EFFECTS OF IMPLANTING STRATEGY AND ZILPATEROL HYDROCHLORIDE ON THE CALPAIN PROTEOLYTIC SYSTEM IN SECTIONED BEEF STEAKS FOR TWO TIME PERIODS

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Effects of implanting strategy and zilpaterol hydrochloride on the calpain proteolytic system in sectioned beef steaks for two time periods

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ABSTRACT

The objective of this study was to evaluate the impact of an anabolic implant and its use with the beta-adrenergic agonist zilpaterol hydrochloride (ZH) the calpain proteolytic system activity across specific areas of the beef strip steak over two aging days. Crossbred heifers were blocked by weight and randomly assigned to one of three treatments: 1) no implant or ZH (CON), 2) implant, no ZH (IMP), and 3) implant and ZH (IMP+ZH). At slaughter, strip steaks were collected and aged for 3 and 14 d. Samples were evaluated for Minolta objective color scores, pH, bioelectrical impedance analysis, and were then cut into lateral, lateral/medial, and medial sections. Protein was extracted from each section, and the calpain proteolytic system was evaluated. A day of aging effect was seen in protein degradation, along with a treatment by section interaction proteolytic activity. No differences were found in pH or color by treatment.

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LIST OF ABBREVIATIONS

ADG.....Average Daily Gain

AMPER.....Ammonium Persulfate

BAAs.....β-Adrenergic Agonists

BIA.....Bioelectrical Impedance

C° .....Degrees Celsius

Ca .....Calcium

CAF.....Calcium activated factor

Calpain .....Calcium dependent protease

Calpain 1 .....μ-Calpain

Calpain 2 .....m-Calpain

cAMP .....Cyclic adenosine monophosphate

CLN.....Clenbuterol

cm.....Centimeters

CON .....Control Treatment

CREB .....cAMP response binding element

d.....Day

DES .....diethylstilbestrol

E2 .....Estradiol

EDTA.....ethylenediamine tetraacetic acid

FDA.....Food and Drug Administration

g.....Gravitational Units

g.....Grams

HCW .....Hot Carcass Weight

HSP 70 .....Heat Shock Protein 70

IGF-I .....insulin-like growth factor I

IMP .....Implanted Treatment

kg.....Kilogram

km .....Kilometer

KPH.....Kidney-Pelvic-Heart Fat

LAT.....Lateral

LD .....Longissimus Dorsi

m .....Meters

M.....Molar

MCE.....Mercaptoethanol

MED.....Medial

mg .....Milligram

MID.....Lateral/Medial

mL.....Milliliter

N.....Nitrogen

nm .....Nanometers

Pa.....Phase Angle

PBS .....Phosphate Buffered Saline

pH.....Potential Hydrogen

ppm .....Parts per Million

R.....Resistance

RAC .....Ractopamine

RNA .....Riboneucleic Acid

SAS .....Statistical Analysis Software

SDS .....sodium dodecyl sulfate

SDS-PAGE .....sodium dodecyl sulfate polyacrylamide gel electrophoresis

SSF.....Slice Shear Force

TBA.....Trenbolone acetate

TCA.....Trichloroacetic Acid

TMED .....tetramethylethylenediamine

TnT.....Troponin-T

Tris .....tris(hydroxymethyl)aminomethane

Tween-20 .....polyoxyethylene sorbitan monolaurate

US .....United States

USDA.....United States Department of Agriculture

V.....Volts

vol .....Volume

WBSF.....Warner-Bratzler Shear Force

wt.....Weight

X.....Reactance

Z .....Impedance

ZH .....Zilpaterol Hydrochloride

α .....Alpha

β .....Beta

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Introduction

Beta-adrenergic agonists (BAAs) and anabolic implants are growth promoting technologies that are commonly used in beef production. Anabolic implants were approved earlier than beta-adrenergic agonists, and in 1956, one year after approval, diethylstilbestrol (DES) was being implanted in approximately 50% of all feedlot cattle (Raun and Preston, 1997) and in 1999 more than 96% of all feedlot cattle were implanted at least once (Scheffler et al., 2003). However, implanting has been shown to reduce beef tenderness (Roeber et al. 2000, Johnson et al. 2008, Scheffler et al., 2003). Likewise, the two FDA-approved beta-adrenergic agonists, zilpaterol hydrochloride (ZH) and ractopamine (RAC) may also cause a decrease in tenderness (Arp et al. 2013, Scramlin et al. 2010). When beta-adrenergic agonists are utilized in conjunction with implants, the combination tends to yield less tender meat than just implanting, and at d 14 of postmortem aging the areas of the longissimus dorsi (LD) cross-sections exhibit unique tenderness characteristics (Ebarb et al., 2016). The mechanism behind these changes in tenderness when these technologies are used in combination is not well established, therefore, the primary objective of this study was to identify if postmortem protein degradation and the modulation of the calpain system is the reason for decreased tenderness in beef cattle that are implanted and fed ZH.

Meat Quality

Meat quality is a set of variables that vary depending to what segment of the industry or the individual consumer responds to positively. Because quality of the overall eating experience is somewhat subjective, there have been methods developed to correlate objective measures with

meat quality (Warner, 1929, Honikel, 1998). The first and most economically impactful determination of beef quality is performed by a USDA grader and takes into account the whole carcass, including the amount of intramuscular fat, also known as marbling, visible on the face of the LD at the 12^{th} and 13^{th} rib, firmness and color of the lean, firmness and color of the fat, and skeletal ossification (USDA, 1996). This system places the highest value on young carcasses with the most marbling, and assigns value to carcasses to help consumers decide what beef to purchase.

Beyond the USDA quality assessments, meat quality is also based on the actual eating experience; tenderness, flavor, juiciness and also as aroma and color that the consumer experiences. Savell et al. (1987 and 1989) determined that tenderness is the most important palatability factor influencing consumer satisfaction. Therefore, a great deal of research has been conducted to objectively determine beef tenderness (Destefanis et al., 2008, Honikel, 1998, Huffman et al., 1996, Guillemin et al., 2011, Picard et al., 2014, Savell et al 1987, Savell et al., 1989, Zapata et al., 2009) and to determine how tenderness can be improved. Two common ways to measure tenderness include trained sensory panels and Warner-Bratzler shear force (WBSF). Warner-Bratzler shear force is a mechanical test that measures the force to shear through a core of cooked meat against the grain of the muscle fibers, which is essentially the measure of the least tender bite a consumer can take (Honikel, 1998). Warner-Bratzler shear force is widely used and has been shown to correlate well with consumer ability to determine tenderness (Destefanis et al., 2008, Huffman et al., 1996). Tenderness in beef is highly dependent on postmortem aging of the carcass (Hoagland et al., 1917). The aging process consists of storage of meat, either as a whole carcass or in primal cuts, in refrigerated conditions to allow for increased tenderness through protein degradation. Gruber et al. (2006) demonstrated

the improvement in tenderness due to postmortem aging when the tenderness of 17 different beef muscles over an aging period of 28 d was evaluated and it was found that in carcasses grading USDA Choice, all of the evaluated muscles with the exception of the teres major improved in tenderness a minimum of 1.0 kg of force from 2 d to d 28 of aging.

Bioelectrical Impedance Analysis

Another possibility for a physical method of determining meat quality is that of bioelectrical impedance analysis. Bioelectrical impedance analysis (BIA) is a measurement of impedance (Z) through tissue. Impedance is based on resistance (R), reactance (X), and phase angle (Pa), and is a characteristic of alternating current. As current moves through the body, cellular membranes work as capacitors, fats respond as insulators, and other factors such as extracellular fluids and electrolytes also impact the flow of current (Swantek et al., 1992). Bioelectrical impedance has been utilized with success in humans as a means to determine percent body fat (Lukaski et al., 1985), and has been demonstrated to be more precise than body mass index when determining composition (Roubenoff et al., 1995). The same concept has been applied to the whole carcasses of swine (Swantek et al., 1992), lambs (Berg and Marchello. 1994), beef (Marchello and Slanger, 1994), as well as primal beef cuts (Slanger and Marchello, 1994) and even ground beef and pork (Marchello et al., 1999) to determine fat content. In the conversion of muscle to meat, muscle cell membranes may fragment (Pearce et al., 2011) and allow current to pass through cells without any capacitance effects (Yang et al., 2013). Because not all carcasses age at the same rate, and not all animals have the same maximum tenderness, a method to predict tenderness such as bioelectrical impedance would be beneficial to the industry by way of reducing aging periods of already tender carcasses, and identifying carcasses that will yield more tender cuts. Lepetit and Hamel (1998) found that measuring fiber resistance on one (r

= 0.8) or two (r = 0.9) days postmortem accurately allows for prediction of proper aging period and maximum tenderness. While most research concerning bioelectrical impedance and meat is focused on whole-carcass analysis to determine percent lean, the use of this technology on retail cuts to determine quality such as fat content in the form of marbling, and time spent aging has not been documented in the literature.

Calpain and Calpastatin

Aging of meat has long been known to improve tenderness. Hoagland et al. (1917) hypothesized proteolysis was occurring during aging and was a component to tenderness. First described in relation to myofibrillar integrity by Cheng and Parrish (1977), the calcium-dependent protease (calpain) system, then known as calcium activated factor (CAF), has been identified as a major component in postmortem tenderization (Koohmaraie, 1992a, Koohmaraie, 1992b). In the calpain system, the first two enzymes to be purified and described were calpain 2 (m-calpain) and calpain 1 (μ-calpain) (Dayton et al., 1976, Kishimoto et al., 1981, Yoshimura et al., 1983). Calpain is active postmortem in refrigerated temperatures, and is pH sensitive (Koohmaraie, 1992b). To achieve the most tender meat, an intermediate rate of pH decline to an ultimate pH of approximately 5.5 to 6.0 is required (Pike et al., 1993). A rapid pH decline, or a minimal decline from lack of glycogen will negatively impact tenderness, as well as flavor and color (Hwang and Thompson, 2001). Compounding the circumstances of calpain activity, calpain autolyzes in the presence of Ca^{2+} creating products with reduced proteolytic ability (Edmunds et al., 1991, Boehm et al., 1998). Calpastatin is an endogenous calcium dependent protease inhibitor that inhibits calpain to prevent tissue damage from excessive proteolysis in the living animal. Both calpain and calpastatin are essential for growth and development, yet counteract each other (Murachi, 1983), sometimes with inverse amounts of activity (Barnoy et

al., 1996). Like calpain, calpastatin activity also decreases with increased time postmortem, approximately a 40% reduction at d 1 postmortem (Boehm et al., 1998) and has shown to be a substrate for calpain. Additionally, it has been demonstrated that animals with more calpastatin activity postmortem have higher WBSF values (O'Connor et al., 1997).

Protein Markers for Tenderness

The next logical step after recognizing protease activity is using protein analysis to objectively determine tenderness qualities. A number of different protein markers that are utilized to predict tenderness because their presence or integrity is correlated to protein degradation, and in turn, tenderness measurements such as WBSF values (Guillemin et al., 2011, Zapata et al., 2009). Troponin-T (TnT) is a commonly used marker for protein degradation in skeletal muscle. When the TnT protein degrades under proteolysis, a 30 kDa polypeptide is released as a product, a phenomena that led Cheng and Parrish (1977) to hypothesize that CAF had a role to play in tenderness. The accumulation of this 30 kDa product increases over time spent aging (Ho, et al., 1994) and correlates to WBSF values (Koohmaraie et al., 1984). This degradation of TnT in postmortem muscle is comparable to the degradation of skeletal muscle proteins by purified calpain 1 at low temperatures and pH (Huff-Lonergan et al., 1996), indicating that the primary proteinase at play in meat tenderization is calpain 1. Geesink et al. (2006) supported this with calpain 1 knockout mice where casein zymography was used to confirm the absence of calpain 1 and western blots showed a highly significant ($P < 0.005$) decrease in postmortem TnT degradation, as well as in other proteins.

Other proteins that are not directly tied to the caplain system are also utilized as markers for protein degradation postmortem. Heat shock protein 70 (HSP 70) is a protein that has been used to predict tenderness, as well as monitor animal welfare. There are various heat shock

proteins, with many functions, such as protein folding and translocation of polypeptides across the membrane of cells (De Maio, 1999). However, HSP 70 is most commonly associated with stress, and its expression has been shown to correlate well with chronic stress (Turner et al., 2013, Valros et al., 2013). Alternatively, short term stress, such as transportation, may increase HSP 70 expression in some tissues (Zhang et al., 2011). Some tissues are more expressive of HSP 70 than others as a result of stress, but brain, heart, lung, kidney, and muscle have been shown to exhibit some HSP 70 response due to transportation stress (Shim et al., 2009). In addition to stress, the amount of HSP 70 present can be considered as a marker for tenderness in beef cattle (Picard et al., 2014), or more accurately, toughness markers (Guillemin et al., 2012). Carvalho et al. (2014) demonstrated this correlation with work in Nellore cattle, where animals with low WBSF values had lower expressions of HSP 70 in skeletal muscle than animals with high expression of HSP 70, an overall 16.6% difference in HSP 70.

Introduction to Growth-Promoting Technologies

As world population increases, there is a correlating increase in demand for high quality proteins in the form of meats (Godfray et al., 2010). The production of livestock in the past century has already undergone massive changes in method and scale, but improvements are still needed to supply a rapidly increasing human population with enough food. Due to the fact that land and resources devoted to agriculture are at best static, and at worst, shrinking, efficiency with our current resources must be maximized in order to provide increased production and sustainability (Capper and Hayes, 2012).

Energy partitioning in the livestock industry is studied as a way to reduce waste and improve efficiency in relation to growth and development of animals. Obviously, modifying current methods of raising livestock to create more product with the same or less inputs is

desirable. β-Adrenergic agonists (BAAs) are a relatively recent breakthrough and represent a possible way to control energy partitioning. β-Adrenergic agonists at the functional level vary widely in what they influence, but are known to play a role in energy, and metabolism of lipids, carbohydrates, and proteins (Yang and McElligott, 1989). Additionally, anabolic implants are commonly used in conjunction with BAAs to improve gains and efficiency, and implanted cattle represent a large portion of beef slaughtered in the US (Bartle et al., 1992, Duckett and Andrae, 2001, Scheffler et al., 2003).

Mechanisms of β-Adrenergic Agonists

Chemically, BAAs are organic molecules that bind to β-adrenergic receptors on the surface of nearly all mammalian cells. When bound to the receptor, the BAA activates the $G_{s\alpha}$ protein, which in turn activates adenylyl cyclase, which produces cyclic adenosine monophosphate (cAMP). Cyclic adenosine monophosphate is an intracellular signaling molecule that controls the catalysis for phosphorylation of intracellular proteins. Some of these proteins are enzymes, which may activate or deactivate with phosphorylation (Mersman, 1998), such as acetyl-CoA carboxylase, which is inactive under phosphorylation, and is responsible for long-chain fatty acid biosynthesis (Browsnsey et al., 1997). Another result of cAMP production is increased transcription of some genes in the cell due to the cAMP response binding element (CREB) (Mersman, 1998). Because of the diversity of synthetic BAAs, the possible applications and true effects for many of these compounds are yet unknown and their use is debated.

Under natural conditions however, the physiological BAAs produced by the animal are norepinephrine and epinephrine (Mersmann, 1998). These two hormones carry out various signaling roles in the sympathetic nervous system. When either of these signaling molecules are bound to an adrenergic receptor, a response will be issued pending the type of receptor. The first

differentiation of receptors was noted by Ahlquist (1948), in which alpha (α) receptors were noted as issuing responses opposite to beta (β) receptors. For example, α receptors were associated with vasoconstriction and stimulation of the uterus, both excitatory responses, while β receptors were associated with inhibitory responses such as vasodilation and inhibition of uterine musculature. Over time the adrenergic system has continued to be explored and been discovered to contain multiple subtypes for both α- and β-adrenoreceptors. Of importance, skeletal muscle primarily relies on the β-adrenoreceptor group for signals to grow via hypertrophy (Yang and McElligott, 1989).

Emery et al. (1984) demonstrated that BAAs have some ability to increase muscle mass and decrease body fat. These findings opened up a new field of BAA work apart from the established use of BAAs as airway dilators to treat conditions such as asthma (Fernandes et al., 2004), and allowed for the development and use of BAAs in livestock to improve feed efficiency and growth.

Efficiency in Feeding β-Adrenergic Agonists

In animals fed BAAs, there are variations in performance on feed. Typically, changes in live body weight of animals fed BAAs compared to control groups do not occur (He et al., 1993, Higgins et al., 1988, Miller et al., 1988, Morgan et al., 1989, Reeds et al., 1986, Walker et al., 2010, Wheeler and Koohmaraie, 1992). However, Scramlin, et al. (2010) showed that RAC and ZH do increase body weight in steers in the last 33 d of feeding by 7.3 kg and 3.1 kg, respectively. While body weight changes may be often insignificant, feed efficiency is much more pronounced. Increased gain to feed ratios have been widely reported in cattle and swine. He et al. (1993) found that with the inclusion of ractopamine in pig diets that feed:gain decreased from 3.44 to 3.14. Miller, et al. (1988), found that heifers feed:gain decreased, from 12.9 to 8.29,

with the use of clenbuterol. In steers, gain:feed improved from a control of 0.107 to 0.131 and 0.128 with ractopamine and ZH respectively (Scramlin et al., 2010). Wheeler and Koohmaraie (1992) recorded improved gain:feed ratios over 6 weeks in steers fed L-$_{644,969}$. However, Ricks et al. (1984) found the feeding of clenbuterol (CLN) depressed daily feed efficiency during a large portion of the study, along with reporting a lower average daily gain (ADG) and lower average daily feed consumption. This may have been a result of overfeeding CLN as one treatment group was receiving 50 times the recommended dosage. Other work has shown insignificant change in rate of gain (Miller et al., 1988, Reeds et al., 1986, Walker et al., 2010, Wheeler and Koohmaraie, 1992). Reeds et al. (1986) found that CLN given to rats did not change intake, and would only result in improvements in final weight with mild dosage. At 10 mg/kg and 50 mg/kg inclusions in the diet, clenbuterol yielded final body weights that were no better than the control. Walker et al. (2010) fed ractopamine to steers and heifers at 200 mg/d and found no differences in gain:feed or ADG. The differences in reported outcomes may be the result of different BAAs, or other variables such as diet, environment, or genetics. However, the findings of increased gains on the same amount of feed is economically advantageous to producers and merits further consideration.

β-Adrenergic Agonist's Impact on Adiposity

The role of fat tissue in livestock is complex. In certain roles, fat has value, however, it is generally considered to represent waste and an inefficient use of nutrients by the animal. For that reason, it is highly desirable to shift an animal's energy from depositing fat to building muscle (Miller et al., 1988). Research has been conducted to compare the amount of fat produced by animals on traditional diets to those that have been administered BAAs. Miller et al. (1988) found that beef heifers fed CLN had nearly identical live and carcass weights compared to the

control group, but yielded carcasses with less kidney-pelvic-heart (KPH) fat and lower 12th rib adjusted fat thicknesses. At the cellular level, the concentrations of adipose cells were increased in heifers given CLN while the adipocyte diameter and volume had both decreased. The explanation for this change in adiposity was found in a depression of lipogenic enzyme activity and a significant decrease in fatty acid-binding protein activity. In another study, inclusion of CLN in the diet was found to improve USDA yield grade by 1.0 point on average, and these animals were found to have at minimum, 13% more protein and 20% less fat compared to their control group counterparts (Ricks et al., 1984). This work indicated that CLN partitions energy away from fat deposits, instead using the energy to increase muscle hypertrophy as evidenced by an increased muscle fiber diameter as well as a greater LD muscle weight.

These findings are not entirely supported by Wheeler and Koohmaraie (1992) when investigating L-$_{644,969}$, a BAA, in beef steers. While increased muscle area and lower yield grades were noted, adjusted fat thickness and KPH did not differ among treatments. However, a strong effect has been observed in hogs fed RAC. Hogs fed RAC had greater lean and less fat, primarily subcutaneous fat, on a percentage basis when compared to hogs on the same diet excluding RAC (He et al., 1992). This is a promising result because subcutaneous fat is typically heavily trimmed and represents a significant portion of waste. A similar reduction in subcutaneous adiposity was recorded in steers fed CLN. At the 12th rib, external fat depth was decreased by up to 42% (Ricks et al., 1984). A body fat reduction was observed in broiler chickens fed the BAA cimaterol, including a reduction in leg muscle fat (Morgan et al., 1989). Cattle fed ZH or RAC had increased hot carcass weights (HCW) as well as decreased total fat compared to control carcasses, with ZH having the most pronounced effect (Scramlin et al., 2010, Wheeler and Koohmaraie, 1992,). This phenomena has been well documented and is

suggested to be the result of repartitioning of energy away from fat in areas that are not included in the carcass in addition to the well-known decrease in adiposity on the carcass. The weighing of hides has revealed that those origination from ZH cattle tend to weigh less than those from RAC or control animals, indicating a reduction in fat content on and within the hide (Scramlin et al., 2010).

While typically fat is a negative aspect on a carcass, intramuscular fat, or marbling, is a positive aspect and has a large economic role due to the well-established correlation between marbling and palatability, particularly in beef cattle (Wheeler et al., 1994). If a BAA were to diminish adiposity within the muscle itself, this would be a severe cost that could outweigh the decreased subcutaneous fat and increased muscle mass. Wheeler and Koohmaraie (1992) found the BAA L-$_{644,969}$ had no impact on marbling scores when fed to cattle. Scramlin et al. (2009) also reported no differences in marbling between control, RAC-fed, or ZH-fed cattle. However, Arp et al. (2012) found that levels of ZH in the diet higher than a 200 mg dosage per head per day resulted in a decrease in marbling score from 429.01 to 407.50 on average, which decreased the number of carcasses in the upper 2/3 of the Choice USDA quality grade. The use of CLN has shown no impact on marbling by Ricks et al. (1984), however this was contradicted by Miller et al. (1988) where heifers on CLN were found, on average, to have a lower marbling score (slight^{90} compared to traces^{80}). The variation in reports is possibly due to different dosages and different BAAs. However, the trend appears to be that intramuscular fat is less affected by BAAs when compared to the effects on subcutaneous or KPH fat.

As previously mentioned, BAAs are incredibly diverse and not all share the same physiological impact. However, in most studies, BAAs are associated with reduction of body fat and increased energy expenditure, which is thought to be the result of BAAs binding to adipose

tissue, resulting in the β-receptors activating adenylate cyclase, cyclic AMP levels, the protein kinase cascade, and eventually the activation of lipase resulting in triacylglycerol hydrolysis (Yang and McElligott, 1989). These effects have been successfully simulated *in vitro* (P. Mauriège et al., 1988).

Protein Turnover and Accretion

It has been established that muscle proteins are constantly degrading and being replaced, in addition to new growth. It is estimated that 15-22% of total energy use of an animal is in this muscle protein turnover (Reeds et al., 1985). One of the most promising ways to impact energy partitioning in relation to this turnover during growth appears to be the use of BAAs. This component of the energy partitioning effects of BAAs in the context of meat animal production has a synergistic relationship with the decrease in adiposity.

As BAAs promote growth via muscular hypertrophy, an increase in protein density is expected. Ricks et al. (1984) found lower blood urea N levels in animals fed CLN, suggesting increased N retention and increased protein construction, which was similar to a trend reported by Walker et al. (2009). By utilizing measurements of urinary N content of cattle on L-$_{644,969}$ fractional degradation rate of myofibrillar proteins was found to be lower and neither muscle protein synthesis figures nor fractional accretion rates were greater than control animals. However, when fractional synthesis rate was calculated it was found to not differ between BAA and control animals. This strongly points to the conclusion that the primary mechanism of muscle hypertrophy in BAA-fed animals is one of decreased degradation, not that of increased synthesis. This was confirmed by Wheeler and Koohmaraie (1992) who determined that steers fed L-$_{644,969}$ had lower muscle protein degradation, higher muscle protein accretion, yet no difference compared to the control group in muscle protein synthesis by studying urinary N

content. This data indicates a significant increase in hypertrophy without a change in how much new protein is actually being synthesized. Once the calpain proteolytic systems in these animals was evaluated it was discovered that although calpain 1 and 2 activities did not differ among treatments, calpastatin, the direct inhibitor of calpains, activity was strongly increased (348%) as a result of feeding the BAA (Wheeler and Koohmaraie, 1992). This data suggests that protein accretion for many BAAs is a result of a decrease in protein turnover by inhibition of calpains, not a result of increased protein synthesis rate. This is supported by work with sheep fed CLN (Higgins et al., 1988). As expected, there was a significant increase in LD weight and cross sectional area, along with an increase in protein content. Calpastatin activity was increased in the BAA-fed lambs from 68 units/g extractable protein to 115 units/g extractable protein, along with calpain 2, which increased from 30 units/g extractable protein up to 61 units/g extractable protein (Higgins et al., 1988). This increase in calpain 2 is in contrast to the work done by Wheeler and Koohmaraie (1992) and is likely the result of either a species difference or a reflection of the variability of BAA form and function. Investigation into CLN's effect in rats further supports the hypothesis of decreased protein degradation leading to hypertrophy. Reeds et al. (1986) reported that over time there was no increase in protein synthesis in skeletal muscle of rats, regardless if expressed as a fractional rate or against total RNA. The researchers came to the conclusion that CLN greatly reduced the rate of muscle protein degradation. Supporting evidence has also been observed in chickens fed cimaterol. In these poultry, there was no significant difference in fractional protein synthesis rate between treatments, as well as no differences in DNA concentration, indicating that muscle growth was not a result of satellite cell division (Morgan et al., 1989).

Tenderness with β-Adrenergic Agonists

The data showing an increase in muscle protein due to inhibition of calpain activity is strongly supported by measurements of tenderness due to the role of calpain activity in postmortem tenderization of muscle. In cattle fed RAC and ZH, there is an increase in Warner-Bratzler Shear Force (WBSF) values as well as scoring lower in a trained sensory panel for tenderness (Arp et al., 2013). The similar results for the WBSF and sensory panel are important as they demonstrate that the consumer can detect changes in tenderness that are reflected in a WBSF. A similar increase in WBSF values has been reported in animals fed CLN (Miller et al., 1988). Wheeler and Koohmaraie (1992) found that while control carcasses had an expected decline in WBSF values over the aging period, the $L_{644,969}$-fed animals did not yield any decrease in WBSF values from 1 to 14 d postmortem. When Scramlin et al. (2010) evaluated WBSF values of cattle fed a control, RAC, or ZH, it was found that values for all treatments decreased over aging as is expected, yet ZH and RAC were significantly less tender at d 3 when compared against the control. By aging d 14, RAC and the control were equal, but ZH was still yielding significantly higher WBSF values and it was not until d 21 that the values for ZH were the same as the control values from d 3. This work suggests that RAC and ZH differ in the magnitude of their effects. In poultry fed cimaterol, WBSF values have been reported as much as 39.0% higher than control birds, suggesting a significant reduction in tenderness as a result of the inclusion of BAAs in the diet (Morgan et al., 1989). The significance of decreased tenderness as a result of utilizing BAAs was well put by Arp et al. (2013), “Beta-agonists may be a necessary tool to improve productivity of the beef industry, but these compounds likely achieve this at the expense of eating satisfaction”.

Implant Strategies

Implants are a commonly used strategy in beef production. These implants are frequently used in the finishing stage to promote growth and efficiency (Duckett et al., 1999, Johnson, et al., 1996, Roeber et al., 2000). First approved by the FDA in 1991, there was rapid industry acceptance and by 1999 more than 96% of all feedlot cattle were implanted (Scheffler et al., 2003) due to improved ADG, up to 20% with certain implants and strategies (Bartle et al., 1992, Duckett and Andrae, 2001), which helps combat high feed costs. There are two main hormones administered through these implants, either alone, or in combination: estradiol (E_2) and trenbolone acetate (TBA). TBA is a steroid agonist with up to a 5 times greater affinity for the androgen receptor than testosterone, a naturally occurring hormone in all cattle (Bauer et al., 2000, Ankley et al., 2003). E_2 is known to increase overall growth hormone (GH) in various species (Grigsby and Trenkle, 1986). When both E_2 and TBA are combined and implanted the result is substantial improvements in growth and gains by way of increased insulin-like growth factor I (IGF-I), which may in turn increase muscle growth (Johnson et al., 1996b). The combination of these two hormones results in superior animal performance, which is reflected in the amount and variety approved and sold in the US (Table 1).

However, the benefits of implanting are accompanied by some draw backs, primarily a decrease in marbling, as well as more advanced skeletal maturity (Bartle et al., 1992, Duckett and Andrae, 2001, Duckett et al., 1999, Scheffler et al., 2003, Roeber et al., 2000). Often, the improved rate of gains and HCW (Bartle et al., 1992, Thompson et al., 2008) make up for the discounts that lower marbling scores bring, but tenderness may decrease with use of anabolic implants. Roeber et al. (2000) reported trends of increasing WBSF values with implant use. Scheffler et al. (2003) found significantly increased WBSF values in only the most aggressive

Table 1.1. Anabolic implants approved for use in finishing beef cattle.[1]

Brand name	Hormone Content
Estrogenic (E)	
Component E-S with Tylan[2]	20 mg estradiol benzoate + 200 mg progesterone + tylosin
Compudose 200[2]	25.7 mg estradiol
Encore (Compudose 400)[2]	43.9 mg estradiol
Magnum[3]	72 mg estradiol
Ralgro[3]	36 mg estradiol
Synovex-S[4]	20 mg estradiol benzoate + 200 mg progesterone
Androgenic (A)	
Component T-H with Tylan[2]	200 mg TBA[5] + tylosin
Component T-S with Tylan[2]	200 mg TBA + tylosin
Finaplix-H[3]	200 mg TBA
Finaplix-S[3]	140 mg TBA
Combination (C)	
Component E-H with Tylan[2]	20 mg estradiol benzoate + 200 mg testosterone propionate + tylosin
Component TE-G[2]	8 mg estradiol + 40 mg TBA
Component TE-G with Tylan[2]	Component TE-G + tylosin
Component TE-IH with Tylan[2]	8 mg estradiol + 80 mg TBA + tylosin
Component TE-IS[2]	16 mg estradiol + 80 mg TBA
Component TE-IS with Tylan[2]	Component TE-IS + tylosin
Component TE-S[2]	24 mg estradiol + 120 mg TBA
Component TE-S with Tylan[2]	Component TE-S + tylosin
Component TE-200[2]	20 mg estradiol + 200 mg TBA
Component TE-200 with Tylan[2]	Component TE-200 + tylosin
Revalor-G[3]	4 mg estradiol + 40 mg TBA
Revalor-H[3]	14 mg estradiol + 140 mg TBA
Revalor-IH[3]	8 mg estradiol + 80 mg TBA
Revalor-IS[3]	16 mg estradiol + 80 mg TBA
Revalor-S[3]	24 mg estradiol + 120 mg TBA
Revalor-XS[3]	40 mg estradiol + 200 mg TBA
Revalor-200[3]	20 mg estradiol + 200 mg TBA
Synovex-Choice[4]	14 mg estradiol benzoate + 100 mg TBA
Synovex-H[4]	20 mg estradiol benzoate + 200 mg testosterone propionate
Synovex-Plus[4]	28 mg estradiol benzoate + 200 mg TBA
Synovex-T120[4]	20 mg estradiol + 120 mg TBA
Synovex-T40[4]	8 mg estradiol + 40 mg TBA
Synovex-T80[4]	16 mg estradiol + 80 mg TBA

[1] FDA, 2013. Adapted from Duckett and Pratt, 2014. J. Anim. Sci. 92:3-9.
[2] Elanco Animal Health, Greenfield, IN.
[3] Merck Animal Health, Summit, NJ.
[4] Zoetis, Inc., Florham Park, NJ.
[5] TBA = trenbolone acetate

implanting strategies. Other workers have noted differences in shear force early in the aging period (5 d), but no differences after a longer period of aging (21 d) (Johnson et al., 2008).

These differences in shear force are reflected in work done by Igo et al. (2011), where WBSF showed no differences, but slice shear force (SSF) did reveal differences in some implant treatments, but once again, these differences lost significance after a longer aging period. A consumer acceptance panel confirmed these measurements were detectable by the consumer. Roeber et al. (2000) also found that consumers rated implanted beef as being less tender than the control. Because consumers place value on tenderness, the benefits of implanting, such as increased HCW and ADG, must be weighed against possible decreases in tenderness and marbling.

Growth Promotant Conclusions

BAAs and anabolic implants are a possible ways to create better efficiency in livestock and modify traits in meat animals to create economic benefits. However, these benefits may come with costs such as lower marbling scores and decreased tenderness depending on growth promotants selected and dosage used. In the US there are many various implants available, but currently only two BAAs are approved for use in food animals by the FDA: ZH and RAC. More research of BAAs and anabolic implants and their mechanisms could reveal alternative compounds and shed more light on the true usefulness and possible roles of growth promoting technologies in livestock production.

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CHAPTER 2. EFFECTS OF IMPLANTING STRATEGY AND ZILPATEROL HYDROCHLORIDE ON THE CALPAIN PROTEOLYTIC SYSTEM IN SECTIONED BEEF STEAKS AGED FOR TWO TIME PERIODS

Introduction

Beta-adrenergic agonists and anabolic implants are growth promoting technologies commonly used in beef production. In 1991 implants with a combination of TBA and E_2 were approved by the FDA, and in 1996 more than 96% of all feedlot cattle were implanted (Scheffler et al., 2003). However, implanting has been shown to reduce beef tenderness (Roeber et al. 2000, Johnson et al. 2008, Scheffler et al., 2003). Likewise, the two FDA-approved beta-adrenergic agonists, zilpaterol hydrochloride (ZH) and ractopamine (RAC) may also cause a decrease in tenderness (Arp et al. 2013, Scramlin et al. 2010). When beta-adrenergic agonists are utilized in conjunction with implants, the combination yields less tender meat than just implanting at d 14 of postmortem aging, and the areas of the longissimus dorsi (LD) cross-sections exhibit unique tenderness characteristics (Ebarb et al., 2016). The mechanism behind these changes in tenderness when these technologies are used in combination is not well established, therefore, a primary objective of this study was to identify if postmortem protein degradation and changes in activation of the calpain system is the reason for decreased tenderness in beef cattle fed ZH and implanted. The hypothesis for this study was that ZH and anabolic implants have an effect on the calpain proteolytic system in finishing beef heifers.

Materials and Methods

Experimental Design

Crossbred heifers (n = 15; initial BW 464±6 kg) were blocked by weight and randomly assigned to one of three treatments: 1) no implant and no ZH (CON), 2) implant, no ZH (IMP),

and 3) implant and ZH (IMP+ZH). Ten days prior to the initiation of the study, to allow the animals to acclimate to their new environment, cattle were moved into individual pens located in an enclosed barn at the Kansas State University Beef Cattle Research Center. Each pen was 4.64 m^2 and contained metal pipe sidewalls, slatted floors for waste removal, an individual waterer, and a 75 × 51 cm feed bunk. Animals were fed a similar diet (Table 2.1) and feed was delivered once daily to allow *ad libitum* access to feed. Bunks were managed to leave a minimum amount of unconsumed feed daily. On d 0, heifers (IMP and IMP+ZH) were given an implant containing 200 mg trenbolone acetate and 20 mg estradiol (Component TE-200; Elanco Animal Health, Greenfield, IN). On d 50, 8.3 ppm zilpaterol hydrochloride (ZH; Zilmax, Merk Animal Health) was included in the finishing ration (IMP + ZH) for 21 d with a 3-d withdrawal prior to slaughter. The mean final body weight for the CON heifers was 530.9 kg, 541.8 kg for the IMP heifers, and 541.9 kg for the ZH heifers. Final body weight did not significantly different by

Table 2.1. Diet percentages (DM basis) for crossbred heifers subjected to two exogenous growth promoting programs

	Treatments		
Ingredient, %	Control	Implant[1]	Implant/Zilpaterol[2]
Steam-flaked corn	53.39	53.39	53.39
Corn gluten feed	35.00	35.00	35.00
Ground alfalfa hay	4.00	4.00	4.00
Ground wheat straw	3.00	3.00	3.00
Vitamin/mineral supplement[3]	2.25	2.25	2.25
Feed additive premix[4]	2.16	2.16	2.16

[1]Heifers were implanted with Component TE-200 on d 0 of feeding.
[2]Heifers were implanted with Component TE-200 on d 0 of feeding and supplemented with 8.3 ppm of zilpaterol for the last 20 d of feeding, followed by a 3-d withdrawal period.
[3]Formulated to provide 0.7% calcium, 0.7% potassium, 0.3% salt, 0.1 ppm cobalt, 10 ppm copper, 60 ppm manganese, 0.3 ppm selenium, 60 ppm zinc, 2,200 KIU/kg vitamin A, and 22 IU/kg vitamin E on a DM basis.
[4]Formulated to provide 300 mg/day monensin and 90 mg/day tylosin (Elanco Animal Health, Greenfield, IN) per animal in a ground corn carrier.

treatment ($P < 0.82$). Heifers were shipped 430 km to be harvested in a commercial abattoir (Tyson Fresh Meats, Holcomb, KS) on d 75. Following a 36-hr chill period, strip loins (Institutional Meat Purchase Specifications 180) were removed from one side of the carcass from each heifer and were transported back to the Kansas State University Meats Laboratory for further fabrication.

Collection of Muscle Samples and Preparation

Strip loins that had aged for 3 and 14 d at 4°C were cut into steaks, frozen, and shipped on dry ice overnight from Kansas State University (Manhattan, KS) to the North Dakota State University muscle biology laboratory (Fargo, ND). Steaks were thawed overnight at 4°C. After performing bioelectrical impedance analysis (BIA) and objective color, samples were then cut into lateral, lateral/medial, and medial sections and immediately underwent protein extraction for analysis.

Color and pH

Before sectioning of steaks, objective color (L*, a*, and b* values) was determined with a portable colorimeter (CR-410 Chroma Meter, Konica Minolta, NJ, USA) using illuminant D64, a 5 cm aperture, and a 2° standard observer. Two readings were taken directly on the face of each steak after a 15 minute bloom period. pH was measured using an IQ150 pH meter with a PH57-SS non-glass probe (IQ Scientific Instruments, Carlsbad, CA, USA) calibrated to pH range 4-7. The pH was measured with the steak wrapped around the probe to avoid damage to the steak that could possibly effect bioelectrical impedance analysis.

Bioelectrical Impedance Analysis

Bioelectrical Impedance Analysis (BIA) was performed on the whole strip steak prior to sectioning using an EFresh® Analyzer (CellMetRx Corp. Harrisville, MI). The analyzer was connected to a tetra-polar probe array with stainless steel probes set to 0.635 cm in depth and spaced at a 1.27 cm, 5.08 cm, 1.27 cm, for an overall array length of 8.89 cm. The steak was laid out on a plastic tray and the probe array set into the steak so that the probes were parallel to the muscle fiber direction and two readings were taken on each steak.

Extraction of Protein

The sarcoplasmic fraction for heated calpastatin activity assays and casein zymography was extracted from steak samples that were collected and had been frozen at 3 d and 14 d of postmortem aging (Rowe et al., 2004, Melody et al., 2004). Five g of muscle tissue from each section of each steak was minced and then homogenized in 20 mL of extraction buffer [10 m*M* EDTA; 100 m*M* Tris; pH 8.3, 0.1% (vol/vol) 2-mercaptoethanol; 2 μ*M* E-64; 0.1 mg/mL trypsin inhibitor, 0.4 m*M* phenylmethylsulfonylfluoride] using a Polytron Kinematica (10/35 with controller and PTA 10S generator; Brinkmann, Westbury, NY) on wet ice until tissue is completely ground (3 to 5, 10-sec bursts). The homogenate was clarified by centrifugation (21,100 x *g*) at 4°C for 30 min (Allegra 25R centrifuge with TA-14-50 rotor, Beckman Coulter, Fullerton, CA). After centrifugation, the supernatant was filtered through cheesecloth seated into a plastic funnel positioned on top of a 25-mL glass graduated cylinder. The volume of the supernatant (sarcoplasmic fraction) was collected, recorded, and stored at -80°C until protein determination.

Whole muscle protein extraction for Western Blot analysis was also performed according to Huff-Lonergan et al. (1996). Approximately 5 g or muscle tissue was placed in a glass tissue

grinder (Potter-Elvegjem) with 5 mL of whole muscle extraction buffer and homogenized with a serrated pestle attached to a mechanical homogenizer (Eberbach, Ann Arbor, MI) until well ground. The sample and buffer was transferred into a 15 mL conical tube and centrifuged (1500 x *g*) for 15 m at 22°C (Allegra 25R centrifuge with TA-14-50 rotor, Beckman Coulter, Fullerton, CA). After centrifugation, protein concentration was determined for each cleared sample using the BioRad DC Protein Assay Kit (Lowry) and following the DC Protein Assay [Lowry method] (Mircroplate Method) instructions.

Casein Zymography

To prepare protein gel samples, sarcoplasmic protein samples (stored at -80°C) and Sample Tracking Dye [187.5 mM Tris, pH 6.8; 25% (vol/vol) glycerol; 0.125 mg/mL bromophenol blue] containing 0.80% (vol/vol) MCE were calculated to allow for loading of 250 µg of protein into each lane using a ratio of 60:40 sample to tracking dye. Purified in-house calpain 1 and calpain 2 from beef lung (Maddock et al., 2005) were run on each gel as a standard references. Samples for casein zymography were run on 1.5 mm separating gels containing 11% acrylamide [375 mM Tris, pH 8.8; 2.1 mg/mL casein; 0.05% (wt/vol) AMPER; 0.05% (vol/vol) TEMED] and 4% acrylamide stacking gels [0.125 M Tris, pH 6.8; 0.075% (wt/vol) AMPER; 0.125% (vol/vol) TEMED] with 100:1 acrylamide:bis cross-linking in a running buffer [25 mM Tris, pH 8.3; 192 mM glycine; 1 mM EDTA; 0.1% (vol/vol) MCE] (Melody et al., 2004; Raser, et al., 1995). Electrophoresis was carried out on a Hoefer SE 268 unit with cooling at 4°C (Hoefer, Inc., Hollison, MA). Gels were pre-run for 20 min at 100 V, loaded with 250 µg of protein per lane, and run overnight at 65 V until sample tracking dye ran off (14 to 18 h total electrophoresis time). Gels were then removed from glass sandwiches and incubated in 100 mL of Tris-Casein Gel Incubation Buffer [50 mM Tris; 5 mM CaCl2, pH 7.5; 0.1% (vol/vol) MCE]

for 20 min. The buffer was decanted and the washing step was repeated 2 additional times before replacing with fresh Tris-Casein Gel Incubation Buffer and incubated overnight at room temperature with rocking. The next morning, the buffer was decanted from the gels and replaced with R-250 Coomassie Blue Gel Stain [0.1% (wt/vol) coomassie blue, 45% (vol/vol) methanol, 10% (vol/vol) glacial acetic acid]. The gels were allowed to stain for 1 h at room temperature with rocking after decanting and replacing with Coomassies Blue Destain Solution [10% (vol/vol) methanol, 10% (vol/vol) glacial acetic acid in water] for 30 min. This destain step was repeated 2 additional times, destain was replaced and continued for approximately 3 h. The gels were then rinsed with water and then photodocumented on a FluorChem Imager using a F2.8 28-70 mm zoom lens camera (Alpha Innotech Corp.). Spot clearing densities were analyzed with AlphaEaseFC software (Alpha Innotech Corp.) using 1-D multi with rolling disc baseline.

SDS-PAGE Electrophoresis

Samples in sample buffer/tracking dye for troponin-T (TnT) and heat shock protein 70 (HSP 70) were run on 1.5 mm thick, 15% acrylamide separating gels (acrylamide for Tnt: N, N'-bis-methylene acrylamide = 37.5:1 [wt/wt], acrylamide for HSP 70: N, N'-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.05% (TEMED), 0.05% [wt/vol] ammonium persulfate, and 0.37 M Tris, pH 8.8) with 5% acrylamide stacking gels [acrylamide: N, N'-bis-methylene acrylamide = 37.5:1 (wt/vol), 0.1% (wt/vol) SDS, 0.125% TEMED, 0.075% (wt/vol) ammonium persulfate, and 0.125 M Tris, pH 6.8] in a running buffer [25 mM Tris, 192 mM glycine, 2 mM EDTA, pH ≈ 8.3, 0.1% (wt/vol) SDS] (Melody et al., 2004). Electrophoresis was carried out on a BioRad Mini-PROTEAN Tetra Cell system (BioRad Laboratories, PA). Gels for TnT were loaded with 3.28 μg of protein per lane and run at a constant voltage of 140 V for

approximately 3 h. Gels for HSP 70 were loaded with 8 μg of protein per lane and run at a constant voltage of 120 for 2.5 h.

Transfer Conditions

Both TnT and HSP 70 proteins were transferred onto Millipore Immobilon-P polyvinylidene diflouride transfer membranes (Millipore Corporation, Bedford, MA) using a TE22 Might Small Transphor electrophoresis unit (Hoefer Scientific Instruments; Holliston, MA) at a constant voltage of 90 V and 0.27 mA for 1.5 h. in a buffer [25m*M* Tris, 1.9 *M* glycine, 0.017 *M* EDTA, pH ≈ 8.3, and 15% (vol/vol) methanol] maintained at 4˚C using a refrigerated circulating water bath.

Western Blotting

Post transfer, all membranes were blocked in PBS-Tween [80 mM disodium hydrogen orthophosphate, 100 mM sodium chloride, 0.1% (vol/vol) polyoxyethylene sorbitan monolaurate (Tween-20), and 5% (wt/vol) nonfat dry milk] for 1 h at room temperature (23˚C). After blocking, TnT membranes were placed in PBS-Tween and incubated overnight at 4˚C with the primary antibody (mouse monoclonal anti-rabbit troponin-T antibody, Catalog No. T6277; Sigma Chemical Co., St. Louis MO) diluted 1:40,000 in PBS-Tween. HSP 70 proteins were placed in PBS-Tween and incubated overnight at 4˚C with the primary antibody (mouse monoclonal anti-bovine heat shock protein 70, Catalog No. ab6535; Abcam, Cambridge UK) diluted 1:50,000 in PBS-Tween. After the overnight incubation, membranes were allowed to warm to room temperature for 20 min and washed 3 times (10 mL/wash) using PBS-Tween. TnT blots were then incubated 1 h at room temperature with the secondary antibody (goat anti-mouse conjugated with horseradish peroxidase, Catalog No. A2554; Sigma Chemical Co.) diluted at 1:100,000 in PBS-Tween, and HSP 70 blots were incubated 1 h at room temperature with the

secondary antibody (goat anti-mouse conjugated with horseradish peroxidase, Catalog No. A2554; Sigma Chemical Co.). Upon completion of secondary antibody incubation, all membranes were washed 3 times (10 mL/wash) with PBS-Tween at room temperature to achieve a total wash rinse time of 30 min before chemiluminescence detection (Melody et al., 2004). Chemiluminescence was initiated using premixed reagents (ECL Prime kit; Amersham Pharmacia Biotech, Pascataway, NJ). Chemiluminescence was detected using a F2.8 28-70 mm zoom lens camera (Alpha Innotech Corp., San Leandro, CA). Densitometry measurements were done using the AlphaEaseFC software (Alpha Innotech Corp.).

Heated Calpastatin Activity Assay

The activity of calpastatin was determined by using casein as a substrate as performed by Koohmaraie (1990) and Dayton et al. (1976). Sarcoplasmic portions of samples in 15 ml conical tubes were removed from storage at -80°C and allowed to thaw to approximately 4˚C in a refrigerator overnight. The samples were then dialyzed (Spectra/Por dialysis membrane; 3.3 mL/cm; MCWO 10,000; Spectrum 132-119; Rancho Dominguez, CA) in 40 times the volume of the samples 1X TE (1 m*M* EDTA, 40 m*M* Tris, pH 7.4) at 4˚C overnight. The samples were then placed in a 100˚C water bath for 15 minutes to denature calpains. After heating, the samples were placed into an ice bath for 15 minutes to cool, after which the samples were stirred to release proteins from the sides of the tubes and then centrifuged (2000 x g) for 30 minutes at 4˚C. The supernatant was filtered through cheesecloth into a 15 mL conical tube and could be stored safely at 4˚C. Glass borosilicate test tubes (13 x 100 mm) were then prepared for the assay. Tubes labeled as blanks contained 1 mL of 1X TE, 1 mL of assay media [100 m*M* Tris; 7 mg/mL Casein (Hammerstein grade, MP Biomedical, Catalog No. 101289), 1 m*M* NaN_3, 0.2% (vol/vol) 2-mercaptoethanol], and 100 μL of 100 m*M* $CaCl_2$. Tubes labeled as positive controls

contained 1 mL of 1X TE, 1 mL of assay media, 100 µL of 100 m*M* $CaCl_2$, and m-calpain (0.4 units/tube). The reaction mixture consisted of a serial dilution of the sample in 1X TE, 1 mL of assay media, 100 µL of 100 m*M* $CaCl_2$, and m-calpain (0.4 units/tube). Another series of tubes contained the sample, 1 mL of assay media, m-calpain (0.4 units/tube), and 100 µL of ETDA (ethylenediamine tetraacetic acid). The tubes were then mixed and allowed to incubate at room temperature (20 to 22˚C) for an hour, at which point 2 mL of 5% TCA (final concentration in the reaction mix is 2.5%) was added to stop the reaction. Tubes were centrifuged at 2000 x *g* for 20 minutes at 20˚C and then analyzed using a spectrophotometer set at a wavelength of 278 nm.

Statistics

Data were analyzed using the random statement of the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) with repeated measures for aging day. The experimental unit for this study was the individual animal. Interactions between day of aging, treatment, and location on the cross section of the LD were evaluated. Pairwise comparisons were made between least squares means when main effects or interactions were significant at $P \leq 0.05$.

Results and Discussion

Color and pH

Minolta L*, a*, b* values (Table 2.2) were not different ($P > 0.19$) between treatments. This result is contrasting with work by Hope-Jones et al. (2012) where L* values were higher and a* color values were lower in cattle fed ZH at both 24 h and 14 d of aging when compared to control groups. Hilton et al. (2009) found differences in a* and b*, but not L* in the strip steaks of beef steers fed ZH when measured 36 h after slaughter. The results of the current study may have differed due to the fact that heifers were used rather than steers, and that these other studies used larger sample sizes. The present results also showed no difference in pH between

treatments (P = 0.13), which is supported by Hilton et al. (2009), where no difference in LD muscle pH was found. Additionally, pH did not differ between steak samples d 3 or d 14 of postmortem aging (P = 0.27). Of interest are claims made in popular press that ZH can result in animal welfare issues (Thomson et al., 2015) that would possibly result in stress to the animal. Voisinet et al. (1997) linked stress in cattle to darker color of lean, which negatively impacts carcass values. The lack of difference in the color results (Table 2.2) indicate ZH cattle may not have differing strip loin color scores or an increase in percentage of dark cutters, which can be an indicator of stress.

Table 2.2. Treatment means of Minolta L*, a*, b* values and pH of beef strips steaks from heifers (n = 15) treated with growth promotant technologies during finishing.

	Treatment				
Item[1]	Control	Implant[2]	Implant/ZH[3]	SEM[4]	P - Value[5]
L*					
Day 3	37.76	37.43	37.87	1.20	0.873
Day 14	38.75	37.85	38.21	1.20	
a*[6]					
Day 3	18.84	17.65	18.51	0.67	0.194
Day 14	20.88	19.67	19.54	0.67	
b*					
Day 3	8.43	7.69	8.26	0.56	0.484
Day 14	9.10	8.49	8.65	0.56	
pH					
Day 3	5.49	5.72	5.55	0.15	0.137
Day 14	5.23	5.59	5.47	0.15	

[1]Minolta L*, a*, and b* taken on strips steaks after 15 min bloom time.
[2]Heifers were implanted with Component TE-200 on d 0 of feeding.
[3]ZH = zilpaterol hydrochloride. Heifers were implanted with Component TE-200 on d 0 of feeding and supplemented with 8.3 ppm of ZH for the last 20 d of feeding, followed by a 3-d withdrawal period.
[4]Standard error of mean.
[5]Treatment P – Value.
[6]Day of aging, $P < 0.05$.

Troponin-T

Troponin-T is a myofibrillar regulatory protein involved in muscle contraction. In living muscle, the troponin complex of proteins is attached to tropomyosin and regulates the binding of actin to myosin (Ebashi and Endo, 1968). Troponin-T is the protein that connects the troponin complex to tropomyosin. This protein is degraded during postmortem aging by the calpain proteolytic system and degrades into various products with distinct molecular weights (Koohmaraie, 1992). In the current study, six immunoreactive bands for TnT were analyzed from both 3 d and 14 d of aging, ranging from 40 kDa to 27 kDa. The amount of intact TnT, as well as the total degradation into these products is an indicator of overall protein breakdown and can be correlated to WBSF (Sun, et al., 2014, Ho, et al., 1994, Huff-Lonergan, et al., 1996). The present results, as seen in Table 2.3, showed significant day of aging effect for all bands ($P < 0.05$), a result that confirms that aging results in increased protein degradation and increased tenderness. For band 2, there was a treatment effect, indicating that that ZH and implanting had a negative impact on TnT degradation ($P < 0.05$). Band 6 had a location interaction ($P < 0.05$) within the LD cross section and band 3 had a day of aging x treatment x location interaction ($P < 0.05$) which shows a possible difference in postmortem proteolysis based on location.

Table 2.3. Interactive least squares means of troponin-T Western blot bands (percentage of total density) of sectioned beef strips steaks from heifers treated with growth promotant technologies during finishing.

	Treatment									
	Medial			Medial/Lateral			Lateral			
Item[1]	Control	Implant[2]	Implant/ZH[3]	Control	Implant	Implant/ZH	Control	Implant	Implant/ZH	SEM[3]
Band 1 (40 kDa)[4]										
Day 3	46.15	67.48	54.45	53.35	57.20	53.84	44.03	56.94	53.51	8.81
Day 14	29.50	26.36	45.46	25.86	41.45	47.37	26.04	38.94	30.78	
Band 2 (38 kDa)[4,5]										
Day 3	9.97	12.11	19.08	10.71	13.43	17.13	13.20	16.28	19.34	2.45
Day 14	6.78	7.35	14.31	5.62	11.26	14.83	6.78	10.64	12.87	
Band 3 (36 kDa)[4,7]										
Day 3	14.99	6.44	6.41	11.42	10.35	10.19	12.89	9.05	8.28	2.36
Day 14	14.52^{ab}	17.86^{a}	9.58^{b}	17.64^{a}	14.54^{ab}	10.64^{b}	17.78^{a}	12.39^{b}	14.21^{ab}	
Band 4 (34 kDa)[4]										
Day 3	16.16	9.53	13.61	16.17	13.88	16.38	18.05	13.26	14.12	4.66
Day 14	22.14	28.49	24.38	30.42	22.84	24.75	23.51	23.70	33.10	
Band 5 (30 kDa)[4,6]										
Day 3	9.17	2.62	4.40	6.01	3.17	1.10	8.93	2.58	2.66	3.93
Day 14	18.23^{a}	14.60^{a}	2.44^{b}	14.06^{a}	6.38^{ab}	1.27^{b}	13.00	10.26	6.10	
Band 6 (27 kDa)[4,6,8]										
Day 3	2.05	0.71	1.01	1.06	0.78	0.72	1.37	0.61	1.23	0.96
Day 14	6.91^{a}	2.74^{b}	1.87^{b}	4.79^{a}	1.63^{b}	0.64^{b}	3.66^{ab}	2.26^{ab}	1.84^{b}	

[1]Heifers (n = 15) were implanted with 200 mg trenbolone acetate and 20 mg estradiol on d 0 of feeding.
[2]ZH = zilpaterol hydrochloride. Heifers were implanted with 200 mg trenbolone acetate and 20 mg estradiol on d 0 of feeding and supplemented with 8.3 ppm of ZH for the last 20 d of feeding, followed by a 3-d withdrawal period.
[3]Standard error of mean.
[4]Day of aging, $P < 0.05$.
[5]Treatment, $P < 0.05$.
[6]Day of aging x treatment, $P < 0.05$.
[7]Day of aging x treatment x location, $P < 0.05$.
[8]Location, $P < 0.05$. [a,b]Means within a row differ, $P < 0.05$.

The implication resulting from the differences in LD sections indicates that protein degradation does not occur at the same rates through a single cross section of the muscle, which is often within a single retail cut. This is likely the result of the rate of cooling the muscle postmortem as the interior section of the muscle demonstrated less degradation product than the two outer sections. Pomponio and Ertbjerg (2012) showed that calpains activate more quickly at higher temperatures, which in turn would lead to more protein breakdown in more interior muscle areas that cool more slowly postmortem. Band 6 density (Table 2.3) was significantly ($P < 0.016$) higher in the medial section compared to the middle section and tended to be increased over the lateral section ($P < 0.060$). This is likely due to differences in cooling rates and temperatures across the muscle, but the current study did not include a way to quantify this effect. While all TnT bands had an effect of aging day ($P < 0.0002$), where bands 1 and 2 as a percentage of total lane density decreased and bands 3, 4, 5, and 6 percentages increased with days. Bands 5 and 6 had a day of aging x treatment interaction ($P < 0.05$) where the CON and IMP treatments increased in total lane density, but the ZH treatment did not suggesting lower levels of proteolysis after d 3 for the ZH treatment groups. In the companion paper to the present study (Ebarb et al., 2016), there was a location effect over all days of aging. Additionally, the MID location had higher WBSF values than MED and LAT locations, while MED and LAT did not differ. Ebarb et al. (2016) also showed that CON and IMP treatments had similar WBSF values, but the ZH treatment had higher WBSF values over the CON, and tended to have higher WBSF values over IMP samples. These findings support those in the current work where band 6 was found to have a location effect ($P < 0.042$) and within band 6 ZH samples exhibited less density ($P < 0.05$) than the CON samples in the MED and MID locations. The WBSF values also support the treatment effect ($P < 0.02$) found in band 5.

Heat Shock Protein 70

There was no effect ($P > 0.05$) on either of the 65 or 67 kDa bands of HSP 70 by either treatment or aging day (Table 2.4). Due to ZH being linked to fatigued cattle syndrome (FCS) by popular media (Thomson et al., 2015), a means to objectively quantify stress in ZH-fed animals would be useful. Heat shock protein 70 (HSP 70) quantity would, in theory, be a possible protein marker for animal stress (Shim et al., 2009, Turner et al., 2013, Valros et al., 2013), should animal welfare issues have been present in regards to the animals in the current study. In live cattle, it has been reported that ZH does not affect HSP 70 serum concentration (Burson, 2014), which corroborates the lack of difference in muscle postmortem as seen in the current results. However, there has been work done suggesting that different tissues yield different amounts of HSP concentration as a stress response (Valros et al., 2013). It is possible that analyzing muscle and blood samples for HSP 70 quantity is not a suitable way to determine increased HSP70 expression.

Table 2.4. Treatment means (± standard error of mean) of HSP 70 in beef strips steaks from heifers treated with growth promotant technologies during finishing.

	Treatment		
Item[1]	Control	Implant[2]	Implant/ZH[3]
HSP 70 Band 1			
Day 3	0.67 ± 0.09	0.74 ± 0.08	0.66 ± 0.09
Day 14	0.57 ± 0.09	0.61 ± 0.08	0.67 ± 0.08
HSP 70 Band 2			
Day 3	0.49 ± 0.07	0.48 ± 0.06	0.49 ± 0.07
Day 14	0.41 ± 0.07	0.48 ± 0.06	0.42 ± 0.06

[1]HSP 70 immunoreactive bands from western blots: Band 1 = 67 kDa, Band 2 = 65 kDa
[2]Heifers were implanted with 200 mg trenbolone acetate and 20 mg estradiol on d 0 of feeding.
[3]ZH = zilpaterol hydrochloride. Heifers were implanted with 200 mg trenbolone acetate and 20 mg estradiol on d 0 of feeding and supplemented with 8.3 ppm of ZH for the last 20 d of feeding, followed by a 3-d withdrawal period.

It is well-established that ZH usage can impact tenderness (Arp et al., 2013, Ebarb et al., 2016, Scramlin et al., 2010). While HSP 70 is commonly used as a measure of animal welfare tenderness, it has also been utilized as a marker for tenderness in cattle (Carvalho et al., 2014, Guillemin et al., 2012, Picard et al., 2014). A link between stress and tenderness has been established (Voisinet et al., 1997) using animals that are more excitable and therefore more stressed when handled. These animals yielded meat that was tougher when measured by WBSF. Therefore, HSP 70 could be used as an objective measure of both animal stress and product tenderness. Despite data showing ZH will decrease tenderness (Garmyn, et al., 2010) and the results from the troponin-T western blots and calpain zymography in the present study, along with results published in Ebarb et al. (2016), which demonstrates increased WBSF in ZH cattle, the HSP 70 western blot results do not show differences in treatment or aging day. It is possible that differing HSP 70 concentrations in skeletal muscle are too subtle to detect through these methods, or the differences in tenderness from these cattle would not be great enough to result in changes in HSP 70 concentration.

Casein Zymography

Calpain activities did not differ ($P > 0.05$) among treatment, aging days, or section, but there was a treatment by section interaction ($P = 0.027$) in calpain 2 clear zone zymography measurements where the lateral/medial section of IMP+ZH had a smaller clear zone than the lateral/medial sections of both CON and IMP (Table 2.5), meaning a lower activity level. Additionally, the lateral section of CON had a larger calpain 2 clear zone than the medial section of CON and lateral section of IMP+ZH, indicating a higher activity level. This is an unusual result because calpain 2 activity is typically stable postmortem and less impacted by the pH decline than calpain 1 (Thomson et al., 1996, Whipple et al., 1990). It is believed that calpain 1

plays a major role in tenderization postmortem due to the decrease in calpain 1 activity being parallel to the increase in myofibrillar fragmentation (Koohmaraie et al., 1987). Due to calpain 2 not exhibiting great changes in activity postmortem, it is commonly suggested that calpain 2 does not impact postmortem tenderization (Veiseth et al., 2001). Cattle that are fed ZH commonly have increased WBSF values (Avendaño-Reyes, et al., 2006, Garmyn, et al., 2010, Holmer, et al., 2009, J. D. Kellermeier, et al., 2009, Leheska, et al., 2009, Shook, 2009). In the present study, calpain 2 activity tended to be lower in cattle fed ZH while calpain 1 activity was insubstantial by day three in all treatments, which is supported by Whipple et al. (1990) where calpain 1 activity was reduced by nearly half from 0 h to 24 h while calpain 2 activity remained relatively constant. This result is perplexing and is at odds with work by Higgins et al. (1988) where clenbuterol usage in sheep resulted in elevated calpain 2 activity. This result suggests a possible role of calpain 2 in animal growth as a result of ZH use, or possibly an impact of calpain 2 on lower tenderness when ZH is utilized. More research is needed to determine if either of these options have merit.

The effect on calpain 2 activity by area of the muscle is possibly due to differing rates of cooling within the muscle postmortem. Beltrán et al. (1997), found that in beef cattle calpain 2 had a higher activity level than calpain 1 during a longer aging period, supporting Koohmaraie et al. (1987) and Veiseth et al. (2001), and further explained that lower temperatures and pH will reduce binding of calpastatin to calpain. Hwang and Thompson (2001) reported beef carcasses that were chilled more slowly had lower calpain 1 activity 4 h postmortem, however, there was no effect on calpastatin activity by temperature. This is also supported by Pomponio and Ertbjerg (2012) where it was demonstrated that calpains activate more quickly at higher temperature. Koohmaraie (1992) found a decrease in temperature reduced the autolysis rate of calpain 1.

Table 2.5. Interactive least squares means of calpastatin and calpain activity of beef strips steaks from heifers treated with growth promotant technologies during finishing

	Treatment									
	Medial			Medial/Lateral			Lateral			
Item[1]	Control	Implant[2]	Implant/ZH[3]	Control	Implant	Implant/ZH	Control	Implant	Implant/ZH	SEM[4]
Calpastatin										
Day 3	0.97	1.22	1.42	0.99	1.15	1.59	0.89	1.16	1.50	0.36
Day 14	0.86	0.87	1.39	0.78	0.83	1.22	0.88	1.01	1.24	
Calpain 1 (Day 3)										
Band 1	0.004	0.039	0.033	0.006	0.018	0.055	0.005	0.017	0.076	0.022
Band 2	0.00	0.00	0.04	0.00	0.00	0.02	0.00	0.00	0.02	0.02
Calpain 2 (Day 3)[5]	1.01^{b}	0.93^{b}	0.93^{b}	1.09^{ab}	1.09^{ab}	0.80^{c}	1.16^{a}	1.12^{ab}	0.87^{bc}	0.10

[1]Calpastatin activity = units/g sample; calpain activity = area of clear zone from casein zymogram gel.
[2]Heifers were implanted with Component TE-200 on d 0 of feeding.
[3]ZH = zilpaterol hydrochloride. Heifers were implanted with Component TE-200 on d 0 of feeding and supplemented with 8.3 ppm of ZH for the last 20 d of feeding, followed by a 3-d withdrawal period.
[4]Standard error of mean.
[5]Treatment x area, $P = 0.03$.
[a,b]Means within a row differ, $P < 0.05$.

The results in the current research show significant differences of calpain 2 activity between the lateral and medial locations ($P < 0.02$) in the samples. This does align with the TnT data which suggests there are possible differences in rates of cooling resulting in more accelerated calpain 2 activity. The treatment by area affect that was found could possibly be explained by the use of ZH, there is less subcutaneous fat to insulate the muscle (Baxa, et al., 2010, Hilton, et al., 2009), resulting in more rapid cooling in all areas of the LD. The exact mechanism for these differences is yet unknown.

Heated Calpastatin Assays

It has been theorized that increased protein accumulation and decreased tenderness are results of increased calpastatin activity in muscle (Geesink and Koohmaraie, 1999). Wheeler and Koohmaraie (1992) found an increase in calpastatin activity in cattle fed the beta-agonist $L_{646,969}$. Higgins et al. (1998) also recorded increased calpastatin activity in sheep fed the beta-adrenergic agonist clenbuterol. However, work by Hilton et al. (2009) did not uncover increased calpastatin levels in cattle ZH. Rathmann et al. (2009) showed no effect of ZH on calpastatin expression. Work examining calpastatin mRNA concentrations have also yielded no significant effects due to ZH (Baxa 2008). However, some work has been done that has shown a change in calpastatin activity due to ZH use. Strydom et al. (2009) found that ZH increased calpastatin activity in beef LD. Geesink et al. (1993) found higher calpastatin activity postmortem when the beta-adrenergic agonist clenbuterol was utilized. The beta-adrenergic agonist cimaterol has been shown to increase calpastatin activity by 76% in steers (Parr et al., 1992). In lambs, Simmons et al. (1997) reported an increase in calpastatin activity with clenbuterol use. In the current study, calpastatin activity did not differ ($P > 0.05$) among treatment, aging days, or section. While many reports suggest other beta-adrenergic agonists increase calpastatin activity levels, results

on ZH are mixed. Based on the literature and the results from the current study, calpastatin activity alone may not be responsible for all observed changes in tenderness as a result of ZH use in cattle.

Bioelectrical Impedance Analysis

When measuring bioelectrical impedence, four varaibles were analyzed: R, X, Z, and Pa. Resistance (R) is simply the resistance of the material to electrical current. Reactance (X) is the inductive or capacitive property of the material and makes up the non-resistive component of impedance (Z). Impedance is derived from R and X, along with the phase angle (Pa). Phase angle, like impedance, is only a characteristic of alternating current and in the context of capacitance and inductance indicates the lag of the current waves across the material. In the results from these trials, different treatments resulted in a significant difference in R, and by relation, Z ($P < 0.05$) measurements (Table 2.6). All BIA variables had a day of aging effect ($P < 0.05$) and a day by of aging treatment effect ($P < 0.05$). It is well established in the literature that electrical current does not move through all tissues with the same resistance, with fats acting as an insulator (Swantek et al., 1992). In the present study, the strip steaks from the CON treatment had the most R and the ZH treatment had the least. Due to fat acting as an insulator and the fat-reducing properties of ZH, it is possible that the reduction in R between treatments is simply due to a lack of marbling. The literature supports this phenomena when using BIA as a means to determine fat content in ground product (Marchello et al., 1999). Yang et al. (2013) found that moisture content is correlated to Z, which may appear in the present results as the increase of overall Z between d 3 and d 14 of aging, where the longer-aged steak had more time to dry out. However, this does not account for the differences in treatment where it is clear that growth promoting strategy has an impact. The companion paper (Ebarb et al., 2016) did show a

Table 2.6. Treatment means for bioelectrical impedance across treatment and aging day[1]

	Day 3			Day 14			
	Control	Implant[2]	Implant/ZH[3]	Control	Implant	Implant/ZH	SEM[4]
R[5,6,7]	371.70	355.00	266.50	795.90[a]	672.70[a]	529.50[b]	42.02
X[5,7]	-0.40	-0.10	0.30	4.20[a]	2.10[b]	0.40[b]	0.80
Z[5,6,7]	371.70	355.00	306.50	800.90[a]	672.70[b]	529.50[c]	39.75
Pa[5,7]	-0.05	-0.02	0.09	0.30[a]	0.15[b]	0.04[b]	0.06

[1]Bioelectrical impedence as measured by an eFresh device across the steak surface.
[2]Heifers were implanted with 200 mg trenbolone acetate and 20 mg estradiol on d 0 of feeding.
[3]ZH = zilpaterol hydrochloride. Heifers were implanted with 200 mg trenbolone acetate and 20 mg estradiol on d 0 of feeding and supplemented with 8.3 ppm of ZH for the last 20 d of feeding, followed by a 3-d withdrawal period.
[4]Standard error of mean.
[5]Day of aging, $P < 0.05$.
[6]Treatment, $P < 0.05$.
[7]Day of aging x treatment, $P < 0.05$

trend ($P < 0.09$) for decreased marbling in carcasses from IMP and ZH treated heifers compared to the CON treatment, which does not completely support the results in the current study, but does suggest that the reduction in R across treatment may be at least partially related to marbling.

Implications

Overall, the results from the present work indicate a possible change in postmortem protein breakdown due to inclusion of ZH in the diet that correlates with a decrease in tenderness. The data also suggest calpain 2 is affected by ZH and implants. There were no differences in calpain 1 at d 3 of aging. This is supported by the literature that shows a rapid decline in activity within the first 24 h postmortem. Calpastatin activity did not differ between treatments. Despite no significant differences in calpastatin and calpain 1 activity, changes were seen in TnT degradation, indicating changes in protein degradation postmortem. Significant differences in calpain 2 activity between treatments are unusual and are not supported by the

literature. It is unknown what these differences in activity correspond to in the live animal or if this is reflected in postmortem protein degradation. Additionally, the results of the analysis of HSP 70 concentration in skeletal muscle do not suggest reduced tenderness, suggesting that HSP 70 expression at 3 and 14 d of aging may not be as sensitive to changes in tenderness as troponin-T. Alternatively, no treatment effect on HSP 70 does provide a piece of evidence that cattle fed ZH are not subjected to undue stress and are upregulating HSP 70 as a result. In terms of color and pH of the samples, ZH and implantation do not impact the color of strip steaks after aging. The BIA results suggest that there is less fat or some other resistive tissue in implanted cattle and cattle fed ZH. The results of Ebarb et al. (2016) somewhat support this as a change in marbling was observed. However, the differences between aging days in BIA do not have a readily apparent cause, and may be due to either decreased moisture content or a change in cellular membrane integrity with increased aging. Overall, the use of an anabolic implant in conjunction with the BAA ZH resulted in changes to the calpain proteolytic system and differences in postmortem protein degradation that are not readily explained and warrant further study.

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