016/S1161-0301(03)00070-4
- Sillence, M. N., and M. L. Matthews. 1994. Classical and atypical binding sites for β -adrenoceptor ligands and activation of adenylyl cyclase in bovine skeletal muscle and adipose tissue membranes. *Br. J. Pharmacol.* 111: 866-872. doi: 10.1111/j.1476-5381.1994.tb14818.x
- Smith, D. J., and G. D. Paulson. 1997. Distribution, elimination, and residues of [^{14}C] clenbuterol HCl in Holstein calves. *J. Anim. Sci.* 75: 454-461.
- Smith, D. J. 1998. The pharmacokinetics, metabolism, and tissue residues of β -adrenergic agonists in livestock. *J. Anim. Sci.* 76: 173-194.
- Spurlock, M. E., J. C. Cusumano, S. Q. Ji, D. B. Anderson, C. K. Smith II, D. L. Hancock, and S. E. Mills. 1994. The effect of ractopamine on β -adrenoceptor density and affinity in porcine adipose and skeletal muscle tissue. *J. Anim. Sci.* 72: 75-80.
- Strader, C. D., I. S. Sigal, and R. A. F. Dixon. 1989. Structural basis of β -adrenergic receptor function. *FASEB J.* 3: 1825-1832.
- Stitt, T. N., D. Drujan, B. A. Clarke, F. Panaro, Y. Timofeyva, W. O. Kline, M. Gonzalez, G. D. Yancopoulos, D. J. Glass. 2004. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol. Cell.* 14: 395-403. doi: 10.1016/S1097-2765(04)00211-4

- Stoffel, B., and H. H. D. Meyer. 1993. Effects of the β -adrenergic agonist clenbuterol in cows: Lipid metabolism, milk production, pharmacokinetics, and residues. *J. Anim. Sci.* 71: 1875-1881.
- Strosberg, A. D. 1996. G protein coupled R7G receptors. *Cancer Surv.* 27: 65-83.
- Strydom, P. E., L. Frylinck, J. L. Montgomery, and M. F. Smith. 2009. The comparison of three β -agonists for growth performance, carcass characteristics, and meat quality of feedlot cattle. *Meat Sci.* 81: 557-564. doi: 10.1016/j.meatsci.2008.10.011
- Tanaka, Y., T. Horinouchi, and K. Koike. 2005. New insights into β -adrenoceptors in smooth muscle: Distribution of receptor subtypes and molecular mechanisms triggering muscle relaxation. *Clin. Exp. Pharm. Phys.* 32: 503-514. doi: 10.1111/j.1440-1681.2005.04222.x
- Tyler, S. C. 1991. The global methane budget. In: J. E. Rodgers and W. B. Whiteman (Ed.) *Microbial Production and Consumption of Greenhouse Gases: Methane, Nitrogen Oxides, and Halomethanes.* Am. Soc. Microbiol., Washington, D.C.
- Ungemach, F. R. 2004. Ractopamine (addendum). WHO Food Additive Series: 53.
<http://www.inchem.org/documents/jecfa/jecmono/v53je08.htm> Accessed on: 23 October 2017.
- United States Environmental Protection Agency. 2004. National emission inventory – Ammonia emissions from animal husbandry operations. USEPA, Washington, DC.
https://epa.gov/ttn/chief/ap42/ch09/related/nh3inventorydraft_jan2004.pdf Accessed: 20 May 2018.
- Walker, C. E. 2009. Effects of ractopamine hydrochloride are not confined to mammalian tissue: Evidence for direct effects of ractopamine hydrochloride supplementation on

fermentation by ruminal microorganisms (Dissertation). Retrieved from K-REx Repository.

Walker, C. E. and J. S. Drouillard. 2010. Effects of ractopamine hydrochloride are not confined to mammalian tissue: Evidence for direct effects of ractopamine hydrochloride supplementation on fermentation by ruminal microorganisms. *J. Anim. Sci.* 88: 697-706. doi: 10.2527/jas.2009-1999

Walker, D. K., E. C. Titgemeyer, J. S. Drouillard, E. R. Loe, B. E. Depenbusch, and A.S. Webb. 2006. Effects of ractopamine and protein source on growth performance and carcass characteristics of feedlot heifers. *J. Anim. Sci.* 84:2795-2800. doi: 10.2527/jas.2005-614

Walker, D. K., E. C. Titgemeyer, T. J. Baxa, K. Y. Chung, D. E. Johnson, S. B. Laudert, and B. J. Johnson. 2010b. Effects of ractopamine and sex on serum metabolites and skeletal muscle gene expression in finishing steers and heifers. *J. Anim. Sci.* 88: 1349-1357. doi: 10.2527/jas.2009-2409

Ward, B. B. 2013. Nitrification.

https://www.princeton.edu/nitrogen/publications/pdfs/Ward_2015_Nitrification.pdf

Accessed: 15 May 2018.

Yuge, B. T., T. Hase, Y. Takayanagi, T. Kamasuka, K. Amemiya, and M. Horiba. 1984.

Pharmacokinetic studies of mabuterol, a new selective β_2 -stimulant. I: Studies on the absorption, distribution, and excretion in rats. *Arzneim.-Forsch.* 34:1659-1667

Chapter 2

EFFECTS OF RACTOPAMINE HYDROCHLORIDE ON NUTRIENT DIGESTIBILITY AND NITROGEN EXCRETION OF FINISHING STEERS

ABSTRACT

The objective was to quantify the effects of ractopamine hydrochloride (Actogain) on nitrogen excretion and nutrient digestibility. In experiment 1, twelve Simmental × Angus steers were used in a randomized complete block design. Dietary treatments were top-dressed and included: 1) a control without Actogain (CON) or 2) 400 mg·steer⁻¹·d⁻¹ ractopamine hydrochloride (RAC) for 35 d before slaughter. Steers were blocked by weight (BW = 531 ± 16 kg). Diets contained 55% dry rolled corn, 20% corn silage, 15% modified wet distillers grains with solubles, and 10% supplement on a DM basis. For each block, total collection of feed, orts, feces and urine were conducted for two 5 d sampling periods during week 2 and 4 of RAC supplementation. No interaction ($P > 0.10$) between treatment and collection period was observed for any parameter evaluated. Dietary treatment had no effect ($P = 0.38$) on DMI, but RAC-fed steers had decreased fecal DM output (2.1 vs. 2.5 kg DM/d; $P = 0.04$) compared with CON-fed steers. Steers fed RAC had greater apparent total tract DM digestibility (72.8 vs. 68.9%; $P = 0.02$), NDF digestibility (59.2 vs. 53.3%; $P < 0.01$), and ADF digestibility (53.8 vs. 47.9%; $P = 0.05$) than CON-fed steers. Although dietary treatment did not affect nitrogen intake ($P = 0.51$) or nitrogen digestibility ($P = 0.14$), RAC-fed steers excreted less total nitrogen (120.8 vs. 138.2 g/d; $P = 0.02$) than CON-fed steers due to a tendency for decreased fecal nitrogen output (61.5 vs. 71.8 g/d; $P = 0.09$) in RAC-fed steers compared with CON-fed steers. However,

dietary treatment had no effect ($P = 0.31$) on urinary nitrogen output or percentage of urinary nitrogen excreted as urea. Experiment 2 was an in vitro experiment conducted to validate the effects of RAC on nutrient digestibility using a contemporary group of heifers ($N = 19$). Rumen fluid was collected by stomach tube from CON- and RAC-fed heifers to inoculate tubes containing the CON or RAC in a split-plot design. No interaction between rumen fluid source and in vitro substrate was observed ($P = 0.44$). Greater IVDMD (64.0 vs. 58.6%; $P = 0.01$) was observed in rumen fluid from RAC-fed heifers compared with rumen fluid from CON-fed heifers. Inclusion of RAC in the in vitro substrate increased IVDMD (63.1 vs. 59.5%; $P < 0.01$). Overall, feeding RAC impacted microbial digestion of the finishing diet to increase total tract digestion and reduce nitrogen excretion in the 35 d period prior to slaughter.

INTRODUCTION

Improving the environmental sustainability of beef production has come under increasing producer, consumer, and regulatory scrutiny (Johnson and Johnson, 1995; Capper, 2011). Feedlot cattle production systems are considered a major source of excess nitrogen excretion in the environment (Schroder et al., 2003). Ammonia and methane emissions, nutrient runoff, nitrate leaching, and soil denitrification have surfaced as primary targets in the efforts to mitigate and minimize the environmental effects of intensive beef production systems (Hristov et al., 2010; Prados et al., 2016). Indications for beta-adrenergic agonists (β -AA) such as ractopamine hydrochloride (RAC; Actogain, Zoetis, Parsippany, NJ) and other growth-promoting technologies (GPT) include improvements in efficiency of BW gain and carcass leanness. Furthermore, GPT have enabled producers to use fewer land and feed resources while reducing manure output and greenhouse gas emissions compared with steers raised without GPT (Coopridge et al., 2010; Capper and Hayes, 2012). Despite these secondary improvements in

environmental sustainability associated with the use of β -AA, relatively little is known about the direct effects of β -AA on nitrogen excretion, methane emissions, and nutrient digestibility. Beta agonists are proven to increase carcass protein concentration by up to 8% in finished steers, improving retail cut yields (Boler et al., 2009; Hilton et al., 2009). If β -AA can increase carcass protein accretion, then a greater amount of fed nitrogen should be retained, thereby reducing nitrogen excretion into the environment. However, efficaciousness of β -AA is known to decrease over time because of receptor desensitization (Hausdorff et al., 1990). Therefore, the objective was to quantify the effects of RAC and period of RAC inclusion (d 8-13 vs d 22-27) on nitrogen excretion and nutrient digestibility through a pair of in vivo and in vitro experiments.

MATERIALS AND METHODS

Animal procedures were approved by the University of Illinois Institute of Animal Care and Use Committee (IACUC #14278) and followed the guidelines recommended in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010).

Experiment 1: Nitrogen Excretion and Nutrient Digestibility

Twelve Angus-sired, Simmental \times Angus crossbred steers were used in a randomized complete block design to evaluate the effects of ractopamine hydrochloride (Actogain, Zoetis, Parsippany, NJ). Dietary treatments were top-dressed and included: 1) a control without ractopamine hydrochloride (**CON**) or 2) 400 mg·steer⁻¹·d⁻¹ ractopamine hydrochloride (**RAC**) for 35 d before slaughter. Moderately framed steers receiving only a single implant after weaning containing 80 mg trenbolone acetate and 16 mg estradiol (Component TE-IS; Elanco Animal Health, Greenfield, IN) were used, to isolate observations of the potential treatment effects. Steers were split into a heavy (initial BW = 534 \pm 22 kg) and light (initial BW = 529 \pm 8 kg)

block by weight and metabolism trial procedures were conducted sequentially beginning with the heavy block. All steers were fed in separate pens for one week to determine individual feed intake and acclimated to individual feeding behavior and metabolism stalls prior to trial initiation.

The basal diet contained 55% dry rolled corn, 20% corn silage, 15% modified wet distillers grains with solubles, 10% supplement (DM basis, Table 1), and was formulated to meet or exceed NRC (2016) recommendations. The diet was top-dressed with 0.45 kg ground corn which was used as the treatment carrier. Diets were fed once daily with the amount of feed offered continually adjusted throughout the experiment to insure ad libitum intake and complete ingestion of ractopamine as well as to minimize feed wastage. Water was available on an ad libitum basis. Steers were housed in metabolism tie stalls at the University of Illinois Beef Cattle and Sheep Field Research Laboratory in Urbana, IL. Stalls (2.3×1.3 m) were equipped with individual feed bunks and non-siphoning, automatic water bowls housed within gas emission collection chambers (Ruminant Emission Monitoring System [**REMS**]; Urbana, IL). The barn was equipped with heat, ventilation, and air-conditioning systems, providing a controlled environment (18.3° C) for steers on trial.

Sample collection. For each block, two-5 d collection periods were conducted on d 8-13 and d 22-27 with a one week period between collections. Additionally, a one week treatment adaptation period was observed prior to the first collection of each block. Control or RAC diets were fed continuously during the 35 d period including adaptation and rest weeks. Feed samples were collected for each day of the collection period (d 1 – 5). Orts were weighed to determine daily feed intake and were sampled as well. Feed and ort samples were stored at -20° C until analysis.

On d 2 of the collection period, fecal bags were attached to each steer to determine fecal output for the entire 5 d period. Feces were collected in waterproof canvas bags connected to the steer by a leather harness, removed from fecal bags twice daily, and weighed at 0600 h the following morning. A 5% subsample of the total daily fecal weight was added to a composite sample for the entire collection period. Fecal composite samples were stored at -20°C until analysis.

Urine collection funnels were attached to steers on d 1 of the collection period and used to collect urine for the entire 5 d period. Silicone urine funnels were positioned around the steer's sheath. Continuous vacuum suction was applied to the funnel system to aid in collection of urine into 18.9 L plastic collection vessels via plastic hoses. During the 5 d of urine collection, steers were observed to ensure funnels and collection system remained in place and collections accurately represented total urine output. On d 1, urine was collected without acidification in containers placed on ice for analysis of nitrogen species. During the remaining days of urine collection, urine was acidified by adding 175 mL of 6 N HCl in urine containers. Urine was weighed twice daily at 0600 and 1800 hr and a 2% subsample of each weigh periods urine output was added to a composite sample representative of the entire collection period. Urine was stored at -20° C.

Eructation and indirect calorimetry analysis. Head-box style respiration chambers were used to measure respiratory gas exchange for a single continuous 24 h period for each block. Steers were placed into one of six (1 steer per chamber) positively pressurized ventilated hood-type REMS chambers. Features of the individual chambers included thermal environmental control to maintain animal comfort, fresh air supply for carbon dioxide (CO₂) control, and measurement of incoming ventilation volumetric flow rate (Maia et al., 2015). Lastly, gas

sampling was conducted via a solenoid multiplexer to infrared photoacoustic gas analyzer (INNOVA 1412; LumaSense Technologies, Inc., Santa Clara, CA), configured with methane (CH_4), CO_2 , nitrous oxide (N_2O), ammonia (NH_3), and sulfur hexafluoride (SF_6) optical filters. More information regarding the REMS, including system description, operation, sampling integration time, and detection limits of the optical filters are reported by Sun (2013) and Ramirez (2014). The last 5 of 10 gas concentration and thermal environment measurements at each sampling location (6 chambers and barn [incoming]) were averaged every 86 min for approximately 24 h. Before and after each period the steers were in the REMS, a mass recovery test was performed to verify mass measurement was within the expected range for these chambers. Methane emission rates were normalized to 24 h following a trapezoidal integration of the computed ER, resulting in a single CH_4 emission (g/h) for each steer within each period. Feed and water were provided inside the chamber for ad libitum intake.

Laboratory analysis. Feed, orts, and fecal samples were composited within collection period and lyophilized (FreeZone, Labconco, Kansas City, MO) and ground using a Wiley mill (1-mm screen, Arthur H. Thomas, Philadelphia, PA). Ground feed samples, orts, and feces were analyzed for DM (24 h at 105° C), NDF and ADF (Ankom200 Fiber Analyzer, Ankom Technology, Macedon, NY) using the Ankom methods 5 and 6, respectively, nitrogen by combustion (Leco TruMac, LECO Corporation, St. Joseph, MI), fat (Ankom Technology, Macedon, NY) using the Ankom method 2, and total ash (12 h at 500° C; HotPack Muffle Oven 770750, HotPack Corp., Philadelphia, PA). Urine was analyzed for total nitrogen and urea content by the University of Illinois Veterinary Diagnostic Laboratory. Results for total nitrogen analysis were deemed acceptable at a coefficient of variation (CV) of $\leq 5\%$ within duplicates of feed, orts, urine, and feces.

Slaughter and carcass characteristics. On d 35, steers were transported to the University of Illinois abattoir and humanely slaughtered under USDA inspection. At approximately 24 h postmortem, carcasses were evaluated for 12th-rib backfat thickness, LM area, percent KPH, ribeye lean maturity score, and ribeye marbling score by trained personnel. Surface color was evaluated on the exposed LM lean surface at the 12th-13th rib interface using a spectrophotometer (HunterLab Miniscan XE Plus Model 45/0, HunterLab Associates, Reston, VA) with a 2.54 cm diameter aperture, A illuminant, and 10° observer angle. All CIE L*(lightness), a*(redness), and b*(yellowness) measurements were measured on two random locations of the LM and averaged for analyses. Chroma, or saturation index, was used to assess color intensity with greater values indicating greater saturation of the principle hue on the steak (AMSA, 2012). Saturation index (chroma) was calculated using the following equations:

$$\text{Chroma} = (a^{*2} + b^{*2})^{0.5}$$

Experiment 2: In Vitro Dry Matter Disappearance

An in vitro experiment was conducted to validate nutrient digestibility results with a 2 × 2 factorial arrangement of treatments in split-plot design. A contemporary group of heifers (N = 19) were fed the same diet as steers in experiment 1 (Table 1) and treatments were top-dressed including a control without ractopamine hydrochloride (**CON**) or 400 mg·heifer⁻¹·d⁻¹ ractopamine hydrochloride (**RAC**). Heifers were adapted to the diet for 21 d prior to rumen fluid collection. Immediately prior to feeding, rumen fluid was collected by stomach tube from CON- and RAC-fed heifers, strained through 4 layers of cheesecloth, flushed with CO₂, sealed in individual bottles, and transported to the laboratory in a warmed, insulated container to maintain temperature and limit oxygen exposure. Rumen fluid from CON- and RAC-fed heifers was buffered in a 1:2 ratio with McDougall's artificial saliva (McDougall, 1948). The inoculum (30

ml) was added to 50 ml conical tubes fitted with rubber stopper and a one-way valve. Each conical tube contained 0.7 g of the lyophilized, ground diet, with no ractopamine hydrochloride (**IVCON**) added to the substrate or ractopamine hydrochloride added (**IVRAC**). An incubator (HeraTherm Oven, Thermo Scientific, Waltham, MA) was used to maintain the fermentation tubes at 39° C under anaerobic conditions for 24 h. Heifer and in vitro treatment combinations were evaluated in triplicate at 24 h with blanks included for 0 h to correct for DM contained in the ruminal fluid inoculum from each animal. At the end of 24 h, pH of each tube was determined using a benchtop pH meter (Accumet Basic AB15, Fisher Scientific, Hampton, NH) and fermentation was terminated by the addition of 6 N HCl. Samples were filtered and dried at 60° C for at least 24 h before allowed to cool in desiccators and then weighed to determine dry matter disappearance.

Statistical Analysis

Data from experiment 1 were analyzed as a randomized, complete block design with fixed effects of treatment, collection period, their interaction and random effect of block. Experiment 2 was analyzed as a split-plot design with whole plot of heifer treatment and the split-plot of in vitro substrate. Random effects for experiment 2 included block and the interaction between heifer treatment and block. Both experiments were analyzed using the MIXED procedure of SAS (v 9.4; SAS Institute Inc., Cary, NC). Treatment effects and interactions were considered significantly different at $P \leq 0.05$ and trends were discussed at $P \leq 0.10$.

RESULTS

Experiment 1 - Nitrogen Excretion and Nutrient Digestibility

Nitrogen balance and nitrogen species. No interaction ($P \geq 0.59$) between treatment and sampling period was observed for any nitrogen balance parameter evaluated (Table 2). Although RAC inclusion did not affect nitrogen intake ($P = 0.51$), RAC-fed steers excreted less total nitrogen as urine and feces (120.8 vs. 138.2 g/d; $P = 0.02$) than CON-fed steers. This is due in part to a tendency for decreased fecal nitrogen output (61.5 vs. 71.8 g/d; $P = 0.09$) of RAC-fed steers compared with CON-fed steers. Nonetheless, RAC inclusion had no effect ($P = 0.31$) on urinary nitrogen output. There was no difference ($P = 0.44$) in nitrogen retention between RAC- and CON- fed steers, despite the difference in nitrogen excretion; however, numerical differences indicate a 8.8 g/d increase in nitrogen retention of RAC-fed steers compared with CON-fed steers. The discrepancy between nitrogen excretion and nitrogen retention findings is likely a result of the large SEM associated with nitrogen intake in this experiment. No difference ($P = 0.84$) in absorbed nitrogen was observed between RAC- and CON-fed steers. Nitrogen retention findings were unchanged when expressed as a percentage of nitrogen intake ($P = 0.36$) or as a percentage of absorbed nitrogen ($P = 0.56$) with no difference between RAC- and CON-fed steers.

Expressed both in g/d and as a percentage of total urine nitrogen, urea-nitrogen excretion was not affected by RAC inclusion ($P \geq 0.31$) or sampling period ($P \geq 0.19$). No interaction between RAC inclusion and sampling period was observed ($P \geq 0.71$) for urinary urea-nitrogen excretion.

Nutrient digestibility. Although RAC inclusion had no effect ($P = 0.38$) on DMI, RAC-fed steers had decreased fecal DM output (2.1 vs. 2.5 kg DM/d; $P = 0.04$) compared with CON-

fed steers (Table 3). Moreover, RAC-fed steers had greater apparent total tract DM digestibility (72.8 vs. 68.9%; $P = 0.02$), NDF digestibility (59.2 vs. 53.3%; $P < 0.01$), and ADF digestibility (53.8 vs. 47.9%; $P = 0.05$) than CON-fed steers. Further, RAC-fed steers also had greater ($P = 0.04$) OM digestibility than CON-fed steers. Despite the decrease in nitrogen excretion observed in RAC-fed steers, an effect on total tract nitrogen digestibility was not observed ($P = 0.14$).

Sampling period had no effect ($P \geq 0.23$), on DMI, fecal or urinary output, apparent total tract DM or nitrogen digestibility. However, fiber (NDF and ADF) digestibility was greater during the second collection period ($P \leq 0.05$) than the first. Furthermore, there was a tendency for an interaction ($P = 0.10$) between RAC inclusion and sampling period with RAC-fed steers having exhibited substantially greater NDF digestibility (7.35% unit increase) during the second sampling compared with the first, whereas CON-fed steers exhibited a considerably smaller change in digestibility (0.77% unit increase) between sampling periods. Despite a lack of statistical significance ($P = 0.14$), similar results were shown for ADF digestibility with a 10.4% unit increase in ADF digestibility of RAC-fed steers during the second sampling period compared with the first, whereas CON-fed steers exhibited a 1.8% increase between period 1 and 2.

Gaseous emissions and indirect calorimetry. Ractopamine inclusion had no effect ($P = 0.15$) on methane emission via eructation when expressed in g/d (Table 4). Because changes in DMI have been shown to affect methane production (Johnson and Johnson, 1995; Benchaar et al., 2001), emissions were also expressed in g/kg of DMI during the 24 h test period as well as g/kg of DMI averaged over the previous 5 d. No differences ($P = 0.42$) in CH₄ emission were observed when calculated in g/kg of in-chamber DMI. However, when expressed as g/kg of intake 5 d average DMI, there was a tendency ($P = 0.07$) for RAC-fed steers to have greater CH₄ production than

CON-fed steers. However, no differences in CH₄ were observed when expressed as g/kg of digested NDF ($P = 0.22$).

Ractopamine inclusion also had no effect ($P = 0.21$) on ammonia emissions through eructation when expressed in g/d, although RAC-fed steers demonstrated numerically lesser NH₃ production than CON-fed steers. This numerical trend was supported when expressed as a g/kg of in-chamber DMI basis, with RAC-fed steers emitting less ($P < 0.01$) NH₃ through eructation than CON-fed steers. Removing the variability in intake associated with DMI during the 24 test period, when expressed in g/kg of average DMI, there was a tendency ($P = 0.08$) for reduced NH₃ eructation in RAC-fed steers compared with CON-fed steers. As above, when expressed as g NH₃ / kg of NDF digested no differences were observed ($P = 0.14$). No differences ($P \geq 0.41$) were observed in respiratory quotient (RQ), heat production, and moisture production between RAC- and CON-fed steers.

Carcass characteristics. No difference ($P \geq 0.16$) in HCW, dressing %, 12th-rib fat thickness, or LM area of CON- and RAC-fed steers was observed (Table 5). Despite the lack of statistical significance, LM area of RAC-fed steers was 5.03 cm² greater than CON-fed steer. Nonetheless, no differences ($P \geq 0.31$) in calculated USDA yield grade or the percentage of boneless closely trimmed retail cuts (BCTRC) were observed, an expected response given the lack of difference in 12th-rib fat thickness. Interestingly, there was a tendency ($P = 0.06$) for carcasses from RAC-fed steers to have greater KPH than those from CON-fed steers. From a meat quality standpoint, RAC usage had no effect ($P \geq 0.16$) on marbling score, LM lightness (L*), redness (a*), yellowness (b*), reflectance, or chroma values. Despite a lack of statistical difference, lean color at the 12th-rib of RAC-fed steers was numerically redder (2.1 a* units) than

CON-fed steers. In addition, chroma values for RAC-fed steers were numerically greater than CON-fed steers indicating greater color intensity and greater saturation of the primary hue.

Experiment 2 - In Vitro Dry Matter Disappearance

Dietary inclusion of RAC affected IVDMD with tubes containing rumen fluid from heifers fed Actogain (**RAC**) having greater ($P = 0.01$; 64.0 vs 58.6%) DM disappearance than tubes containing rumen fluid from control-fed heifers (**CON**). In vitro inclusion of Actogain also affected IVDMD with greater ($P < 0.0001$; 63.1 vs 59.5%) DM disappearance observed in tubes containing the Actogain added substrate (**IVRAC**) than in those with the control substrate (**IVCON**). No interaction ($P = 0.44$) between dietary RAC inclusion and in vitro RAC inclusion were observed (Figure 1).

DISCUSSION

Recent consumer and policy-maker interest in environmental sustainability has spurred new interest in developing methods to decrease the environmental footprint of multiple industries, including beef production. On average, only 10 – 20% of fed nitrogen is retained in animal tissue with excess nutrient excretion not only reducing profits through forfeited feed costs, but also creating environmental challenges as well. Formation and volatilization of the nitrogenous molecules ammonia (NH_3) and nitrous oxide (N_2O) are major contributors to air, soil, and water pollution in both terrestrial and aquatic ecosystems contributing to increased aerosol formation, soil acidity, and eutrophication (Hristov et al., 2010).

With 50 – 75% of total excreted nitrogen lost to volatilization (Bierman et al., 1999), excess excretion has serious environmental implications. In the present study, RAC usage resulted in a 12.6% decrease in nitrogen excretion. Others have reported similar findings for nitrogen excretion regardless of ractopamine dosage. Carmichael et al. (2018) observed a 10.6%

numerical decrease in nitrogen excretion of steers fed 300 mg·steer⁻¹·d⁻¹ RAC for 28 d and Walker et al. (2007) observed a 10.8% decrease in steers fed 200 mg·steer⁻¹·d⁻¹ RAC. Data have revealed little difference in nitrogen absorption (nitrogen absorbed into bloodstream) of RAC-fed steers (Carmichael et al., 2018; Walker et al., 2007), indicating limited likelihood of absorption-linked effects. Despite the lack of effect observed in absorption, effects of β-AA use on urea kinetics and excretion are less well defined. Representing 60 – 90% of total urinary nitrogen excreted in cattle (Bristow et al., 1992), urea nitrogen is highly susceptible to volatilization as NH₃ given the abundance of ureolytic compounds present in manure and soil (Hristov et al., 2010). Cole et al. (2005) demonstrated urinary N excretion were highly correlated (r = 0.83) to environmental ammonia losses. Like the small numerical decrease in urea nitrogen observed in the present study, Walker et al. (2007) also reported reduced urea nitrogen in urine of RAC-fed steers. However, when standardized for differences in nitrogen intake between treatment urea nitrogen effects are considerably diminished in both studies. Nonetheless, a study evaluating the effects of β-AA on urea recycling reported a tendency for decreased urea-nitrogen entry rate and urea-nitrogen recycled to the gastrointestinal tract in zilpaterol-fed steers when adjusted for nitrogen intake (Brake et al., 2011). Given these findings and the lack of nitrogen absorption effects observed between treatments, data suggest nitrogen repartitioning effects observed in RAC-fed steers may be a result of reduced urea production and recycling.

Steers fed RAC in the current study exhibited a numerical increase in nitrogen retention, similar to the 28% increase in nitrogen retention reported by Walker et al. (2007). Although carcass effects traditionally associated with the use of ractopamine were not observed in the present study, likely a result of the smaller number of animals used in metabolism trials, numerical improvements in LM area were observed suggesting greater nitrogen incorporation

through increased protein accretion. This assumption is further strengthened when considering the observed 35 g daily increase in nitrogen retention over a 35 d period approximately equates to an additional 1.9 kg of carcass protein accretion (assuming a nitrogen conversion factor of 6.25) nearly matching reported treatment differences in HCW.

Walker and Drouillard (2010) revealed grain processing methods, used to improve starch utilization of feed ingredients, may be another important consideration when evaluating nitrogen and urea kinetics in RAC-fed finishing steers. An *in vivo* experiment reported ruminal ammonia concentrations were more affected by RAC supplementation in diets containing dry rolled corn than in diets containing steam-flaked corn (Walker and Drouillard, 2010). Although ruminal ammonia concentrations were not evaluated in the present study, decreased ruminal ammonia concentrations associated with RAC usage when feeding DRC, as observed by Walker and Drouillard, (2010), suggest greater bypass protein or microbial crude protein availability.

In the present study, feeding RAC resulted in an improvement in nutrient digestibility. However, of the relatively few studies evaluating the effects of feeding β -AAs on nutrient digestibility and ruminal fermentation, mixed results have been observed. Several studies evaluated in lambs have shown no difference in DM digestion when fed cimaterol (10 ppm; Kim et al., 1989) or RAC (López-Carlos et al., 2010) compared with control-fed lambs. Strydom et al. (2009) reported steers fed RAC (30 ppm) exhibited DM and CP digestion similar to control-fed steers. More similar to the findings of this study, Walker et al. (2007) reported a 2% improvement in DM digestibility of RAC-fed steers fed 200 mg·steer⁻¹·d⁻¹ RAC over control-fed steers.

Improvements in nutrient digestibilities related to β -AAs have been hypothesized to be a response to the binding of beta-adrenergic receptors present in the gut, inhibiting ruminal contractions, resulting in greater retention time and greater ultimate digestion (Brikas, 1989). Nonetheless, increased fiber digestibilities observed at period 2 (d 22-27) than period 1 (d 8-13) in the current study contrast the expectation that normal rumen motility would resume upon receptor desensitization resulting faster passage rate and decrease digestibility. Increased fiber digestibility at period 2 than 1 is likely a result of greater animal comfortability in metabolism stalls and increased cud-chewing behaviors. However, the tendency for an interaction between sampling period and RAC supplementation indicate an alternative mechanism behind improvements in nutrient digestibility.

An alternative hypothesis is that RAC usage may have a direct effect on non-mammalian cells. Previous in vitro research has confirmed stimulatory effects of catecholamines on bacterial species. Both Lyte and Ernst (1992) and Kinney et al. (2000) demonstrated catecholamines dramatically increased gram-negative bacterial species. Results of a series of in vivo studies evaluating the effects of RAC usage on foodborne pathogens in lambs, pigs, and feedlot cattle indicated RAC may affect gut microflora populations. Edrington et al. (2006a) demonstrated increased *Escherichia coli* O157:H7 fecal shedding in experimentally inoculated lambs administered RAC and decreased *Salmonella* shedding in inoculated pigs administered RAC. In a separate experiment, Edrington et al. (2006b) reported decreased *E. coli* O157:H7 shedding in inoculated feedlot cattle fed RAC, but increased *Salmonella* shedding. A series of in vitro experiments by Walker and Drouillard (2010) provided further evidence for the effects of RAC on ruminal fermentation. Like the results of Exp. 2, Walker and Drouillard (2010) demonstrated greater IVDMD of the ground diet included in tubes inoculated with rumen fluid containing

RAC. However, given the additive effects of dietary and in vitro RAC inclusion in Exp. 2 (Figure 1), it appears improvements in IVDMD may be the result of different mechanisms. Ungemach (2004) reported peak plasma concentration for ractopamine at 0.5 – 2 hr after dosing and elimination half-life at 6 – 7 hr after initial dosing. With rumen fluid having been collected prior to feeding, significant effects observed in the whole plot (heifer) would likely be the result of long-term microbe population selection pressure such that microbes present in rumen fluid of RAC-fed heifers better digested the diet substrate.

Effects of β -AAs on nutrient digestibility may also be somewhat dosage dependent. Walker and Drouillard (2010) observed both greater IVDMD and gas production with increasing RAC concentrations, up to a certain point, lending credence to greater increase in DM digestibility observed in Exp. 1 ($400 \text{ mg} \cdot \text{steer}^{-1} \cdot \text{d}^{-1}$) compared with the Walker et al. (2007) study ($200 \text{ mg} \cdot \text{steer}^{-1} \cdot \text{d}^{-1}$). Given the increase in fermentative gas production with greater RAC concentrations observed by Walker and Drouillard (2010), it is possible changes in ruminal fermentation may slightly increase the amount of eructated methane in vivo. The theory is further supported by the absence of a concomitant increase in VFA production observed by Walker and Drouillard (2010) suggesting methanogen species may be responsible for increased gas production.

Although no differences in total eructed methane and ammonia were observed between control and RAC-fed cattle, methane emissions in beef cattle are dependent on several factors including animal size, dietary composition, and feed intake (Johnson and Johnson, 1995; Beuchemin and McGinn, 2006). As individual feed intake differences were observed during gaseous emission test, data were corrected for DMI during the 24 h test period. On comparison to other respiration calorimetry literature evaluating eructed methane (Hales et al., 2012), elevated

heat production values and depressed respiratory quotient values observed in the present study indicate steers were moderately uncomfortable during the 24 h test period. For this reason, authors chose to also standardize methane and ammonia emission data for DMI of the 5 d prior to the emission test. G NDF intake for the 5 d prior. Further, given RAC-fed steers exhibited greater fiber digestibility, CH₄ and NH₃ production were also expressed as g/kg of NDF digested. Matching total methane production results, no difference in methane standardized for both 5 d average digested DM and NDF was observed between control- and RAC-fed steers.

Results of this study indicate feeding RAC resulted in a 13% reduction in total nitrogen excretion over the 35 d feeding period. Improvements in nutrient digestibility of RAC-fed steers were unexpected given previous research. However, the findings presented were in line with in vitro results of the companion experiment demonstrating greater IVDMD in both tubes incubated with rumen fluid from RAC-fed steers and tubes containing a substrate that included RAC. These findings indicate ractopamine inclusion can impact microbial digestion of the finishing diet to increase nitrogen efficiency in heavy weight finishing cattle.

TABLES AND FIGURE

Table 2.1. Diet and nutrient composition of common feedlot diet

Ingredient, %DM	Inclusion, %DM
Dry rolled corn	55
Corn silage	20
MWDGS	15
Supplement ¹	10
Analyzed nutrient content, % DM	
DM	54.0
CP	14.1
NDF	25.4
ADF	10.6
Ether extract	4.2

¹ Supplement contained: 76.2% ground corn, 15.9% limestone, 6% urea, 0.9% trace mineral salt (20% CaCO₃, 15.43% Availa-4 Zinpro, 14.16% KCl, 8.75% MgO, MnSO₄, 6.74% FeSO₄, 6.55% rice hulls mineral oil, 5.95% S prilled, 4.41% vitamin E, 1.50% Se, 1.03% MgSO₄ & KSO₄, 0.88% CuSO₄, 0.22% vitamin A, 0.13% vitamin D₃ 500, 0.04% Ca(IO₃)₂, yielding 277 mg/kg Co, 5,000 mg/kg Su, 250 mg/kg I, 20,200 mg/kg Fe, 30,000 mg/kg Mn, 150 mg/kg Se, 30,000 mg/kg Zn, 2,205 KIU/kg vitamin A, 662 KIU/kg vitamin D₃, 22,000 IU/kg vitamin E) 0.75% liquid fat, 0.15% Rumensin 90 (200 g monensin/kg DM; Elanco Animal Health, Greenfield, IN), 0.10% Tylan 40 (88 g tylosin/kg DM; Elanco Animal Health)

Table 2.2. Effects of ractopamine hydrochloride on nitrogen balance of finishing steers

Item	CON			RAC			<i>P</i> -value		
	Period 1 d 8-13	Period 2 d 22-27	SEM	Period 1 d 8-13	Period 2 d 22-27	SEM	Treatment ¹	Period	Treatment x Period
DMI, kg/d	7.91	8.27	0.47	7.60	7.74	0.47	0.36	0.60	0.82
Intake, g N/d	183.03	176.40	18.37	176.76	165.64	18.37	0.49	0.49	0.86
Fecal output, g N/d	69.60	73.97	6.13	62.64	60.36	6.13	0.09	0.87	0.59
Urinary output, g N/d	65.61	67.21	8.06	58.17	60.44	8.06	0.31	0.78	0.97
Urea, g N/d	49.77	53.82	7.08	45.73	49.49	7.08	0.51	0.54	0.98
% of urine N as urea	75.29	79.42	2.38	78.65	81.01	2.38	0.31	0.19	0.71
Excretion, g N/d ²	135.11	141.19	7.42	120.81	120.80	7.42	0.02	0.69	0.69
Absorption, g N/d ³	113.43	102.43	14.44	114.13	105.28	14.44	0.84	0.27	0.90
Retention, g N/d ⁴	47.92	35.22	20.02	55.96	44.84	20.02	0.43	0.30	0.94
% of N intake	23.40	19.02	10.42	28.62	25.06	10.42	0.36	0.52	0.95
% of N absorbed	38.15	31.56	16.45	43.91	37.85	16.45	0.56	0.54	0.98

¹ Steers received a top-dress containing no ractopamine hydrochloride (**CON**) or 400 mg·steer⁻¹·d⁻¹ ractopamine hydrochloride (**RAC**) for 35 d before slaughter

² Calculated as Fecal N (g/d) + Urinary N (g/d)

³ Calculated as Intake N (g/d) – Fecal N (g/d)

⁴ Calculated as Intake N (g/d) – Fecal N (g/d) – Urinary N (g/d)

Table 2.3. Effects of ractopamine hydrochloride on total tract digestibility of finishing steers

Item	CON ¹			RAC			P-value		
	Period 1 d 8-13	Period 2 d 22-27	SEM	Period 1 d 8-13	Period 2 d 22-27	SEM	Treatment ¹	Period	Treatment x Period
DMI, kg/d	7.9	8.3	0.5	7.6	7.7	0.5	0.38	0.60	0.82
Daily output									
Fecal output, kg DM/d	2.3	2.7	0.2	2.1	2.1	0.2	0.04	0.36	0.38
Urine output, kg/d	8.4	6.6	1.5	6.7	8.4	1.5	0.99	0.96	0.25
DM digestibility, %	70.8	67.1	1.6	72.7	73.0	1.6	0.01	0.25	0.18
OM digestibility, %	70.7	67.4	1.8	72.5	73.5	1.8	0.04	0.55	0.24
NDF digestibility, %	53.0	53.7	1.9	55.5	62.9	1.9	<0.01	0.04	0.10
ADF digestibility, %	47.0	48.8	4.6	48.6	59.0	4.6	0.05	0.04	0.14
N digestibility, %	62.4	57.7	2.9	64.1	63.0	2.9	0.14	0.23	0.45

¹ Steers received a top-dress containing no ractopamine hydrochloride (**CON**) or 400 mg·steer⁻¹·d⁻¹ ractopamine hydrochloride (**RAC**) for 35 d before slaughter

Table 2.4. Effects of ractopamine hydrochloride on gaseous emissions through eructation and calorimetry of finishing steers

Item	Treatment ¹		SEM	P-value
	CON	RAC		
Methane, g/d	172.52	209.50	16.78	0.15
Methane, g/kg DMI during 24 h test	60.51	50.26	14.54	0.42
Methane, g/kg 5 d avg DMI	27.19	34.54	2.57	0.07
Methane, g/kg 5 d avg DM digested	31.83	38.06	3.42	0.22
Methane, g/kg 5 d avg NDF digested	174.49	206.41	23.22	0.36
Ammonia, g/d	7.54	5.27	2.97	0.21
Ammonia, g/kg DMI during 24 h test	2.25	1.08	0.30	<0.01
Ammonia, g/kg 5 d avg DMI	1.12	0.78	0.33	0.08
Ammonia, g/kg 5 d avg DM digested	1.34	0.94	0.48	0.19
Ammonia, g/kg 5 d avg NDF digested	7.29	4.85	2.28	0.14

¹ Steers received a top-dress containing no ractopamine hydrochloride (**CON**) or 400 mg·steer⁻¹·d⁻¹ ractopamine hydrochloride (**RAC**) for 35 d before slaughter

Table 2.5. Effects of ractopamine hydrochloride on carcass characteristics and meat quality traits of finishing steers

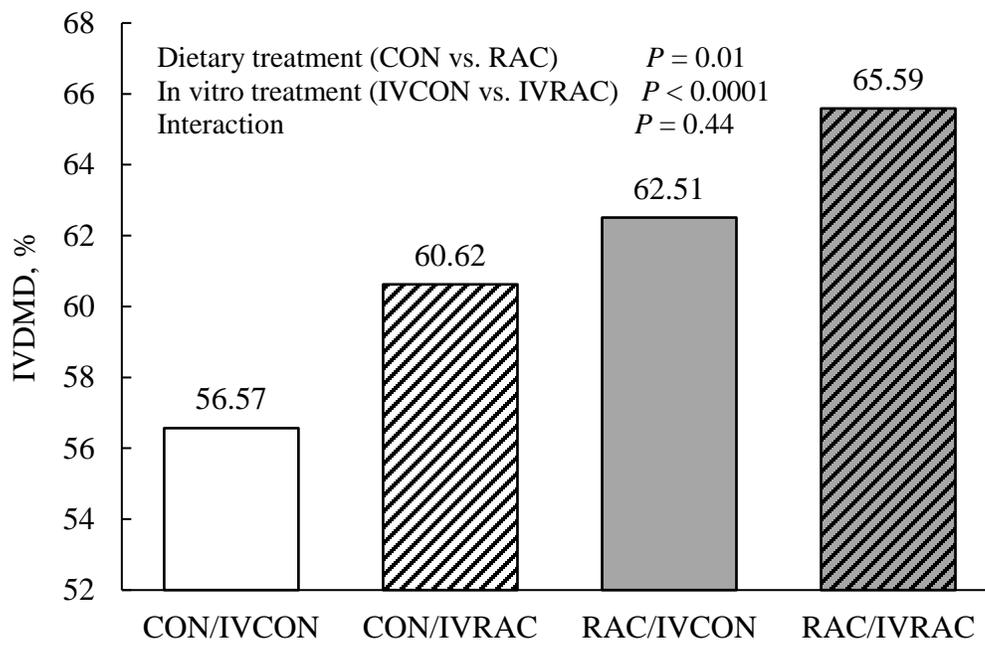
Item	Treatment ¹		SEM	P-value
	CON	RAC		
Final BW, kg	571	572	9.0	0.93
Carcass characteristics				
HCW, kg	353	354	5.4	0.96
Dressing %	64.7	64.3	0.9	0.24
LM area, cm ²	79.2	84.2	2.3	0.16
12 th -rib fat thickness, cm	1.25	1.29	0.12	0.82
KPH fat, %	2.3	2.6	0.1	0.06
USDA yield grade ²	3.12	2.95	0.20	0.57
Meat quality traits				
Marbling score ³	445	452	15.6	0.77
Lightness, L*	42.2	43.8	1.2	0.39
Redness, a*	28.1	30.3	1.7	0.17
Yellowness, b*	20.0	22.4	2.7	0.16
Chroma	34.6	37.7	3.0	0.16

¹ Steers received a top-dress containing no ractopamine hydrochloride (**CON**) or 400 mg·steer⁻¹·d⁻¹ ractopamine hydrochloride (**RAC**) for 35 d before slaughter

² Calculated USDA yield grade = (2.5 + [2.5*12th-rib fat thickness] + [0.2*KPH%] + [0.0038*HCW] - [0.32*LM area])

³ Marbling scores: 400 = Small⁰⁰ (low choice) and 500 = Modest⁰⁰ (average choice)

Figure 2.1. Effects of dietary and in vitro inclusion of ractopamine hydrochloride on in vitro dry matter disappearance (**IVDMD**) of finishing beef heifers



LITERATURE CITED

- Benchaar, C., C. Pomar, and J. Chiquette. 2001. Evaluation of dietary strategies to reduce methane production in ruminants: A modeling approach. *Can. J. Anim. Sci.* 81: 563-574
doi: 10.4141/A00-119
- Bierman, S., G. E. Erickson, T. J. Klopfenstein, R. A. Stock, D. H. Shain. 1999. Evaluation of nitrogen and organic matter balance in the feedlot as affected by level and source of dietary fiber. *J. Anim. Sci.* 77: 1645-1653. doi:10.2527/1999.7771645x
- Boler, D. D., S. F. Homer, F. K. McKeith, J. Killefer, D. L. VanOverbeke, G. G. Hilton, R. J. Delmore, J. L. Beckett, J. C. Brooks, R. K. Miller, D. B. Griffin, J. W. Savell, T. E. Lawrence, N. A. Elam, M. N. Streeter, W. T. Nichols, J. P. Hutcheson, D. A. Yates, and D. M. Allen. 2009. Effects of feeding zilpaterol hydrochloride for twenty to forty days on carcass cutability and subprimal yield of calf-fed Holstein steers. *J. Anim. Sci.* 87: 3722-3729. doi:10.2527/jas.2009-1830.
- Brake, D. W., E. C. Titgemeyer, and M. L. Jones. 2011. Effect of nitrogen supplementation and zilpaterol-HCl on urea kinetics in steers consuming corn-based diets. *J. Anim. Physiol. An. N.* 95: 409-416. doi:10.1111/j.1439-0396.2010.01064.x
- Brikas, P. 1989. The adrenergic receptors in the control of reticulo-rumen myoelectrical activity in sheep. *J. Vet. Med. A.* 36: 402-410. doi: 10.1111/j.1439-0442.1989.tb00747.x
- Bristow, A. W., D. C. Whitehead, and J. E. Cockburn. 1992. Nitrogenous constituents in the urine of cattle, sheep, and goats. *J. Sci. Food Agric.* 59: 387-394.
doi:10.1002/jsfa.2740590316

- Capper, J. L., and D. J. Hayes. 2012. The environmental and economic impacts of removing growth-enhancing technologies from U.S. beef production. *J. Anim. Sci.* 90:3527-3537. doi: 10.2527/jas.2011-4870
- Carmichael, R. N., O. N. Genther-Schroeder, C. P. Blank, E. L. Deters, S. J. Hartman, E. K. Niedermayer, and S. L. Hansen. 2018. The influence of supplemental zinc and ractopamine hydrochloride on trace mineral and nitrogen retention of beef steers. *J. Anim. Sci* *In press*. doi:10.1093/jas/sky177
- Cole, N. A., R. N. Clark, R. W. Todd, C. R. Richardson, A. Gueye, L. W. Greene, and K. McBride. 2005. Influence of dietary crude protein concentration and source on potential ammonia emissions from beef cattle. *J. Anim. Sci.* 83: 722-731. doi:10.2527/2005.833722x
- Coopridge, K. L., F. M. Mitloehner, T. R. Famula, E. Kebreab, Y. Zhao, and A. L. Van Eenennaam. 2010. Feedlot efficiency implications on greenhouse gas emissions and sustainability. *J. Anim. Sci.* 89: 2643-2656. doi: 10.2527/jas.2010-3539
- Edrington, T. S., T. R. Callaway, D. J. Smith, K. J. Genovese, R. C. Anderson, and D. J. Nisbet. 2006b. Effect of ractopamine HCl on *Escherichia coli* O157:H7 and *Salmonella* in vitro and on intestinal populations and fecal shedding in experimentally infected sheep and pigs. *Current Micro.* 53: 82-88. doi: 10.1007/s00284-006-0019-4
- Edrington, T. S., T. R. Callaway, S. E. Ives, M. J. Engler, T. H. Welsh, D. M. Hallford, K. J. Genovese, R. C. Anderson, and D. J. Nisbet. 2006b. Effect of ractopamine HCl supplementation on *Escherichia coli* O157:H7 and *Salmonella* in feedlot cattle. *Current Micro.* 53: 340-345. doi: 10.1007/s00284-006-0200-9

- Hales, K. E., N. A. Cole, and J. C. MacDonald. Effects of corn processing method and dietary inclusion of wet distillers grains with solubles on energy metabolism, carbon-nitrogen balance, and methane emission of cattle.
- Hausdorff, W. P., M. G. Caron, and R. J. Lefkowitz. 1990. Turning off the signal: desensitization of β -adrenergic receptor function. *FASEB J.* 4: 2881-2889.
- Hilton, G. G., J. L. Montgomery, C. R. Krehbiel, D. A. Yates, J. P. Hutcheson, W. T. Nichols, M. N. Streeter, J. R. Blanton Jr., and M. F. Miller. 2009. *J. Anim. Sci.* 87:1394-1406. doi: 10.2527/jas.2008-1170
- Hristov, A. N., M. Hanigan, A. Cole, R. Todd, T. A. McAllister, P. M. Ndegwa, and A. Rotz. 2010. Review: Ammonia emissions from dairy farms and beef feedlots. *Can. J. Anim. Sci.* 91: 1-35. doi: 10.1139/CJAS10034
- Johnson, K. A., and D. E. Johnson. 1995. Methane emissions from cattle. *J. Anim. Sci.* 73: 2483-2492.
- Kim, Y. S., Y. B. Lee, W. N. Garrett, and R. H. Dalrymple. 1989. Effects of cimaterol on nitrogen retention and energy utilization in lambs. *J. Anim. Sci.* 67: 674-681.
- Lopez-Carlos, M. A., R. G. Ramírez, J. I. Aguilera-Soto, C. F. Aréchiga, F. Méndez-Llorente, H. Rodríguez, and J. M. Silva. 2010. Effect of ractopamine hydrochloride and zilpaterol hydrochloride on growth, diet digestibility, intake, and carcass characteristics of feedlot lambs. *Liv. Sci.* 131: 23-30 doi: 10.1016/j.livsci.2010.02.018
- Maia, G. D. N., B. C. Ramirez, A. R. Green, L. F. Rodriguez, J. R. Segers, D. W. Shike, R. S. Gates. 2015. A novel ruminant emission measurement system: Part I. Design evaluation and description. *Trans. ASABE.* 58(3): 749-762.

- McDougall, E. I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochem. J.* 43: 99-109.
- National Academies of Sciences, Engineering, and Medicine. 2016. *Nutrient Requirements of Beef Cattle: 8th Revised Edition*. Washington, DC: The National Academies Press. doi: 10.17226/19014.
- Prados, L. F., M. L. Chizzotti, S. C. V. Filho, F. H. M. Chizzotti, P. P. Rotta, L. F. C. Silva. 2016. Environmental management and prediction of nitrogen and phosphorus excretion by beef cattle. *Nutrient Requirements of Zebu and Crossbred Cattle (BR-CORTE)*. Third Edition. S. C. V. Filho and L. F. C. Silva. (Ed.) Suprema Gráfica Ltda. Brazil.
- Ramirez, B. C. 2014a. Design and evaluation of open-circuit respiration chambers for beef cattle. MS Thesis, University of Illinois at Urbana-Champaign, Department of Agricultural and Biological Engineering, Urbana, IL.
- Ramirez, B. C., G. D. N. Maia, A. R. Green, D. W. Shike, L. F. Rodríguez, and R. S. Gates. 2014b. Design and validation of a precision orifice meter for ventilation rate control in open-circuit respiration chambers. *Trans. ASABE* 57(6):1865–1872.
- Schroeder, A. L., D. M. Polser, S. B. Laudert, and G. J. Vogel. 2003. Effect of Optaflexx on growth performance and carcass traits of steers. *Optaflexx Exchange* No. 1. Elanco Animal Health, Greenfield IN.
- Strydom, P. E., L. Frylinck, J. L. Montgomery, and M. F. Smith. 2009. The comparison of three β -agonists for growth performance, carcass characteristics, and meat quality of feedlot cattle. *Meat Sci.* 81: 557-564. doi: 10.1016/j.meatsci.2008.10.011

- Sun, Y. 2013. Quality assurance project plan for a ruminant emission measurement system. MS Thesis, University of Illinois at Urbana-Champaign, Department of Agricultural and Biological Engineering, Urbana, IL.
- Walker, D. K., E. C. Titgemeyer, E. K. Sissom, K. R. Brown, J. J. Higgins, G. A. Andrews, and B. J. Johnson. 2007. Effects of steroidal implantation and ractopamine-HCl on nitrogen retention, blood metabolites, and skeletal muscle gene expression in Holstein steers. *J. Anim. Physiol. An. N.* 91: 439-447. doi: 10.1111/j.1439-0396.2007.000675.x
- Walker, C. E. 2009. Effects of ractopamine hydrochloride are not confined to mammalian tissue: Evidence for direct effects of ractopamine hydrochloride supplementation on fermentation by ruminal microorganisms (Dissertation). Retrieved from K-REx Repository.
- Walker, C. E. and J. S. Drouillard. 2010. Effects of ractopamine hydrochloride are not confined to mammalian tissue: Evidence for direct effects of ractopamine hydrochloride supplementation on fermentation by ruminal microorganisms. *J. Anim. Sci.* 88: 697-706. doi: 10.2527/jas.2009-1999

Chapter 3

COMPARING PROTEIN ABUNDANCE OF BETA-ADRENERGIC RECEPTOR SUBTYPES BETWEEN DIFFERENT BOVINE TISSUES

ABSTRACT

The objective was to compare the protein abundance of beta-adrenergic receptor (β -AR) subtypes between bovine muscle, organ, and adipose tissues through western-blot analysis using polyclonal antibodies to β 1-AR, β 2-AR, and β 3-AR. Beef muscles (longissimus lumborum and psoas major), organs (lung, heart, kidney, and liver), and adipose tissues (visceral, subcutaneous, and intramuscular) from conventionally-raised, Simmental \times Angus finishing steers (N = 9) were collected at slaughter. Feedlot steers were managed and slaughtered in a manner consistent with commercial industry practices. Intramuscular fat samples were collected at 24 h postmortem, with all other tissues collected at 30 min postmortem. Tissue samples were normalized to equal protein concentrations and protein abundance was determined through western blotting by measuring protein band density relative to a standard sample present on every gel. All three β -AR subtype proteins were detected in all tissues evaluated. Differences in protein abundance between tissues was also observed for all three β -AR subtypes ($P \leq 0.03$). Beta 1-AR protein was more highly enriched in muscle tissue than all other tissue types ($P < 0.001$). Beta 2-AR protein was most prevalent in bovine heart and lung tissue. No differences in β 2-AR protein were observed between muscle tissues ($P = 0.25$) and adipose tissues ($P \geq 0.17$). However, a tendency ($P = 0.06$) was observed for greater β 2-AR protein in subcutaneous fat than longissimus tissue. Although differences in β 3-AR protein abundance were observed between tissues ($P = 0.03$), β 3-AR was not especially enriched in any tissue evaluated. Overall, data verify the presence of all

three β -AR subtypes in the tissues evaluated and provide a first look at β -AR subtype protein abundance between different tissues.

INTRODUCTION

Beta-adrenergic agonists (β -AA) have been used extensively in the beef industry to improve efficiency of BW gain, retail cut yield, protein price competitiveness, and reduce environmental impact per kg of beef produced (Avendano-Reyes et al., 2006; Lawrence and Ibarburu, 2006; Stackhouse-Lawson et al., 2013). Recognized as much for their value to human medicine, β -AA and the receptors they bind (beta-adrenergic receptors; β -AR) have been intensely studied since the late 1970s. Categorized into three subtypes (β 1, β 2, and β 3), β -AR subtypes differ widely in pharmacology and associated physiological effects (Lands et al., 1967; Emorine et al., 1989). Early studies revealed tissues frequently exhibited more than one β -AR subtype. Further, β -AR subtype proportions were revealed to be different between tissues of a single species and between species within a single tissue type (Mersmann et al., 1998; Ursino et al., 2009). Of the limited existing literature evaluating β -AR subtype expression, abundance, and distribution, most has been conducted in mice, rats, and humans. Gene expression (Walker et al., 2007; Baxa et al., 2010) and radioligand binding data in cattle (Sillence and Matthew, 1994) support a hypothesis that β 2-AR abundance would be greater in skeletal muscle tissue than adipose and organ tissue. However, despite almost 50 years of intense research, questions persist regarding β -AR subtype protein abundance and distribution in bovine tissue. Given the fact common physiological responses may be mediated by different β -AR subtypes in different species (Ursino et al., 2009), understanding the relative abundance of β -AR subtypes between different tissues in cattle is of paramount importance to the discovery and development of future β -AR-targeting drug compounds for use in cattle. Therefore, the objective was to compare the

protein abundance of beta-adrenergic receptor subtypes between bovine muscles, organs, and adipose tissues through western-blotting procedures.

MATERIALS AND METHODS

Sample Collection and Protein Extraction

Tissue samples were collected from conventionally-raised finishing steers. Moderately framed steers were managed at the University of Illinois Beef & Sheep Field Laboratory on a conventional diet formulated to meet or exceed NRC (2016) recommendations containing dry rolled corn, corn silage, modified wet distillers grains with solubles, and a dry supplement. Steers were transported to the University of Illinois abattoir and humanely slaughtered under USDA inspection. Steers used in the present study represented a typical range of ending live weights (520 to 661 kg), HCW (332 to 421 kg), BF thicknesses (1.0 to 2.0 cm), and USDA quality grades (Low Choice to High Choice).

Beef organ tissues (lung, heart, kidney, and liver) samples (N = 9) were collected from carcasses 30 minutes postmortem. Lung (**B**) tissue was collected from the right primary bronchus. Heart tissues, including the left atrium (**A**) and ventricle (**V**), were collected from their respective locations. Liver (**L**) tissue samples were collected from the visceral surface of the right anterior section nearest the portal vein and kidney (**K**) tissue was removed from the renal cortex of a single lobe on the right kidney. Muscle and adipose tissue samples were collected after 24 h chilling to improve ease and accuracy in collection. Muscle tissue cores were removed from the posterior end of the right longissimus lumborum (**LD**) and anterior tip of the right psoas major (**PM**). Subcutaneous fat (**SQ**) was removed from core samples where LD tissue was collected and visceral fat (**VF**) was collected from fat surrounding the kidneys. Intramuscular fat (**IM**) tissue samples (N = 6) were dissected from longissimus sections. Collected tissue samples

were flash frozen in liquid nitrogen and held in frozen storage at -80° C. Western blot analysis was conducted following similar methods as described by Huff-Lonergan et al. (1996). Whole muscle buffer (2% SDS wt/vol and 10mM Sodium Phosphate, pH 7.0) was added to minced muscle and organ tissue samples. Chilled tissue protein extraction reagent (T-PER; Thermo Fisher Scientific Inc., Rockford, IL) was added to tissue samples to aid in protein extraction. Protease inhibitor (cOmplete Protease Inhibitor Cocktail Tablets; Sigma-Aldrich, St. Louis, MO) and sodium orthovanadate (NA₃VO₄) was added to tissue buffer to prevent proteolysis of isolated proteins. Tissue samples were disrupted using a tissue homogenizer (TissueLyser II; Qiagen, Hilden, Germany) and centrifuged for 15 min at 3220 × g (Eppendorf 5810 R; Eppendorf, Hamburg, Germany). Protein concentration of the supernatant was quantified using a BCA Protein Assay Kit (Pierce Protein Research Products, Rockford, IL) and standard curve. For adipose tissues exhibiting extremely low protein concentrations, samples were centrifuged in protein concentrator tubes (Pierce Protein Concentrator PES; Thermo Fisher Scientific Inc., Rockford, IL) containing a 10kDa molecular weight cutoff polyethersulfone membrane to increase concentration of proteins within the target molecular weight range. Samples were diluted to a final concentration of 4.0 mg of protein/ml with 0.50 ml of Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA), 0.1 ml beta-mercaptoethanol, and whole muscle buffer. Finished protein samples were denatured by heating for 15 min at 50° C using a heating block (Fisher Scientific, Hampton, NH) before stored frozen at -80°C.

Western Blotting Procedures

To achieve the objective of the present study, gels and membranes were organized such that each gel represented a single steer's tissues, including skeletal muscle, fat, and organ with two gels for each β-AR subtype for a total of six gels per animal. This enabled the direct

comparison of protein abundance between tissues for a single β -AR subtype. For each lane, 40 μ g of each tissue protein sample and a pre-stained protein standard (Invitrogen Novex, Thermo Fisher, Waltham, MA) were loaded into 4-12% Bis-Tris gels (Bolt 4-12%, Bis-Tris Plus Gels, 12-well: Life Technologies, Carlsbad, CA). Gels were electrophoresed at 80 volts for approximately 2 hr until the dye front reached the gel foot using an MES-SDS running buffer in a mini gel tank (Life Technologies) and transferred to a PVDF blotting membrane using the iBlot 2 dry blotting system (Life Technologies). Membranes were blocked for 1 hr using a non-fat dried milk solution and incubated at 4^o C 12-18 hr in primary antibody. Blotting membranes were rinsed 3 times to remove residual primary antibody and then incubated at room temperature for 1 hr in secondary antibody. Blotting membranes were washed another three times to remove residual secondary. Primary and secondary antibodies are described in Table 4.1. A chemiluminescent detection kit (ECL Prime; GE Healthcare, Piscataway, NJ) was used to detect protein bands with a molecular weight of 51 kDa marker for the β 1-AR subtype, 47kDa for the β 2-AR subtype, and 45 kDa for the β 3-AR subtype. After imaging, area density was calculated as a mean density (average intensity of all pixels in the selected region, minus average intensity of background pixels). Density of immunoreactive bands was quantified through densitometry using a ChemiDoc-It 2 Imager (UVP, Upland, CA).

Statistical Analysis

Duplicate gels were run simultaneously in the same electrophoresis tank to minimize variation associated with run. Values for tissue samples were reported as a fold change difference compared to the kidney sample of that individual animal. Data were analyzed using the MIXED procedure of SAS (v 9.4; SAS Institute Inc., Cary, NC). The model for protein abundance included fixed effects of tissue as well as random effects of animal and the interaction

between animal and tissue. For the comparison of tissue protein abundance, steer served as a replicate with gels run in duplicate. Tissue comparisons were only performed in the presence of a significant F-test and were considered different at $P \leq 0.05$, with trends discussed at $P \leq 0.10$. Due to the highly preliminary nature of the data presented in the current study, pair-wise comparisons were not protected.

RESULTS

Beta 1 - AR

Beta-1 AR protein abundance of beef tissues is shown in Figure 3.1. An increase in $\beta 1$ -AR protein was observed in both skeletal muscle tissues (longissimus and psoas major) compared with all other tissues ($P < 0.001$), exhibiting an almost two-fold increase in $\beta 1$ -AR protein abundance compared with kidney and more than a 1.5-fold increase in $\beta 1$ -AR protein compared with subcutaneous fat. Beta-1 AR protein concentrations were similar ($P = 0.64$) between the two muscles evaluated (longissimus and psoas muscle). Further, no differences ($P \geq 0.23$) in $\beta 1$ -AR protein abundance were observed between the three adipose depots evaluated (subcutaneous, visceral, and intramuscular). Additionally, heart (atrium and ventricle), liver, and subcutaneous fat exhibited increased $\beta 1$ -AR protein ($P \leq 0.04$) compared with kidney, the standard in this experiment. Numerically, intramuscular fat exhibited the least $\beta 1$ -AR protein of all sampled tissues but were not statistically different ($P \geq 0.29$) from visceral fat, bronchial, or kidney tissue.

Beta 2 - AR

No difference in $\beta 2$ -AR protein abundance were observed between the two muscle types ($P = 0.25$; Figure 3.2) or within the three adipose depots ($P \geq 0.17$). Despite a tendency ($P = 0.06$) for greater $\beta 2$ -AR protein abundance in subcutaneous fat than longissimus tissue, there was

not a clear difference in β 2-AR protein concentrations between muscle and adipose tissue. Atrium, ventricle, and bronchial tissue were moderately enriched with β 2-AR protein exhibiting an almost 1.5-fold increase in abundance compared with kidney tissue. Longissimus muscle exhibited less β 2-AR protein ($P \leq 0.03$) than atrium, ventricle, and bronchial tissue.

Beta 3 - AR

Although β 3-AR protein was observed in all tissues evaluated, relative differences between tissues were numerically small (Figure 3.3). Nonetheless, a significant F-test ($P = 0.03$) indicated differences in protein abundance between tissues. No difference in β 3-AR protein abundance was observed between muscle tissues (longissimus and psoas) and subcutaneous fat ($P \geq 0.48$), however longissimus, psoas major, subcutaneous fat, as well as bronchial tissue, all exhibited greater β 3-AR protein abundance ($P \leq 0.04$) than visceral and intramuscular fat. No differences ($P \geq 0.29$) were observed between visceral fat, intramuscular fat, atrium, ventricle, liver, and kidney.

DISCUSSION

Previous studies documenting the occurrence of species-specific differences in β -AR subtype abundance between tissues (Krief et al., 1993) draw attention to the need for β -AR data specific to beef cattle tissues. Early methods of measuring β -AR subtype abundance and proportions proved largely unsuccessful. Methods evaluating physiological responses did not provide clear differentiation between β -AR subtypes, largely due to differences in ligand specificity (Mersmann, 1992). Similarly, more direct measurements of competitive binding and radioligand binding have produced mixed results. As an alternative, several studies have evaluated mRNA abundance to determine β -AR subtype abundance and relative proportions. However, these data only provide an approximation of total protein abundance due to the fact

mRNA degradation and translational regulatory checkpoints may prevent equal and consistent translational efficiency. Nonetheless, due to the novelty of the current data, literature evaluating ligand binding and mRNA abundance will be discussed to relate total protein abundance data back to the literature concerning β -AR proportions in different tissues.

Early literature has drawn conclusions about the ubiquitous nature of both β 1- and β 2-AR receptors, as they have been observed in virtually all mammalian tissues evaluated (Stiles et al., 1984). The present study demonstrated the presence of β 1-, β 2-, and β 3-AR in all tissues tested. In a review of the available β 3-AR literature, Ursino et al. (2009) reported β 3-AR presence in skeletal, smooth, and cardiac muscle, adipose, and brain tissues. Findings of the current study help to confirm the likely ubiquitous nature of β 3-AR proteins as well.

Beta-1 AR protein abundance was exceptionally great in muscle tissue compared with adipose and organ tissue. Current findings suggest both longissimus and psoas tissue were highly enriched with more than a 1.5-fold increase in β 1-AR protein relative to adipose tissue. Historically, β 2-AR has been thought of as the predominant β -AR present in muscle. Through radioligand binding, Sillence and Matthews (1994) reported that skeletal muscle and subcutaneous fat were highly enriched with β 2-AR with very little β 1-AR observed in proportion. Further, although Baxa et al. (2010) did observe β 1-AR mRNA presence in skeletal muscle tissue, β 2-AR expression was almost 1,000 times greater than β 1-AR. These findings make sense given the more pronounced effects of β 2-AR selective agonists such as zilpaterol hydrochloride and clenbuterol hydrochloride compared to β 1-AR selective agonists on protein accretion and red meat yield (Arp et al., 2014). However, the present findings compare β 1-AR abundance to that of other tissues evaluated and not to other β -AR subtypes; therefore indicating that although β 1-AR protein was observed to be more abundant in muscle than other tissues, β 1-

AR still may only account for a small proportion of the total β -AR present in muscle. Further, previous studies evaluating the effects of β -AA administration on β -AR expression have reported β -AA use may alter β 2-AR expression in skeletal muscle (Walker et al., 2007; Baxa et al., 2010). Meanwhile, changes to β 1-AR expression associated with β -AA use have not been observed (Baxa et al., 2010). Ultimately, protein abundance findings of the current study and expression differences reported in the literature suggest β 1-AR may be more resistant to changes in expression and downregulation than β 2-AR.

Beta 2-AR was most abundant in heart and lung tissue with muscle and subcutaneous fat tissue being moderately enriched as well. Literature evaluating radioligand binding in humans (Bristow et al., 1986) and mRNA concentrations in pigs (McNeel and Mersmann, 1999) have reported greater β 1-AR mRNA concentrations in heart tissue compared with β 2- and β 3-AR. However, as previously mentioned, one finding does not negate the other as although β 2-AR protein was more abundant in heart tissue, β 2-AR may only account for a small proportion of the total β -AR. Bronchial tissue is considered the prototypical tissue for β 2-AR in humans and pigs (Nelson, 1995; McNeel and Mersman, 1999) with some of the earliest β -AA developed for use in asthma patients, targeting β 2-mediated bronchodilation. In the case of bronchial tissue, it appears protein abundance relative to other tissues compliments relative β -AR subtype proportions. Further, greater β 2-AR abundance in beef heart and lung tissue may also explain some of the off-target effects more typically associated with the use of β 2-AR-selective agonists. Brockway et al. (1987) reported calves exhibited increased heart rate for 1 to 3 days after initial clenbuterol administration. These potential off-target effects have even more serious health implications from a food residue standpoint. Pulce et al. (1991) reported side effects including tremors,

headaches, increased heart rate, and dizziness in cases where humans had eaten meat containing clenbuterol residues of 0.375 to 0.500 ppm.

Lesser β 1-AR protein abundance observed in intramuscular fat compared with other tissue may partially explain the intermediate effect of β 1-AR-selective agonists on marbling compared with the more pronounced effect associated with β 2-AR-selective agonist usage (Lean et al., 2014). Although only numerically different, greater β 2-AR protein abundance observed in subcutaneous fat compared with skeletal muscle, visceral, and intramuscular fat may explain some of the lipolytic effects associated with the use of β 2 agonists. Potent β 2-AR selective agonists have been demonstrated to have a more pronounced effect on 12th-rib fat thickness (Lean et al., 2014) and DP than carcasses from both control-fed cattle and cattle fed ractopamine hydrochloride, a β 1-AR-preferential agonist.

With identification and acceptance of the β 3-AR not occurring until several decades after β 1- and β 2-AR (Emorine et al., 1989), considerably less information exists regarding β 3-AR distribution, relative abundance between tissues, and relative subtype proportions in cattle. Brown adipose tissue in rats has historically been identified as the prototypical tissue for expression of β 3-AR (Mersmann, 1998). However, brown or thermogenic adipose tissue is only present in fetal and neonate livestock species (Casteilla et al., 1989). Apart from brown adipose, McNeel and Mersmann (1999) reported β 3-AR proportionally comprised less than 1% of β -AR mRNA in heart, lung, liver, and skeletal muscle and 7% in subcutaneous adipose. Ultimately, findings of the current study indicate that although β 3-AR were present in all tissues evaluated, β 3-AR were not particularly enriched in any tissue.

As previously mentioned, western blotting procedures used in the current study enable a direct comparison of the protein abundance of a single β -AR subtype between tissues. This

procedure did not, however, provide an estimate of the β -AR subtypes proportions present within an individual tissue. Because of observed differences in affinity and specificity of the β 1-, β 2-, and β 3-AR primary antibodies, western blotting procedures used in the present study are not appropriate for the evaluation of β -AR subtype protein proportions. Nonetheless, studies reporting differences in β -AR subtype presence and distribution on the same tissue of different species gives even greater credence to the need for β -AR information specific to cattle, especially given the fact common physiological responses may be mediated by different β -AR subtypes in different species (Ursino, 2009). Beta-AR-induced detrusor muscle relaxation is one such example of these differences. Beta-AR are implicated in relaxation of the detrusor muscle, responsible for storage and release of urine (Ursino et al., 2009). Nonetheless, differences in detrusor muscle β -AR subtype presence between humans, mice, rats, and dogs (Yamaguchi et al., 2007; Ursino et al., 2009) indicate β -AR-induced detrusor muscle relaxation is mediated by targeting different receptors in different species. With physiological responses mediated by different receptors in different species, data evaluating β -AR protein abundance between tissues and relative β -AR subtype proportions present in various tissue in beef cattle would aid in the development of highly specific drug candidates.

Conclusions

All three β -AR subtypes were observed in all tissues evaluated in the present study. Observed differences in protein abundance of the β -AR subtypes between tissues provide a baseline for future studies evaluating β -AR subtype proportions present in bovine tissues and should also facilitate greater interpretation of β -AR-mediated physiological responses. Ultimately, the findings of this study should help to better explain the activity and effects of β -AA and β -AR stimulation on multiple economically important tissues in livestock species.

TABLE AND FIGURES

Table 3.1. Western blotting antibodies, dilutions, and molecular weight

Protein	Primary			Secondary		Molecular weight
	Antibody	Manufacturer	Dilution	Antibody	Dilution	
Beta-1 Adrenergic Receptor	rabbit polyclonal to beta 1 adrenergic receptor, igG	(PA1-049; Thermo Fisher Scientific Inc., Rockford, IL)	1:4000	goat anti-rabbit igG (H+L) HRP conjugate	1:32000	51kDa
Beta-2 Adrenergic Receptor	goat polyclonal to beta 2 adrenergic receptor, igG	(ab40834; Abcam Inc., Cambridge, MA)	1:2000	donkey anti-goat igG (H+L) HRP conjugate	1:16000	47kDa
Beta-3 Adrenergic Receptor	goat polyclonal to beta 3 adrenergic receptor, igG	(ab77588; Abcam Inc., Cambridge, MA)	1:4000	donkey anti-goat igG (H+L) HRP conjugate	1:32000	45kDa

Figure 3.1. Beta-1 adrenergic receptor protein abundance in bovine tissue of conventionally-raised finishing steers. Protein abundance reported as fold change relative to kidney. Bars without a common superscript differ ($P \leq 0.05$). Tissues on western blotting image example follow order displayed in the figure.

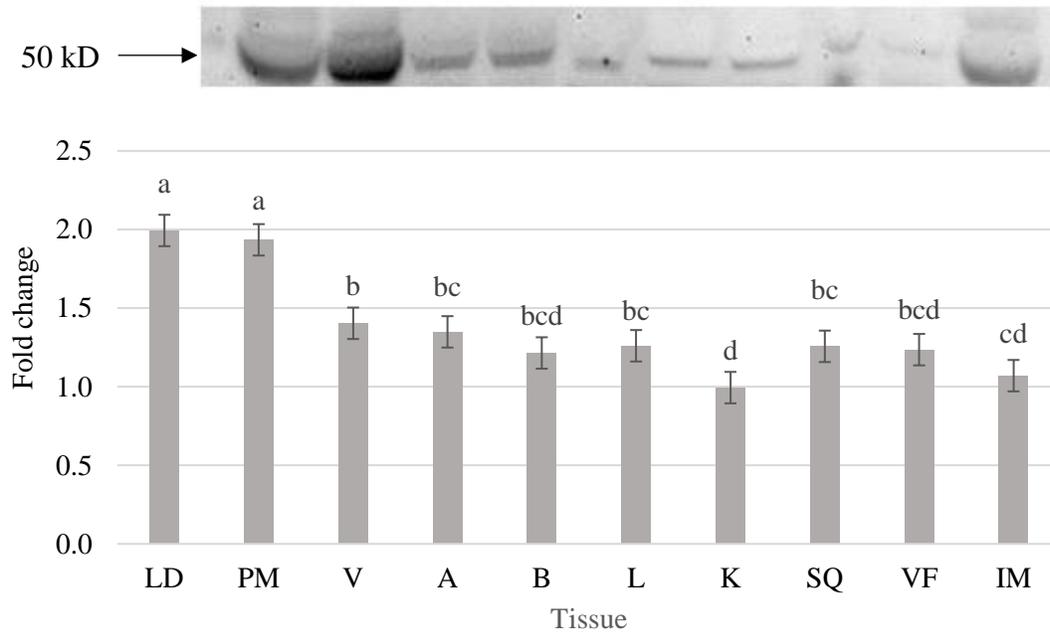


Figure 3.2. Beta-2 adrenergic receptor protein abundance in bovine tissue of conventionally-raised finishing steers. Protein abundance reported as fold change relative to kidney. Bars without a common superscript differ ($P \leq 0.05$). Tissues on western blotting image example follow order displayed in the figure.

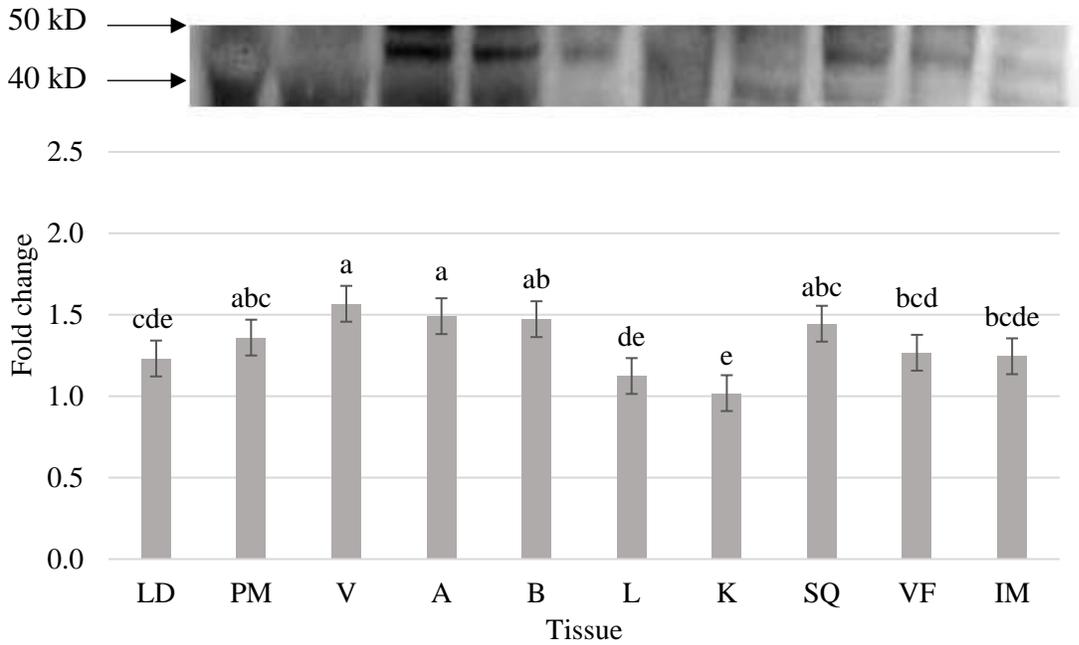
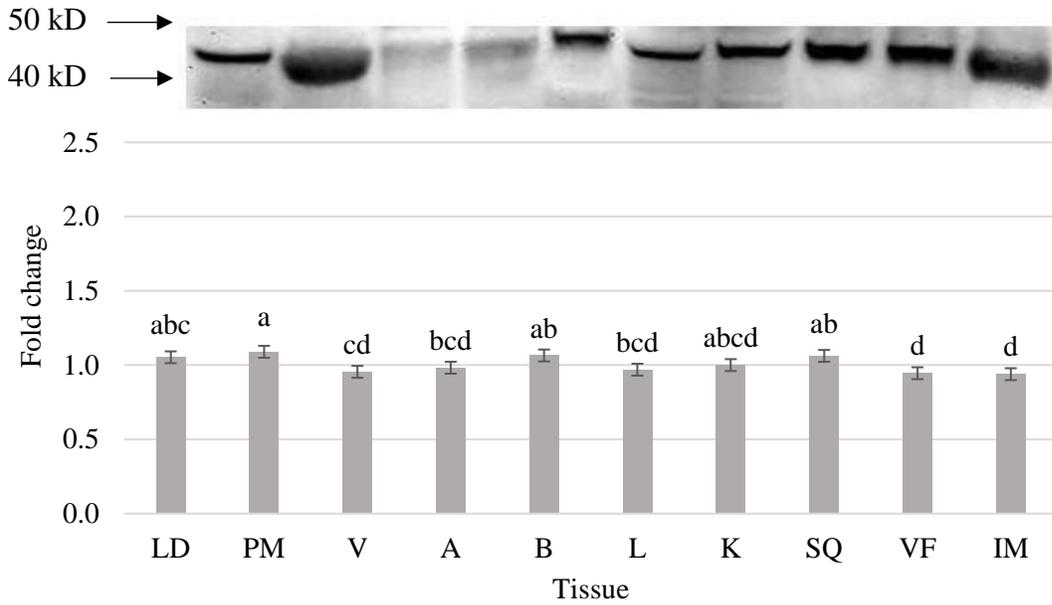


Figure 3.3. Beta-3 adrenergic receptor protein abundance in bovine tissue of conventionally-raised steers. Protein abundance reported as fold change relative to kidney. Bars without a common superscript differ ($P \leq 0.05$). Tissues on western blotting image example follow order displayed in the figure.



LITERATURE CITED

- Arp, T. S., S. T. Howard, D. R. Woerner, J. A. Scanga, D. R. McKenna, W. H. Kolath, P. L. Chapman, J. D. Tatum, and K. E. Belk. 2014. Effects of dietary ractopamine hydrochloride and zilpaterol hydrochloride supplementation on performance, carcass traits, and carcass cutability in beef steers. *J. Anim. Sci.* 92: 836-843. doi: 10.2527/jas2013-7122
- Avendaño-Reyes, L., V. Torres-Rodríguez, F. J. Meraz-Murillo, C. Pérez-Linares, F. Figueroa-Saavedra, and P. H. Robinson. Effects of two β -adrenergic agonists on finishing performance, carcass characteristics, and meat quality of feedlot steers. *J. Anim. Sci.* 84: 3259-3265. doi: 10.2527/jas.2006-173
- Baxa, T. J., J. P. Hutcheson, M. F. Miller, J. C. Brooks, W. T. Nichols, M. N. Streeter, D. A. Yates, and B. J. Johnson. 2010. Additive effects of a steroidal implant and zilpaterol hydrochloride on feedlot performance, carcass characteristics, and skeletal muscle messenger ribonucleic acid abundance in finishing steers. *J. Anim. Sci.* 88: 330-337. doi: 10.2527/jas.2009-1797
- Bristow, M. R., R. Ginsburg, V. Umans, M. Fowler, W. Minobe, R. Rasmussen, P. Zera, R. Menlove, P. Shah, and S. Jamieson. 1986. Beta 1- and beta 2-adrenergic-receptor subpopulations in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective beta-1 receptor down-regulation in heart failure. *Circ. Res.* 59: 297-309. doi: 10.1161/01.RES.59.3.297
- Brockway, J. W., J. C. MacRae, and P. E. Williams. 1987. Side effects of clenbuterol as a repartitioning agent. *Vet. Record* 120: 381-383. doi: 10.1136/vr.120.16.381

- Casteilla, L., O. Champigny, F. Bouillaud, J. Robelin, and D. Ricquier. 1989. Sequential changes in the expression of mitochondrial protein mRNA during the development of brown adipose tissue in bovine and ovine species. *Biochem. J.* 257: 665-671. doi: 10.1042/bj2570665
- Emorine, L. J., S. Marullo, M. M. Briend-Sutren, G. Patey, K. Tate, C. Delavier-Klutchko, and A. D. Strosberg. 1989. Molecular characterization of the human β 3-adrenergic receptor. *Science.* 245: 1118-1121.
- Huff-Lonergan, E., T. Mitsuhashi, F.C. Parrish, and R.M. Robson. 1996. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting comparisons of purified myofibrils and whole muscle preparations for evaluating titin and nebulin in postmortem bovine muscle. *J. Anim. Sci.* 74: 779-785.
- Krief, S., F. Lönnqvist, S. Raimbault, B. Baude, A. Van Spronsen, P. Arner, A. D. Strosberg, D. Ricquier, and L. J. Emorine. 1993. Tissue distribution of β 3-adrenergic receptor mRNA in man. *J. Clin. Invest.* 91: 344-349.
- Lands, A. M., A. Arnolds, J. P. McAuliff, F. P. Luduena, and T. G. Brown, Jr. 1967. Differentiation of receptor systems activated by sympathomimetic amines. *Nature.* 214: 597-598.
- Lawrence, J. D. and M. A. Ibarburu. 2006. Economic analysis of pharmaceutical technologies in modern beef production. <http://www2.econ.iastate.edu/faculty/lawrence/pharmaeconomics2006.pdf> Accessed: 5 June 2018
- Lean, I. J., J. M. Thompson, and F. R. Dunshea. 2014. A meta-analysis of zilpaterol and ractopamine effects on feedlot performance, carcass traits and shear strength of meat in cattle. *PLOS ONE.* 9: e115904. doi: 10.1371/journal.pone.0115904

- Mersmann, H. J. 1998. Overview of the effects of β -adrenergic receptor agonists on animal growth including mechanisms of action. *J. Anim. Sci.* 76: 160-172.
- Mersmann, H. J. 1992. Regulation of porcine adipose tissue lipolytic activity by adrenergic agonists and antagonists. *Comp. Biochem. Physiol.* 102C: 407-411.
- National Academies of Sciences, Engineering, and Medicine. 2016. Nutrient Requirements of Beef Cattle: 8th Revised Edition. Washington, DC: The National Academies Press. doi: 10.17226/19014.
- Nelson, H. S. 1995. Beta-adrenergic bronchodilators. *N. Engl. J. Med.* 333: 499-507. doi: 10.1056/NEJM199508243330807
- Pulce, C., D. Lamaison, G. Keck, C. Bostvironnois, J. Nicolas, and J. Descotes. 1991. Collective human food poisonings by clenbuterol residues in veal liver. *Vet. Human Toxicol.* 33: 480-481.
- Sillence, M. N., and M. L. Matthews. 1994. Classical and atypical binding sites for β -adrenoceptor ligands and activation of adenylyl cyclase in bovine skeletal muscle and adipose tissue membranes. *Br. J. Pharmacol.* 111: 866-872. doi: 10.1111/j.1476-5381.1994.tb14818.x
- Stackhouse-Lawson, K. R., M. S. Calvo, S. E. Place, T. L. Armitage, Y. Pan, Y. Zhao, and F. M. Mitloehner. 2013. Growth promoting technologies reduce greenhouse gas, alcohol, and ammonia emissions from feedlot cattle. *J. Anim. Sci.* 91: 5438-5447. doi: 10.2527/jas.2011-4885
- Stiles, G. L., M. G. Caron, and R. J. Lefkowitz. 1984. Mechanisms of physiological regulation. *Physiol. Rev.* 64: 661-743.
- Strosberg, A. D. 1996. G protein coupled R7G receptors. *Cancer Surv.* 27: 65-83.

- Ursino, M. G., V. Vasina, E. Raschi, F. Crema, and F. De Ponti. 2009. The β_3 -adrenoceptor as a therapeutic target: Current perspectives. *Pharmacol. Res.* 59: 221-234. doi: 10.1016/j.phrs.2009.01.002
- Walker, D. K., E. C. Titgemeyer, E. K. Sissom, K. R. Brown, J. J. Higgins, G. A. Andrews, and B. J. Johnson. 2007. Effects of steroidal implantation and ractopamine-HCl on nitrogen retention, blood metabolites, and skeletal muscle gene expression in Holstein steers. *J. Anim. Physiol. An. N.* 91: 439-447. doi: 10.1111/j.1439-0396.2007.000675.x
- Yamaguchi, O., and C. R. Chapple. 2007. Beta3-adrenoceptors in urinary bladder. *Neurourol. Urodyn.* 26: 752-756.

Chapter 4

EFFECTS OF A MULTI-ELEMENT TRACE MINERAL INJECTION AND VITAMIN E SUPPLEMENTATION ON PERFORMANCE, CARCASS CHARACTERISTICS, AND COLOR STABILITY OF STRIP STEAKS FROM FEEDLOT HEIFERS

ABSTRACT

The objective was to evaluate the interaction of a trace mineral (TM) injection (Multimin 90) and a supranutritional concentration of dietary vitamin E (VITE) on performance, carcass characteristics and color stability of strip steaks from feedlot heifers. Prior to trial initiation, Angus × Simmental cross heifers (N = 48) were managed on a common diet supplemented to meet NRC recommendations (NRC, 2000). Heifers were stratified by BW and allotted to a 2 x 2 factorial arrangement: 1) no supplemental vitamin E and saline injection (CONT+SAL), 2) 1000 IU vitamin E/heifer·d⁻¹ and saline injection (VITE+SAL), 3) no supplemental vitamin E and TM injection (CONT+MM), or 4) vitamin E and TM injection (VITE+MM). Trace mineral injection contained 15, 10, 5, and 60 mg/mL of Cu, Mn, Se, and Zn, respectively and TM injection or saline injection (1 mL/68 kg BW) were given on day 0 of the 89 d finishing period. All heifers were fed a common diet containing a basal concentration of 19.8 IU/kg DM vitamin E. Heifers were slaughtered and loins sections were collected. Strip steaks were cut and placed in overwrap trays for evaluation of color stability for 16 d. Data were analyzed using the MIXED procedure of SAS. Color stability data were analyzed as repeated measures. Neither TM injection nor VITE had an effect on final BW, DMI, or G:F ($P \geq 0.12$). There was a tendency ($P = 0.09$) for TM injection to increase ADG. A tendency ($P = 0.08$) was observed for TM injection

to increase DMI of heifers receiving supranutritional VITE. Trace mineral injection and VITE had no effect on HCW, yield grade, 12th- rib backfat thickness, or ribeye area ($P \geq 0.34$). Marbling scores tended to increase ($P = 0.08$) in VITE heifers compared with control-fed heifers. Vitamin E supplementation decreased final lipid oxidation (1.00 vs. 1.97 μg MDA/g fat, $P = 0.03$) and total visual discoloration (15.82 vs. 33.96%, $P = 0.04$) of steaks compared with steaks from non-supplemented heifers. Heifers fed supranutritional VITE produced steaks that maintained retail color longer shown by lower hue angle values (38.17 vs. 38.66°, $P < 0.01$) than non-supplemented heifers. A TM injection \times vitamin E \times day interaction ($P < 0.01$) revealed by d 16 steaks from the CONT+MM heifers exhibited greater discoloration than VITE+SAL and VITE+MM steaks with CONT+SAL intermediate. Overall, VITE improved color stability and TM injection appeared to increase discoloration of strip steaks from feedlot heifers after d 14 of display.

INTRODUCTION

Fresh meat color is one of the driving forces behind consumer purchasing intent. As a result, premature discoloration of steaks and roasts results in annual revenue losses of \$1 billion (Smith et al., 2000). Therefore, pre-slaughter strategies with potential to slow visual discoloration would decrease losses associated with discounted, re-worked, or discarded product (Zerby et al., 1992). Although the effects of vitamin E supplementation on color-stability of beef products have been well established (Faustman et al., 1998), the potential for vitamin E and trace mineral (TM) supplementation to additively extend color stability is unknown.

Oxidative challenges associated with transit and handling (Genther-Schroeder and Hansen, 2015) and dietary inclusion of feedstuffs high in polyunsaturated fatty acids (Gill et al., 2008) may decrease color stability of steaks from finishing cattle. Recently, a trace mineral

injectable has been increasingly used to overcome issues such as dietary antagonists, TM deficiencies in feedstuffs and forages, and variation in TM intake. Trace mineral injection has been demonstrated to increase the activity of TM-containing antioxidant enzymes such as Mn-superoxide dismutase (Genther and Hansen, 2014b) and Se-containing glutathione peroxidase (Pogge et al., 2012). Selenium deficiencies routinely identified in Midwestern U.S. soils and forages (USDA, 2006), increase the likelihood these antioxidant systems may be compromised in deficient cattle. The hypothesis was that supranutritional vitamin E and trace mineral injection would have little effect on growth performance however, they may additively improve production of antioxidant enzymes, thereby decreasing oxidative stress and improving color stability of strip steaks. The objective was to evaluate the interaction of TM injection and supranutritional concentration of dietary vitamin E on growth performance, carcass characteristics, and color stability of strip steaks from feedlot heifers.

MATERIALS AND METHODS

All protocols were approved by the University of Illinois Institutional Animal Care and Use Committee (Protocol #15008).

Experimental Design and Cattle Management

A total of 48 Angus × Simmental heifers were used in a randomized complete block design with a 2 × 2 factorial arrangement with treatment factors including daily dietary inclusion of the control, basal vitamin E (19.8 IU/kg DM) or targeting 1,000 IU vitamin E/heifer·d⁻¹ (109.8 IU/kg DM [dl-alpha-tocopheryl acetate]) and subcutaneous injection of a saline or trace mineral injection (Multimin90; Multimin USA, Fort Collins, CO) at a dosage of 1 mL/68 kg BW. In total, 4 treatment combinations were used (n = 12): (1) no supplemental vitamin E and saline injection (**CONT+SAL**), (2) vitamin E and saline injection (**VITE+SAL**), (3) no supplemental

vitamin E and trace mineral injection (**CONT+MM**), or (4) vitamin E and trace mineral injection (**VITE+MM**). Trace mineral injection delivered 15 mg Cu/mL (as copper disodium EDTA), 10 mg Mn/mL (as manganese disodium EDTA), 5 mg Se/mL (as sodium selenite), and 60 mg Zn/mL (as zinc disodium EDTA).

Prior to trial initiation, heifers were managed as a group on a common diet fed to meet the 2000 NRC requirements (NRC, 2000) containing 50% high-moisture corn, 20% corn silage, 20% modified wet distillers grains with solubles, and 10% supplement. Diets prior to trial initiation contained a basal vitamin E concentration of 19.8 IU/kg DM. Heifers were administered 140 mg trenbolone acetate and 14 mg estradiol (Component TE-H; Elanco Animal Health, Greenfield, IN) on d 0 of the trial. After being weighed on d -1 and 0, heifers were stratified by BW and allotted across 8 pens (6 heifers per pen). Injection treatment was applied to the individual heifer on d 0 of the trial. Each pen housed heifers receiving both injection treatments (SAL and MM). Dietary vitamin E treatment was applied to pen, however individual animal intakes were collected using a GrowSafe feeding system (GrowSafe Systems Ltd., Airdrie, AB Canada). Heifers were weighed at 28 d intervals and fed for ad libitum intake at 1000 h daily for a total of 89 d on feed. Heifers were housed in 4.88 m × 4.88 m pens in a confinement barn with slatted, concrete floors covered by interlocking rubber matting.

Feed Management

Diets were the same for both dietary treatments with the exception of vitamin E inclusion in feed supplements. Diets were formulated to meet or exceed NRC (2000) recommendations and contained 20% corn silage, 35% modified wet distillers grains with solubles (**MWDGS**), 35% dry rolled corn, and 10% supplement (DM basis; Table 1). Dry supplement (10%) served as the carrier for vitamin E to ensure uniform mixing in diets. The basal diet was formulated to

provide 44 mg Mn/kg DM (as manganese sulfate and Availa-4 [Zinpro Performance Minerals; Zinpro Corp, Eden Prairie, MN]), 67 mg Zn/kg DM (as zinc sulfate and Availa-4), 9 mg Cu/kg DM (as copper sulfate), and 0.4 mg Se/kg DM (as sodium selenite; Table 1). The MWDGS used in this study contained 8.5% fat. Percentage of MWDGS inclusion was intentionally higher to challenge color stability and increase the likelihood of observing treatment effects should they exist. Feed samples were collected every 2 weeks throughout the duration of the trial. Samples were stored in a -20 °C freezer until further analysis. Equal proportions from each collection were composited for laboratory analysis. All samples were dried and ground through a Wiley mill (1-mm screen, Arthur H. Thomas, Philadelphia, PA). Ingredients were analyzed for DM (24 h at 103°C), NDF and ADF (using Ankom Technology method 5 and 6, respectively; Ankom200 Fiber Analyzer, Ankom Technology), CP (Leco TruMac, LECO Corporation, St. Joseph, MI), fat (ether extract, Ankom method 2; Ankom Technology), and ash (600°C for 2 h; Thermolyte muffle oven Model F30420C; Thermo Scientific, Waltham, MA).

Slaughter and Sample Preparation

On d 90, heifers were transported approximately 300 km to a commercial slaughter facility and were humanely slaughtered under USDA inspection. Two heifers were removed from the trial, one prior to slaughter due to laminitis and another for pericarditis observed during slaughter, leaving 46 heifers for use in the experiment. At approximately 24 h postmortem, carcasses were evaluated for 12th rib backfat thickness, ribeye area (**REA**), percent KPH, ribeye lean maturity score, and ribeye marbling score via camera grading. A 5 cm section of longissimus lumborum was excised between the 12th and 13th rib section by facility staff, stored on wet ice, and transported to the University of Illinois Meat Science Laboratory. Two 1.9 cm-thick steaks were cut using a gravity slicer. The first was used for initial lipid oxidation and

proximate analysis and the second for retail display and final lipid oxidation. Steaks for retail display were placed on soaker pads in foam trays (27.3 × 14.9 cm, Dyne-a-pak, Laval, Quebec) and overwrapped with a polyvinyl chloride film (oxygen-permeable polyvinyl chloride fresh meat film; 1,629 mL O₂/m²·d⁻¹ at 23 °C) to be serially evaluated for color stability beginning on d 2 postmortem. Steaks were rotated daily to minimize the effect of location and light exposure. Lighting was provided by 122 cm long 32 W fluorescent bulbs (Ecolux with Starcoat, 3000K, General Electric, Boston, MA). All steaks were removed from retail display and final lipid oxidation was evaluated after the group reached an average of approximately 25% discoloration, at d 16 retail display.

Instrumental Color Analysis

For each day of serial color stability evaluation, surface color was evaluated on steaks, through overwrap film, using a HunterLab Miniscan XE Plus spectrophotometer Model 45/0 (HunterLab Associates, Reston, VA) with a 2.54 cm diameter aperture, A illuminant, and 10° observer angle. All measurements, including reflectance at 580 and 630 nm and CIE L* (lightness), a* (redness), and b* (yellowness), were measured on two random locations of the steak and averaged for analyses. The ratio of reflectance at 630 nm / 580 nm was used to instrumentally measure fresh meat color change with ratios closer to 1.0 indicating greater discoloration due to metmyoglobin formation (Strange et al., 1974). Hue angle was also used to evaluate shifts in color over time toward discoloration with greater values indicating less red and greater metmyoglobin formation (Bernofsky et al., 1959). Chroma, or saturation index, was used to assess color intensity with greater values indicating greater saturation of the principle hue on the steak (AMSA, 2012). Hue angle and saturation index (chroma) were calculated using the following equations: Hue Angle = $\left[\arctan\left(\frac{b^*}{a^*}\right)\right]$ and Chroma = $(a^{*2} + b^{*2})^{0.5}$

For each day of display, the percentage steak surface discoloration was also recorded by two evaluators with extensive experience evaluating meat color changes throughout shelf-life.

Evaluations of discoloration were averaged for analysis.

Lipid Oxidation

Initial and final lipid oxidation of steaks was evaluated using the thiobarbituric acid reactive substances (**TBARS**) assay. Steaks were trimmed of external fat and the entire steak was homogenized in a Waring blender (WaringPro, Torrington, CT) using the pulse function (grind 1 s, rest 3 s) until homogenous to prevent overheating. Duplicate 5 g samples were collected from the ground homogenate of each steak and subjected to extraction by aqueous acidic solution protocol using a modified procedure described by Leick et al. (2010). A standard curve ($R^2 = 0.9989$) was made to represent 0, 1.25, 2.5, 5, and 7.5 mg malondialdehyde (**MDA**)/ml using 25 μ M 1, 1, 3, 3-tetraethoxypropane. After incubation, 150 μ L of sample, blank and standard were pipetted into 96-well flat-bottomed plates (12.8×8.5 cm; ThermoFisher, Rochester, NY 14625) and absorbance measured at 530 nm in a plate reader (Synergy HT Multi-Model Microplate Reader, Bio-Tek, Winooski, VT) to determine sample absorbance. Samples were compared to a standard curve (0 - 7.5 mg MDA/mL), TBARS calculated, and expressed as mg MDA/kg of meat. Lipid oxidation was also corrected for lipid content and expressed as a percentage of weight using data from proximate analysis. Adjusted TBARS, expressed as μ g MDA/g of fat, were calculated using the following equation:

$$\frac{mg\ MDA}{kg\ Meat} * \frac{kg\ Meat}{g\ Fat} * \frac{1000mg\ MDA}{mg\ MDA} = \frac{\mu g\ MDA}{g\ Fat}$$

Proximate Composition

The remaining steak homogenate after initial TBARS analysis (d 0 retail display) was used for moisture and extractable lipid determination. Duplicate 10 g samples were oven dried at

110 °C for at least 24 h and weighed to determine the percentage of moisture content. Dried samples were then washed in warm chloroform-methanol, as described by Novakofski et al. (1989), and weighed to determine the percentage of total extractable lipid.

Statistical Analyses

The experiment was conducted as a 2 × 2 factorial arrangement of treatments in a randomized complete block design with Vitamin E treatment applied to the pen and injection applied to the individual heifer. Data were analyzed using the MIXED procedure of SAS (v 9.4; SAS Institute Inc., Cary, NC). The model for live performance, carcass characteristics, and lipid oxidation included fixed effects of dietary treatment and trace mineral injection, their interaction, as well as the random effect of pen. Heifer served as the experimental unit as individual intake data was collected. Initial and final instrumental color and discoloration parameters were analyzed independently as a 2 × 2 factorial. Color stability over the course of the 16 d retail display period was analyzed as repeated measures using an unstructured covariate structure determined using Akaike's information to minimize variance. The model included treatment, day, and their interaction as well as individual heifer as a random effect. The slice option was used to evaluate the effect of day on instrumental color and color stability data and least square means were calculated. Treatment effects and interactions were considered significantly different at $P \leq 0.05$ and trends were discussed at $P < 0.10$.

RESULTS

Growth Performance

There were no interactions ($P \geq 0.62$) between Vitamin E supplementation and TM injection for BW at any point during the 89 d feeding period (Table 2). Neither vitamin E supplementation ($P \geq 0.79$) nor TM injection ($P \geq 0.51$) had an effect on BW.

From d 0 to d 28 after trial initiation, TM injection increased DMI of VITE+MM heifers whereas it decreased DMI in CONT+MM heifers ($P \geq 0.03$). Unsurprisingly given this DMI interaction, during d 29 to d 56, TM injection resulted in slightly decreased ($P = 0.02$) G:F in VITE+MM heifers and increased G:F of CONT+MM heifers. From d 57 to d 89, heifers receiving TM injection had 8.6% greater DMI (9.11 vs. 8.39 kg/d, $P = 0.04$) and a tendency for greater ADG ($P = 0.07$) than heifers receiving saline injection. Overall (d 0 to d 89) there was a tendency for TM injection to increase (1.37 vs. 1.25 kg/d, $P = 0.09$) ADG compared to saline injection. Further, there was tendency for TM injection to increase DMI of VITE+MM heifers whereas TM injection resulted in decreased DMI in CONT+MM heifers ($P = 0.08$), mirroring the interaction from d 0 to d 28 (Table 2). Vitamin E supplementation had no main effect on growth performance ($P \geq 0.31$).

Carcass Characteristics

There were no interactions ($P \geq 0.49$) between vitamin E supplementation and TM injection on carcass characteristics. Similarly, TM injection had no main effect ($P \geq 0.19$) on any carcass characteristics. Interestingly, there was a tendency for heifers fed supranutritional vitamin E to have greater marbling scores (560 vs. 490, $P = 0.09$) than control-fed heifers. However, there was no difference (8.62% vs. 7.44%, $P = 0.18$) in the percentage of extractible lipid between vitamin E and control-fed heifers.

Instrumental Color and Color Stability

Initially, supplemental vitamin E and TM injection had no main effects on d 1 strip steak lightness (L^*), yellowness (b^*), chroma, or visual discoloration ($P \geq 0.10$). There was a tendency for strip steaks from vitamin E supplemented heifers to exhibit slightly greater 630/580 ratios ($P = 0.09$), however differences were modest (Table 4). Steaks from heifers receiving TM injection

exhibited a tendency for greater hue angles ($P = 0.08$), indicative of greater metmyoglobin formation, than steaks from heifers receiving the control saline injection. Further, there was tendency for TM injection to increase strip steak redness of VITE+MM heifers whereas TM injection decreased redness of steaks from CONT+MM heifers ($P = 0.09$).

On d 16, when steaks were removed from retail display, the average percentage of visual discoloration was 25%. Despite the initial interaction for redness between supplemental vitamin E and TM injection, no interaction ($P = 0.25$, Table 4) was demonstrated at d 16. Nonetheless, there was a main effect of supranutritional vitamin E on redness at d 16, with strip steaks from heifers receiving supplemental vitamin E exhibiting greater surface a^* values (23.52 a^* units vs. 21.42 a^* units, $P = 0.04$) than control fed heifers. Steaks from Vitamin E supplemented heifers also exhibited lesser hue angles (39.56° vs. 41.82°, $P < 0.0001$) and visual discoloration (15.82% vs. 33.96%, $P = 0.02$) than those from control-fed heifers. Trace mineral injection had no effect ($P \geq 0.47$) on d 16 instrumental color, 630/580 nm ratio, hue angle, chroma, or visual discoloration. Furthermore, there were no interactions ($P \geq 0.13$) between Vitamin E supplementation and TM injection at d 16.

Similar findings were shown when color stability was evaluated across the full 16 d duration of retail display using repeated measures. As expected, there was an interaction between day of retail display and vitamin E supplementation with steaks from heifers receiving supranutritional Vitamin E having decreased visual discoloration at later days of retail display ($P < 0.0001$, Fig. 3B) than steaks from control-fed heifers. In support of this finding, steaks from Vitamin E heifers also exhibited smaller hue angles at later days of retail display ($P < 0.01$, Fig. 4A). Because no interactions between vitamin E supplementation and TM injection were shown ($P \geq 0.12$) LS means for the interactions between day and vitamin E supplementation were

statistically separated by day. Steaks from heifers receiving supranutritional vitamin E had smaller hue angles at d 15 and 16 ($P < 0.05$, Fig. 4B) and decreased visual discoloration compared with the control-fed heifers at d 14-16 of retail display ($P < 0.05$, Fig. 3).

Although no main effect of TM injection was ($P \geq 0.70$) demonstrated, an interaction between day of retail display and TM injection was shown ($P = 0.02$, Fig. 3B) for visual discoloration. However, TM injection had increased visual discoloration at later days of retail display than steaks from heifers receiving saline injection. When evaluated by day, steaks from heifers receiving TM injection had a tendency for greater visual discoloration at d 14 ($P = 0.09$) and greater discoloration at d 15 (20.20% vs. 10.74%, $P = 0.04$) of retail display than steaks from heifers receiving saline injection.

The three-way interaction of supplemental vitamin E, trace mineral injection, and day of retail display resulted in a significant effect on the stability of steak surface redness ($P = 0.04$) and extent of visual discoloration ($P < 0.01$). The three-way effect on steak surface redness was primarily driven by changes at late retail display (d 11-16) with steaks from the CONT+SAL and VITE+MM treatments demonstrating a moderate decline in a^* value from d 11 to d 16 ($\Delta a^* = 7.06$ & 5.42 units, respectively), CONT+MM steaks experiencing a greater decline ($\Delta a^* = 9.13$ units) and VITE+SAL treatment maintaining redness ($\Delta a^* = 3.94$ units) from d 11 to d 16 (Fig. 2).

The three-way effect of supplemental vitamin E, trace mineral injection, and day of retail display on visual discoloration was driven by differences between treatments in both point at which initial discoloration occurred and the total extent of discoloration at the end of retail display. No differences in visual discoloration were shown until d 14 of retail display, however steaks from the CONT+MM treatment showed numerically greater discoloration by d 13 of retail

display indicating an earlier point of initial discoloration than the other three treatments (Fig. 3). At d 14 and 15 of retail display the CONT+MM treatment showed greater discoloration ($P < 0.01$) than the other three treatments (Fig. 4). By d 16, there was a tendency ($P = 0.08$) for discoloration of steaks from the CONT+SAL treatment to be intermediate to the CONT+TM and VITE+SAL treatments.

DISCUSSION

The relatively limited number of studies evaluating the effects of vitamin E supplementation on growth performance in feedlot cattle demonstrate considerable variation in response. A meta-analysis conducted by Cusack et al. (2009) indicated feeding vitamin E at concentrations greater than the 1996 NRC recommendation of 15 – 60 IU/kg DM does not improve feedlot performance, mirroring much of the research in feedlot cattle showing little effect of vitamin E supplementation on final BW, ADG, DMI, and G:F (Arnold et al., 1992; Garber et al., 1996). However, a previous review (Secrist et al., 1997) reported vitamin E supplementation improved ADG by 2.9%. Although both reviews suggest the performance response associated with vitamin E is likely dependent on supplementation concentration (Secrist et al., 1997; Cusack et al., 2009), supplemented heifers in this study received Vitamin E well in excess of the industry's average inclusion of 30 IU/kg DM in finishing diets as reported in the feedlot survey of Samuelson et al. (2016) for 90 d prior to slaughter and demonstrated no greater performance traits than non-supplemented heifers.

Considerably less research assessing the potential for TM injection to improve performance of feedlot cattle exists. Nonetheless, numerical differences in the current study show slight improvements in ADG of TM injected heifers compared with saline injected heifers. Arthington et al. (2014) reported a tendency for TM injection to increase ADG of growing

heifers. Genter and Hansen (2014) also demonstrated a tendency for TM supplementation to improve carcass adjusted ADG of steers in the feedlot setting. In a group of steers managed prior to trial initiation on a trace mineral maintenance diet, numerical differences in ADG of saline and TM injected steers amounted to a 3.9% improvement in ADG (Genter and Hansen, 2014). However, when steers were managed on a TM deficient diet prior to trial initiation, those receiving TM injection demonstrated a 17.7% improvement in ADG compared with saline injected steers (Genter and Hansen, 2014). Although not statistically different, the improvement shown in the present study amounted to a 9.6% increase in ADG of feedlot heifers receiving TM injection. This finding suggests that although the heifers in the current study were not managed to be TM deficient, improvements in ADG associated with TM injection may have been the result of improved in TM status in a group of heifers with lesser TM stores, however TM status was not assessed via liver biopsy.

Interestingly, heifers supplemented with vitamin E had increased marbling score and is in contrast to the majority of the literature showing little effect of vitamin E supplementation on marbling deposition (Arnold et al., 1992; Liu et al., 1996; Burken et al., 2012). Although it should be noted the increase of extractible lipid was not statistically different between supplemented and non-supplemented heifers in this study. Even so, the magnitude of difference in extractible lipid between vitamin E and control-fed heifers was 1.18 units. This change in lipid content is representative of the difference between Modest (average choice) and Moderate (high choice) marbling scores according to Savell et al. (1986). To add further credence to this finding, 76.3% of the variability ($R^2 = 0.76$) in camera-assigned marbling score was explained by the percentage of extractible lipid observed in the cut surface of the steaks cut from the ribbed carcass. It should be noted, the Secrist et al. (1997) review reported marbling score increased

numerically with greater vitamin E supplementation. However, increased lipid deposition of vitamin E supplemented cattle observed by Secrist et al. (1997) is less surprising given the concurrent improvements in performance of those cattle.

Of the limited research evaluating effects of TM injection on carcass characteristics, there is considerable variation of response. A study by Genter and Hansen (2014) reported greater REA and marbling scores in feedlot steers receiving TM injection 90 d prior to slaughter. As expected, those steers that were maintained on a TM deficient diet prior to trial initiation had greater REA response to TM injection than steers that were control-fed (Genter and Hansen, 2014). The same study reported TM injection also resulted in greater marbling scores and a shift in distribution to higher quality grades, however the marbling score change between TM injected and saline injected steers amounted to less than one quality grade improvement (Genter and Hansen, 2014). In another study by Genter-Schroeder and Hansen (2015), TM injection had no effect on HCW, BF, REA, or marbling score. The current study agrees with the findings presented previously with little effect of TM injection on carcass characteristics. As it is generally difficult to quantify the TM status of feedlot cattle upon receiving from an industry standpoint, differences in response to TM injection are likely due to differences in TM status on arrival.

Any technology that adds value to one or more segments of the industry with minimal risk has the potential for large-scale adoption. Specific to the retailer, management strategies with the potential to slow the rate of visual discoloration in the retail case would decrease losses associated with discounted, re-worked, or discarded product (Zerby et al., 1992). It's been established vitamin E supplementation results in greater retail shelf life by decreasing the rate of visual discoloration in both whole muscle cuts and ground products (Arnold et al., 1992, Zerby et

al., 1999, Bloomberg et al., 2012) and the results of this study support those findings. However, common finishing diets have not historically included dietary vitamin E concentrations great enough to elicit greater retail shelf life (Samuelson et al., 2016), likely a result of poor investment returns in the form of greater associated diet costs coupled with limited performance benefits.

Trace minerals included in the TM injection used in this study all play roles in the production and activity of antioxidants important in counteracting oxidative stress. Markers of oxidative stress and inflammation were shown to be greater during periods of intense stress such as animal transit (Chirase et al., 2004), making receiving cattle more prone to disease challenge and poor performance. Selenium is a component of the antioxidant enzyme glutathione peroxidase and has an important role in the conversion of superoxide radicals to hydrogen peroxide, protecting cellular membranes from free radical damage (Kincaid, 1995). Given the selenium deficiencies of many Midwestern U.S. soils and forages (USDA, 2006), there is a greater likelihood these antioxidant systems may be compromised in deficient cattle. Another such group of antioxidant enzymes are the superoxide dismutases which are dependent on manganese, copper, and zinc status (Weisiger and Fridovich, 1973; Xin et al. 1991). Steers that received TM supplementation via injection exhibited greater Mn-superoxide dismutase (Genther and Hansen, 2014b) and glutathione peroxidase activity (Pogge et al., 2012) in red blood cell lysate than saline injected steers, adding credence to the idea TM injection might provide additional oxidative stability to the adipose tissue of strip steaks.

Despite the hypothesis vitamin E and TM injection would additively improve color stability and delay the point of initial discoloration, steaks from control-fed heifers that received TM injection discolored both at an earlier point in retail display and to a greater extent than their

saline injected counterparts. In a study of product acceptability and discoloration in the retail case, Gill and Jones et al. (1994) reported products were rated as undesirable after reaching >20% surface discoloration. Applying this measure of acceptability to the current study, steaks from the CON+MM treatment would have been rated as undesirable three days faster than steaks from the other three treatments. A potential explanation for the inability of an injectable trace mineral to improve color stability is the low concentrations of selenium and zinc maintained in skeletal muscle compared with liver or kidney (Jensen-Waern et al., 1998; Lawler et al., 2004)

Further, the detrimental effect of TM injection to retail color stability may be attributable to copper supplementation. Engle and Spears (2000) reported that dietary copper supplementation in finishing steers altered lipid metabolism. This same study showed greater concentrations of polyunsaturated fatty acids and a tendency for decreased saturated fatty acids concentrations in the longissimus muscle of steers receiving copper supplementation (Engle and Spears, 2000). Trace mineral injection has been demonstrated to increase liver copper and selenium concentrations as well as plasma selenium concentrations of both control and trace mineral deficient steers (Genther and Hansen, 2014a). A possible explanation for changes in discoloration of steaks from heifers receiving TM injection is that bolus delivery may also be increasing tissue deposition thereby influencing the initial point of discoloration.

Given dietary inclusion of distillers grains with solubles can also increase the concentration of less oxidatively stable polyunsaturated fatty acids (Gill et al., 2008), one hypothesis is that compounded effects of TM injection and 35% MWDGS inclusion resulted in decreased oxidative and color stability in this study. However, it is important to note MWDGS used in the present study contained only 8.5% fat, the lower end of the range in distiller's grains crude fat content (USGC, 2012). More interesting yet is the observation that steaks from

VITE+MM heifers did not experience the same decline in color stability shown in steaks from CONT+MM heifers. This explanation is supported by the observation that steaks from the VITE+MM treatment actually discolored to the same extent as steaks from the VITE+SAL treatment. Therefore, it appears as though vitamin E supplementation had a stabilizing effect on the cellular membranes of heifers that received MM injection. This explanation is supported by the observation of numerical differences in lipid oxidation in which steaks from the VITE+MM treatment were intermediate to the CON+MM and VIT+SAL treatments.

Conclusions

Vitamin E supplementation and TM injection had little effect on growth performance of feedlot heifers. Although there was a tendency for minor improvements in growth of feedlot heifers receiving TM injection, this response may be greater in finishing cattle with subacute TM deficiencies. As expected, VITE supplementation improved color stability and resulted in greater retail display life than steaks from control-fed heifers. When used in conjunction with a diet higher in MWDGS inclusion, TM injection appeared to increase discoloration of strip steaks from feedlot heifers after 14 d of retail display.

TABLES & FIGURES

Table 4.1. Ingredient and nutrient composition of diets fed for 89 d before slaughter

Ingredient	Dietary treatment	
	Control	Vitamin E ¹
	% of diet DM	
Dry-rolled corn	35	35
Modified wet distillers grains ²	35	35
Corn silage	20	20
Control Supplement ³	10	--
Vitamin E Supplement ⁴	--	10
	% of diet DM	
Analyzed nutrient content		
CP, %	14.61	14.72
NDF, %	26.39	26.32
ADF, %	10.03	10.19
Fat, %	4.82	4.88
Calculated nutrient content ⁵		
Vitamin E, IU/kg DM	19.8	109.8
S, % of diet DM	0.31	0.31
Cu, mg/kg DM	9.32	9.31
Mn, mg/kg DM	43.54	43.51
Se, mg/kg DM	0.43	0.42
Zn, mg/kg DM	66.53	66.44

¹ Heifers were supplemented with no additional vitamin E in the diet (Cont) or 1,000 IU of vitamin E/heifer·d⁻¹(VitE) for 89d prior to slaughter.

² Modified wet distillers grains contained 8.5% fat.

³ Supplement contained: 75.4% ground corn, 22.7% limestone, 0.9% trace mineral salt (20% CaCO₃, 15.43% Availa-4 Zinpro, 14.16% KCl, 8.75% MgO, 8.00% MnSO₄, 6.74% FeSO₄, 6.55% rice hulls mineral oil, 5.95% S prilled, 4.41% vitamin E, 1.50% Se, 1.03% MgSO₄ & KSO₄, 0.88% CuSO₄, 0.22% vitamin A, 0.13% vitamin D₃ 500, 0.04% Ca(IO₃)₂, yielding 277 mg/kg Co, 5,000 mg/kg Cu, 250 mg/kg I, 20,200 mg/kg Fe, 30,000 mg/kg Mn, 150 mg/kg Se, 30,000 mg/kg Zn, 2,205 KIU/kg vitamin A, 662 KIU/kg vitamin D₃, 22,000 IU/kg vitamin E) 0.8% liquid fat, 0.15% Rumensin 90 (200 g monensin/kg DM; Elanco Animal Health, Greenfield, IN), 0.10% Tylan 40 (88 g tylosin/kg DM; Elanco Animal Health)

⁴ Supplement contained: 70.9% ground corn, 22.7% limestone, 4.5% vitamin E supplement (20,000 IU/kg dl- α -tocopheryl acetate; ADM Alliance Nutrition, Quincy, IL), 0.9% trace mineral salt, Rumensin 90 (200 g monensin/kg DM; Elanco Animal Health, Greenfield, IN), 0.10% Tylan 40 (88 g tylosin/kg DM; Elanco Animal Health)

⁵ Nutrients were calculated using NRC values (NRC, 2000)

Table 4.2. Effects of trace mineral injection and dietary vitamin E supplementation on growth performance of feedlot heifers¹

Item	Treatment ^{2,3}				SEM	P-value		
	Cont+Sal	Cont+MM	Vit E+Sal	Vit E+MM		Diet	Injection	Diet × Injection
Total head, n	12	12	11	11				
BW, kg								
Initial	425	422	427	421	9.1	0.98	0.66	0.89
d 28	469	464	464	469	9.7	0.99	0.95	0.62
d 56	500	499	502	503	9.5	0.79	0.99	0.93
Final	538	543	536	544	10.3	0.98	0.51	0.91
Total BW gain, kg	112	121	110	123	6.4	0.94	0.09	0.72
d 0 to 28								
ADG, kg/d	1.53	1.44	1.31	1.64	0.14	0.96	0.36	0.12
DMI, kg/d	8.7 ^{ab}	8.3 ^{ab}	7.5 ^b	8.9 ^a	0.5	0.67	0.27	0.03
G:F	0.17	0.17	0.16	0.18	0.01	0.98	0.48	0.44
d 29 to 56								
ADG, kg/d	1.09	1.26	1.33	1.22	0.12	0.48	0.73	0.12
DMI, kg/d	8.8	8.7	8.6	9.5	0.4	0.55	0.23	0.13
G:F	0.12 ^b	0.14 ^{ab}	0.16 ^a	0.13 ^{ab}	0.01	0.31	0.74	0.02
d 57 to 89								
ADG, kg/d	1.17	1.37	1.07	1.29	0.12	0.47	0.07	0.93
DMI, kg/d	8.6	8.8	8.2	9.4	0.3	0.81	0.04	0.17
G:F	0.14	0.15	0.13	0.14	0.01	0.47	0.29	0.49
d 0 to 89								
ADG, kg/d	1.26	1.36	1.23	1.38	0.07	0.94	0.09	0.73
DMI, kg/d	8.7	8.6	8.1	9.3	0.4	0.96	0.12	0.08
G:F	0.15	0.16	0.15	0.15	0.01	0.80	0.29	0.23

¹Trace mineral injection included 15 mg/mL of Cu, 10 mg/mL of Mn, 5 mg/mL Se, and 60 mg/mL Zn.

²Heifers were supplemented with no additional vitamin E in the diet (Cont) or 1,000 IU of vitamin E/heifer·d⁻¹(VitE) for 89 d prior to slaughter. Heifers received saline (Sal) or trace mineral injection (TM) at a dosage of 1 mL/68 kg BW at d 1 of the 89 d feeding period.

³Treatments within day lacking common superscripts differ ($P < 0.05$).

Table 4.3. Effects of trace mineral injection and vitamin E supplementation on carcass characteristics of feedlot heifers¹

Item	Treatment ²				SEM	P-value		
	Cont+Sal	Cont+MM	Vit E+Sal	Vit E+MM		Diet	Injection	Diet × Injection
Ending live wt, kg	538	543	536	544	10	0.98	0.51	0.91
HCW, kg	340	340	338	341	6	0.99	0.83	0.79
Carcass yield %	63.2	62.5	63.0	62.7	0.4	0.95	0.19	0.65
REA, cm ²	82.8	84.0	82.0	84.6	2.1	0.97	0.35	0.72
12 th -rib fat, cm	1.2	1.3	1.3	1.3	0.1	0.65	0.96	0.49
KPH, %	2.2	2.1	2.2	2.3	0.1	0.09	0.31	0.81
Calculated yield grade	3.1	3.1	3.2	3.0	0.1	0.85	0.56	0.62
Marbling score ³	480	500	565	550	35	0.08	0.89	0.54
Extractible lipid, %	7.1	7.7	8.5	8.7	0.8	0.18	0.58	0.75

¹Trace mineral injection included 15 mg/mL of Cu, 10 mg/mL of Mn, 5 mg/mL Se, and 60 mg/mL Zn.

²Heifers were supplemented with no additional vitamin E in the diet (Cont) or 1,000 IU of vitamin E/heifer·d⁻¹(VitE) for 89 d prior to slaughter. Heifers received saline (Sal) or trace mineral injection (TM) at a dosage of 1 mL/68 kg BW at d 1 of the 89 d feeding period.

³Marbling scores: 400 = Small⁰⁰ (low choice) and 500 = Modest⁰⁰ (average choice).

Table 4.4. Effects of trace mineral injection and vitamin E supplementation on color stability of strip steaks from feedlot heifers at d 1 and d 16 of retail display¹

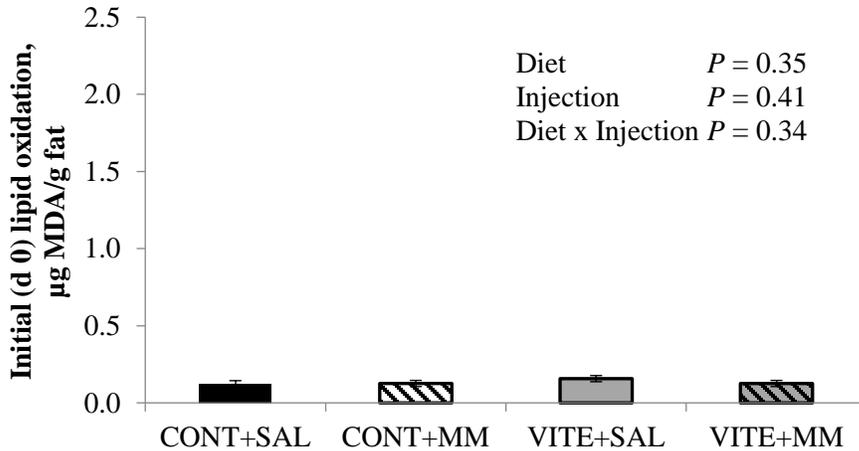
Item	Treatment ²				SEM	P-value		
	Cont+Sal	Cont+MM	Vit E+Sal	Vit E+MM		Diet	Injection	Diet × Injection
Initial, d1 retail display								
Lightness, L*	43.3	42.1	43.4	44.3	1.0	0.23	0.88	0.29
Redness, a*	30.0	29.6	28.4	30.3	0.8	0.63	0.28	0.09
Yellowness, b*	22.0	22.2	20.7	23.2	0.9	0.89	0.11	0.15
630/580 nm	5.8	6.1	5.2	5.7	0.3	0.09	0.15	0.71
Chroma	37.2	36.9	35.2	38.2	1.1	0.75	0.17	0.10
Hue angle	36.2	36.8	35.8	37.4	0.6	0.92	0.08	0.40
Visual discoloration, %	0.0	0.0	0.0	0.0	--	--	--	--
Final, d16 retail display								
Lightness, L*	43.1	41.9	43.6	45.3	1.3	0.11	0.85	0.24
Redness, a*	22.3	20.5	23.3	23.8	1.0	0.04	0.52	0.25
Yellowness, b*	19.8	18.3	19.1	19.8	0.7	0.64	0.61	0.13
630/580 nm	2.8	2.6	3.0	3.2	0.2	0.10	0.98	0.47
Chroma	29.8	27.5	30.1	30.9	1.2	0.13	0.54	0.19
Hue angle	41.7	41.9	39.4	39.7	0.5	<0.0001	0.62	0.85
Visual discoloration, %	28.0	40.0	16.0	15.0	8.0	0.02	0.47	0.41

¹Trace mineral injection included 15 mg/mL of Cu, 10 mg/mL of Mn, 5 mg/mL Se, and 60 mg/mL Zn.

²Heifers were supplemented with no additional vitamin E in the diet (Cont) or 1,000 IU of vitamin E/hd·d⁻¹(VitE) for 89 d prior to slaughter. Heifers received saline (Sal) or trace mineral injection (TM) at a dosage of 1 mL/68 kg BW at d 1 of the 89 d feeding period.

Figure 4.1. Effects of trace mineral injection (containing Cu, Mn, Se, and Zn) and supranutritional dietary vitamin E supplementation on (A) initial lipid oxidation at d 2 postmortem, and (B) final lipid oxidation at d 16 postmortem, of beef strip steaks as determined by the thiobarbituric acid reactive substances assay (TBARS). Oxidation was quantified in μg malondialdehyde (MDA)/g fat to correct for differences in extractable lipid content.

A.



B.

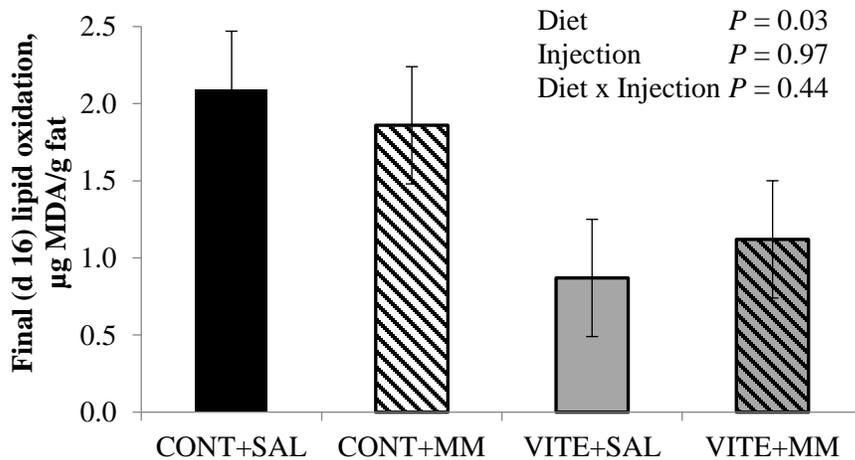


Figure 4.2. Effects of trace mineral injection (containing Cu, Mn, Se, and Zn) and supranutritional dietary vitamin E supplementation on surface redness (a* value) of beef strip steaks across 16 d of retail display. Asterisks indicate significance within day with treatments lacking common superscripts differing ($P < 0.05$).

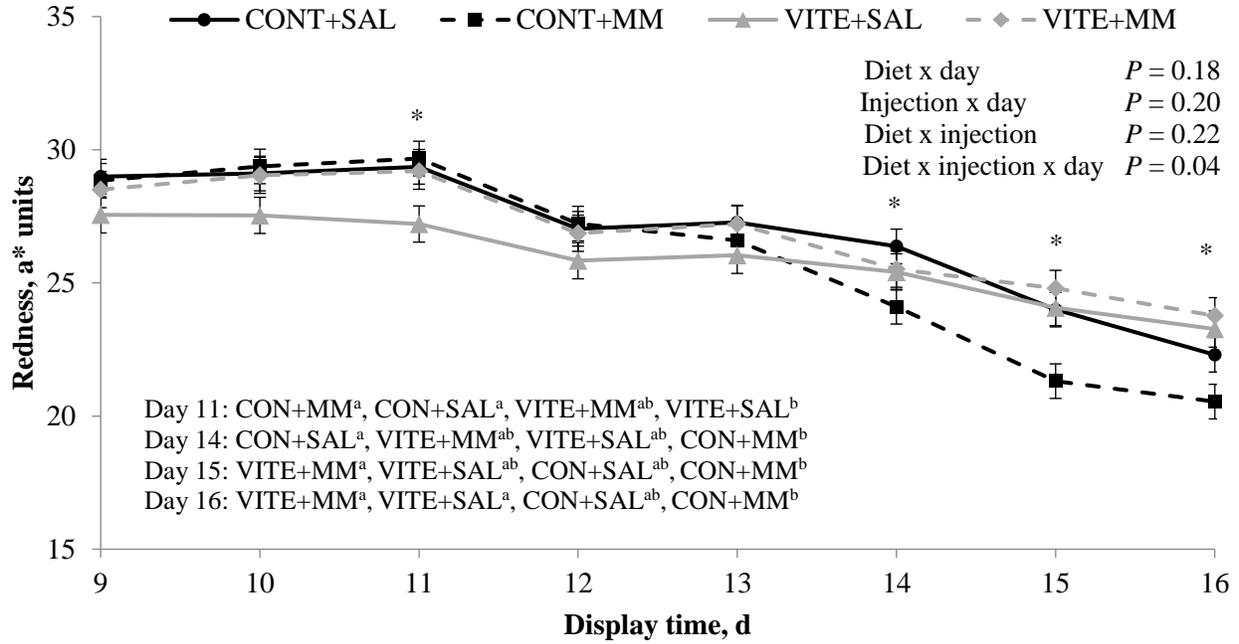


Figure 4.3. Effects of supranutritional dietary vitamin E supplementation on calculated hue angle of beef strip steaks across 16 d of retail display. Treatments within day lacking common superscripts differ ($P < 0.05$).

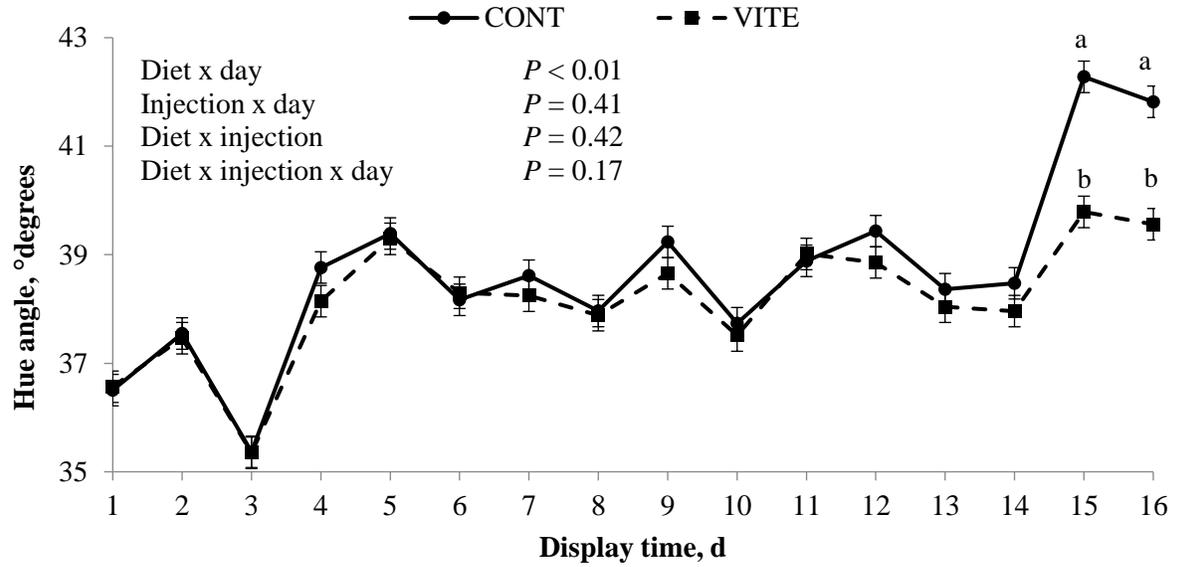
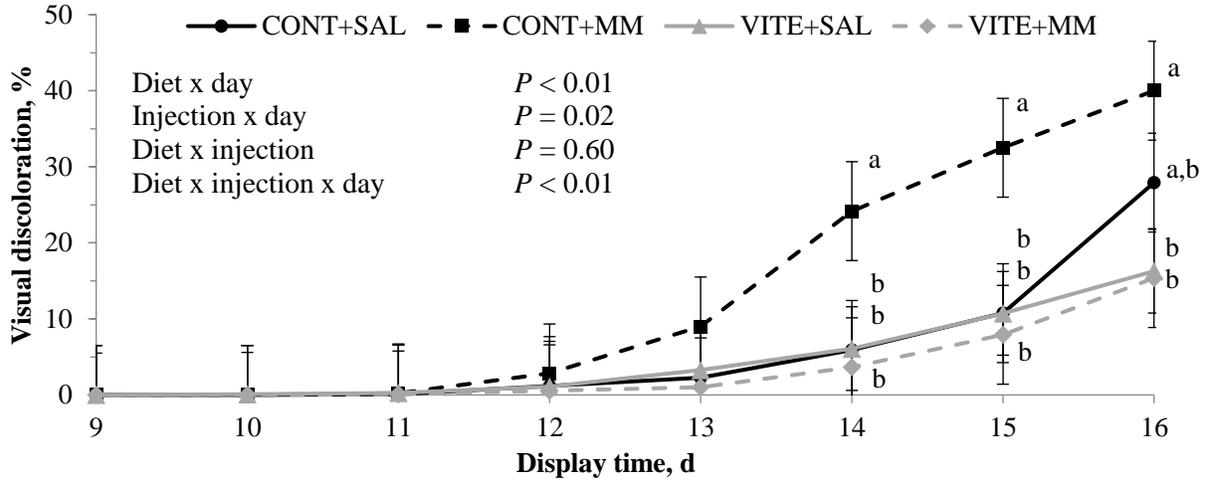


Figure 4.4. Effects of trace mineral injection (containing Cu, Mn, Se, and Zn) and supranutritional dietary vitamin E supplementation on visual surface discoloration of beef strip steaks across 16 d of retail display. Treatments within day lacking common superscripts differ ($P < 0.05$).



LITERATURE CITED

- AMSA. 2012. Meat color measurement guidelines. 2nd ed. Am. Meat Sci. Assoc., Champaign, IL. http://www.meatscience.org/docs/default-source/publications-resources/Hot-Topics/2012_12_meat_clr_guide.pdf?sfvrsn=0. Accessed: 6 October 2017
- Arnold, R. N., K. K. Scheller, S. C. Arp, S. N. Williams, D. R. Buege, and D. M. Schaefer. 1992. Effect of long- or short-term feeding of α -tocopherol acetate to Holstein and crossbred beef steers on performance, carcass characteristics, and beef color stability. *J. Anim. Sci.* 70: 3055-3065. doi:10.2527/1992.70103055x
- Arthington, J. D., P. Moriel, P. G. M. A. Martins, G. C. Lamb, and L. J. Havenga. 2014. Effects of trace mineral injections on measures of performance and trace mineral status of pre- and postweaned beef calves. *J. Anim. Sci.* 92: 2630-2640. doi:10.2527/jas2013-7164
- Bernofsky, C., J. B. Fox, and B. S. Schweigert. 1959. Biochemistry of myoglobin. VII. The effects of cooking on myoglobin in beef muscle. *Adv. Food Res.* 24:339.
- Bloomberg, B. D., G. G. Hilton, K. G. Hanger, C. J. Richards, J. B. Morgan, and D. L. VanOverbeke. 2011. Effects of vitamin E on color stability and palatability of strip loin steaks from cattle fed distillers grains. *J. Anim. Sci.* 89: 3769-3782. doi:10.2527/jas.2011-3843.
- Burken, D. B., R. B. Hicks, D. L. VanOverbeke, G. C. Hilton, J. L. Wahrmond, B. P. Holland, C. R. Krehbiel, P. K. Camfield, and C. J. Richards. 2012. Vitamin E supplementation in beef finishing diets containing 35% wet distillers grains with solubles: Feedlot performance and carcass characteristics. *J. Anim. Sci.* 90: 1349-1355. doi:10.2527/jas.2011-3833.

- Chirase, N. K., L. W. Green, C. W. Purdy, R. W. Loan, B. W. Auvermann, D. B. Parker, E. F. Walborg, D. E. Stevenson, Y. Xu, J. E. Klaunig. 2004. Effect of transport stress on respiratory disease, serum antioxidant status, and serum concentrations of lipid peroxidation biomarkers in beef cattle. *Am. J. Vet. Res.* 65: 860-864. doi:10.2460/ajvr.2004.65.860.
- Cusack, P., N. McMeniman, A. Rabiee, I. Lean. 2009. Assessment of the effects of supplementation with vitamin E on health and production of feedlot cattle using meta-analysis. *Prev. Vet. Med.* 88: 229-246. doi:10.1016/j.prevetmed.2008.12.002.
- Engle, T. E., and J. W. Spears. 2000. Dietary copper effects on lipid metabolism, performance, and ruminal fermentation in finishing steers. *J. Anim. Sci.* 78:2452-2458.
- Garber, M. J., R. A. Roeder, W.M. Pumfrey, G. T. Schelling, and R. M. Davidson. 1996. Dose-response effects of vitamin E supplementation on growth performance and meat characteristics in beef and dairy steers. *Can. J. Anim. Sci.* 76: 63-72. doi:10.4141/cjas96-009.
- Genther, O. N., and S. L. Hansen. 2014a. A multielement trace mineral injection improves liver copper and selenium concentrations and manganese superoxide dismutase activity in beef steers. *J. Anim. Sci.* 92: 695-704. doi:10.2527/jas2013-7066.
- Genther, O. N., and S. L. Hansen. 2014b. Effect of dietary trace mineral supplementation and multi-element trace mineral injection on shipping response and growth performance of beef cattle. *J. Anim. Sci.* 92: 2522-2530. doi:10.2527/jas2013-7426.

- Genther-Schroeder, O. N., and S. L. Hansen. 2015. Effect of a multielement trace mineral injection before transit stress on inflammatory response, growth performance, and carcass characteristics of beef steers. *J. Anim. Sci.* 93: 1767-1779. doi:10.2527/jas2014-8709.
- Gill, R. K., D. L. VanOverbeke, B. Depenbusch, J. S. Drouillard, and A. DiCostanzo. 2008. Impact of beef cattle diets containing corn or sorghum grains on beef color, fatty acid profiles, and sensory attributes. *J. Anim. Sci.* 86: 923-935. doi:10.2527/jas2007-0244.
- Gill, C. O., and T. Jones. 1994. The display life of retail-packaged beef steaks after their storage in master packs under various atmospheres. *Meat Sci.* 38: 385-396. doi:10.1016/0309-1740(94)90065-5.
- Jensen-Waern, M., L. Melin, R. Lindberg, A. Johannisson, L. Petersson, and P. Wallgren. 1998. Dietary zinc oxide in weaned pigs – effects on performance, tissue concentrations, morphology, neutrophil function, and faecal microflora. *Res. Vet. Sci.* 64: 225-261.
- Kincaid, R. 1995. The biological basis for selenium requirements of animals. *Prof. Anim. Sci.* 11:26-29. doi:10.15232/S1080-7446(15)32546-8.
- Lawler, T. L., J. B. Taylor, J. W. Finley, and J. S. Caton. 2004. Effects of supranutritional and organically bound selenium on performance, carcass characteristics, and selenium distribution in finishing beef steers. *J. Anim. Sci.* 82: 1488-1493. doi:10.2527/2004.8251488x
- Leick, C. M., C. L. Puls, M. Ellis, J. Killefer, T. R. Carr, S. M. Scramlin, M. B. England, A. M. Gaines, B. F. Wolter, S. N. Carr, and F. K. McKeith. 2010. Effect of distillers dried grains with solubles and ractopamine (Paylean) on quality and shelf-life of fresh pork and bacon. *J. Anim. Sci.* 88:2751-2766. doi:10.2527/jas.2009-2472.

- Liu, Q., K. K. Scheller, S. C. Arp, D. M. Schaefer, and S. N. Williams. 1996. Titration of fresh meat color stability and malondialdehyde development with Holstein steers fed vitamin E-supplemented diets. *J. Anim. Sci.* 74: 117-126. Doi:
- Novakofski, J., S. Park, P. J. Bechtel, and F. K. McKeith. 1989. Composition of cooked pork chops – Effect of removing subcutaneous fat before cooking. *J. Food Sci.* 54:15-17. doi: 10.1111/j.1365-2621.1989.tb08556.x
- NRC. 2000. *Nutrient Requirements of Beef Cattle*. 7th ed. Natl. Acad. Press, Washington, D.C.
- Pogge, D. J., E. L. Richter, M. E. Drewnoski, and S. L. Hansen. 2012. Mineral concentration of plasma and liver after injection with a trace mineral complex differ among Angus and Simmental cattle. *J. Anim. Sci.* 90: 2692-2698. doi:10.2527/jas2012-4482.
- Savell, J. W., H. R. Cross, and G. C. Smith. 1986. Percentage of ether extractable fat and moisture content of beef longissimus muscle as related to USDA marbling score. *J. Food Sci.* 51:838-840. doi: 10.1111/j.1365-2621.1986.tb13946.x
- Samuelson, K. L., M. E. Hubbert, M. L. Galyean, and C. A. Löest. 2016. Nutritional recommendations of feedlot consulting nutritionists: The 2015 New Mexico State and Texas Tech University survey. *J. Anim. Sci.* 94: 2648-2663. doi: 10.2527/jas2016-0282.
- Secrist, D. S., F. N. Owens, D. R. Gill, L. J. Boyd, and J. E. Oldfield. 1997. Effects of vitamin E on performance of feedlot cattle: A review. *Prof. Anim. Sci.* 13: 47-54. doi:10.15232/S1080-7446(15)31844-1.
- Smith, G. C., K. E. Belk, J. N. Sofos, J. D. Tatum, and S. N. Williams. 2000. Economic implications of improved color stability in beef. *Antioxidants in muscle foods: Nutritional strategies to improve quality*, Wiley Interscience, New York. Pg. 397 - 426.

- Strange, E. D., R. C. Benedict, R. E. Gugger, V. G. Metzger, and C. E. Swift. 1974. Simplified methodology for measuring meat color. *J. Food Sci.* 39: 988-992.
- USDA. 2006. Miscellaneous minerals: Illinois.
https://www.nrcs.usda.gov/wps/portal/nrcs/detail/il/home/?cid=nrcs141p2_030620.
Accessed: 6 October 2017.
- U.S. Grains Council (USGC). 2012. A guide to distiller's dried grains with solubles. Third edition: U.S. Grains Council. www.ethanolrfa.org/wp-content/uploads/2015/11/2012_DDGS_Handbook-1.pdf. Accessed: 3 March 2018.
- Weisiger, R. A., and I. Fridovich. 1973. Superoxide dismutase. *J. Biol. Chem.* 248: 3582-3592.
- Xin, Z., D. F. Waterman, R. W. Hemken, and R. J. Harmon. 1991. Effect of copper status on neutrophil function, superoxide dismutase, and copper distribution in steers. *J. Dairy Sci.* 47: 3078-3085. doi:10.3168/jds.S0022-0302(91)78493-2.
- Zerby, H. N., K. E. Belk, J. N. Sofos, L. R. McDowell, and G. C. Smith. 1999. Case life of seven retail products from beef cattle supplemented with alpha-tocopheryl acetate. *J. Anim. Sci.* 77: 2458-2463. doi:10.2527/1999.7792458x.