INDEPENDENT METHODS TO IMPROVE MEAT QUALITY INCLUDING GENETIC TECHNOLOGIES, MODIFIED PROCESSING, AND GROWTH PROMOTANTS IN BEEF

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Independent methods for improvement of meat quality including genetic technologies, modified processing, and growth promotants in beef

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Three independent studies were done to investigate how genetic factors, carcass management, and exogenous growth promotant programs influence meat quality attributes, palatability, and protein expression in beef. The first study involved Igenity® genetic profiling of myostatin sequence variants C313Y and Q204X of the myostatin gene in Piedmontese-crossbred heifers. Muscle from heifers having 1 or 2 copies of myostatin variants had decreased fat deposition, increased marbling, and minimal influence on quality attributes measured (tenderness and color), indicating a relationship between disruptive myostatin gene alterations and carcass and meat quality traits.

Next, to improve the quality of the under-utilized beef round muscle, a modified hot-boning technique was utilized early post mortem to measure effects on meat palatability and myofibrillar protein degradation in deep and superficial portions of the beef *semimembranosus* (SM). Randomized treatments included deep SM hot-boned (DH) or cold-boned (DC), and superficial SM hot-boned (SH) or cold-boned (SC). Post mortem temperature and pH decline were monitored every 10 min for 24 h, and at 45 min, 3 h, and 24 h, respectively, in superficial and deep portions of the SM muscle. The deep SM had a slower chill rate and a more rapid pH decline when compared with the superficial SM, regardless of hot-boning treatment, which resulted in increased calpain 1 autolysis in deep SM when compared with superficial SM locations. Muscle from both deep SM treatments was less red, however, tenderness was not different among all treatments. Altering proteolytic activity and meat palatability is contingent upon the ability of the modified hot-boning technique to significantly alter temperature and pH decline in the deep portion of the SM.

Lastly, proteomic technologies (two-dimensional in-gel electrophoresis coupled with mass spectrometry) were used to identify differentially abundant sarcoplasmic and myofibrillar proteins in the *longissimus lumborum* (LL) muscle from beef cattle treated with ractopamine hydrochloride with or without anabolic implant treatment during the finishing period. Exogenous growth promoting programs altered the protein profile of beef LL muscle, influencing the abundance of glycolytic enzymes and proteins possessing oxidative resistance, protective, regenerative, recovery, and anti-apoptotic properties that potentially effect meat quality or meat aging.

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DEDICATION

To the best guy I know, my loving husband

Nathan Scott Hayes

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LIST OF ABBREVIATIONS

˚C .....degrees Celsius

µL.....microliter

µm .....micrometer

%CH..... percent choice

2D.....two dimensional

ADG..... average daily gain

ADP..... adenosine diphosphate

ALDH ..... aldehyde dehydrogenase

ATP .....adenosine triphosphate

BF..... *biceps femoris*

bp.....base pairs

BSA.....bovine serum albumin

BW .....body weight

Ca .....calcium

CAIII .....carbonic anhydrase III

calpain 1 ..... µ-calpain

calpain 2 .....m-calpain

CHCA .....α-cyanohydroxy cinammic acid

cm.....centimeter

COOH .....carboxylic acid

Cy2..... cyanine 2 yellow dye

Cy3..... cyanine 3 red dye

Cy5..... cyanine 5 blue dye

CyDye ..... cyanine dye

d..... day

Da..... Dalton

DC..... deep cold-boned

DH..... deep hot-boned

2D DIGE ..... 2 dimensional difference in-gel electrophoresis

DIGE..... difference in-gel electrophoresis

DJ-1..... protein deglycase DJ-1

DM..... dry matter

DTT..... DL-dithiothreitol

E_2..... estradiol

EDTA..... ethylenediamine tetraacetic acid

eIFs..... eukaryotic translation initiation factors

eIF5A ..... eukaryotic translation initiation factor 5A

eNOS..... nitric oxide synthase

Fe..... iron

FT..... 12th-rib fat thickness

g..... gravity

g..... gram

GDF-8 ..... growth and differentiation factor-8

GDP..... guanosine 5'-diphosphate

GP ..... growth promoting technologies

GPCR..... G protein-coupled receptor

h..... hour

HCW ..... hot carcass weight

HPLC ..... high pressure liquid chromatography

Hsp ..... heat shock protein

IEF..... isoelectric focusing

IGF ..... insulin growth factor

IMF ..... intramuscular fat

In ..... inches

IPG ..... immobilized pH gradient

kg..... kilogram

KPH..... kidney, pelvic, and heart fat

LC ..... liquid chromatography

LD ..... *longissimus dorsi*

LEA..... loin eye area

LL..... *longissimus lumborum*

LM..... *longissimus* muscle

LT..... *longissimus thoracis*

M ..... meter

MALDI ..... matrix-assisted laser desorption/ionization

MARB..... marbling score

MBPH ..... myosin binding protein H

Mg..... magnesium

mg ..... milligram

mh ..... myostatin homozygous

min ..... minute

mL ..... milliliter

m*M* ..... millimolar

mo ..... month

mRNA ..... messenger ribonucleic acid

MS ..... mass spectrometry

MSB ..... myofibrillar solubilizing buffer

MW ..... molecular weight

NAD ..... nicotinamide adenine dinucleotide

NADPH ..... nicotinamide adenine dinucleotide phosphate-oxidase

pH ..... potential hydrogen

pI ..... isoelectric point

ppm ..... parts per million

PRDX2 ..... peroxiredoxin 2

PRDX6 ..... peroxiredoxin 6

RAC ..... ractopamine hydrochloride

REA ..... rib-eye area

ROS ..... reactive oxygen species

RT-PCR ..... reverse transcription polymerase chain reaction

SAS ..... Statistical Analysis Software

SC ..... superficial cold-boned

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

SH ..... superficial hot-boned

SM..... *semimembranosus*

SS ..... *supraspinatus*

TBA..... trenbolone acetate

TEND.....tenderness

TEMED.....tetramethylethylenediamine

TGF-β.....transforming growth factor beta

TCA.....tricarboxylic acid cycle

TnT..... troponin-T

UniProt.....Universal Protein resource

USDA.....United States Drug Administration

V..... volt

VL ..... *vastus lateralis*

vol .....volume

WBSF.....Warner-Bratzler shear force

WHC ..... water holding capacity

wk..... week

wt.....weight

YG.....yield grade

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Introduction

With the world population increasing at a rapid rate, the demand for nutritious protein is imminent. Our goal as an industry will be to provide high quality protein to maintain adequate food supply, and meat is a complete source of protein, B-vitamins, and other macronutrients essential to a balanced diet (Murphy et al., 2011; McNeil and Van Elswyk, 2012). New scientific knowledge, technology, and innovative assessments are crucial to face the daunting challenge of providing enough food to support a global population that will grow from 7 billion to over 9 billion by 2050. Efficiency in all areas of production, from breeding and farming to processing and transparency, is needed for future success.

Demand and quality are 2 major factors that will determine how the beef industry performs over the next several years, especially as beef production starts to increase. Consumers are central to the success of the United States beef industry, and with U.S. beef production expected to decline (USDA, 2015), consumers have and will continue to face high prices. Furthermore, the demand for beef has been growing globally for several years, resulting in concern for consumers' ability to pay rising prices. Therefore, the industry needs economic strength to support Americans and provide them with the expenditure to purchase beef. The current situation described here challenges meat scientists, beef producers, and industry professionals to continue to improve beef quality in order to deliver consumers a product worth paying for.

Consumers define meat quality based on their initial response to appearance, particularly color and marbling, followed by their consumption of beef, dependent upon texture and flavor attributes. Genetic factors, animal nutrition, animal management, and carcass treatment all play

significant roles in meat quality traits. The application of established methods to improve beef quality including genetic technologies, modified processing techniques, and growth promotant treatment programs will be discussed in this dissertation. Considering genetic factors, the full impact of myostatin genotype on meat quality traits requires further investigation. Past studies of double-muscled cattle indicate variation in meat quality traits when compared with that from normal cattle, however, many of these studies exhibit differences in myostatin genotype, muscle location, and animal breed (Oliván et al., 2004; Aldai et al., 2006). The number of copies of mutated alleles expressed may also cause variation in influence observed on carcass traits and beef palatability.

While genetics and breeding may be the first step towards an efficient animal, slaughter and processing techniques heavily influence ultimate product quality. Compared with traditional boning of chilled carcasses, modified hot-boning early post mortem has the potential to enhance the under-utilized beef round muscle. Few investigations have examined how partial hot-boning of the SM muscle impacts tenderness and palatability attributes.

Genetic factors and processing techniques, while essential to high quality beef, will have little impact without sufficient nutrition and optimal growth during the finishing period. Growth promoting technologies, in particular, anabolic implants and β-adrenergic agonists, increase animal performance during the beef cattle finishing period. However, the use of these programs has been shown to negatively impact meat tenderness and quality traits (Culp et al., 2013; Arp et al., 2014; Bohrer et al., 2014; Martin et al., 2014). Cellular mechanisms and biochemical processes governing biochemical and physical responses observed in cattle treated with growth promoting technologies remain unclear. Through the application of proteomic technologies, proteins differentially expressed in bovine LL from beef cattle fed ractopamine hydrochloride

with or without anabolic implant treatment during the finishing period will be identified and discussed in the last chapter of this dissertation. The use of genetics, post-harvest processing, and production are 3 ways meat scientists are attempting to create the high quality beef consumers demand while also increasing the efficiencies of meat production.

Meat Quality Parameters

Meat quality is defined differently across several areas of the industry. Generally, scientists and meat industry professionals define meat from 2 different perspectives. The first view of meat quality is through the perspective of the carcass and refers to the amount of marbling, the firmness and color of lean muscle, the firmness and color of the fat, and size, shape, and ossification of the bone (USDA, 1996). Marbling is considered an indicator of meat quality (Smith, 2005). There are 9 degrees of marbling given to beef cuts ranging from practically devoid to abundant. It is generally accepted that as the marbling increases, net quality increases.

A high quality lean is characterized by a very fine texture of muscle that is velvety to the feel. The texture or grain of beef muscle is evaluated in a similar manner to that used in evaluating the grain of wood. As the texture becomes coarser, typically as the carcass matures, the quality becomes less desirable (Smith, 2005). A high quality lean should be very firm to the touch and not soft, watery, or gummy. The use of the *longissimus* muscle at the 12th-13th rib interface is often used as an indicator of color, texture, and marbling quality.

The color of lean is a very important factor in this initial definition of meat quality. From a physiological standpoint, if the color of meat is off-putting, but all other quality characteristics indicate that it is a high-quality product, meat will often still be rejected by the majority of consumers (Smith, 2005). The overall appearance has a very strong influence on purchasing

decision and eating satisfaction. The desired color for beef is cherry-red and is dependent upon several factors including the age of the animal at the time of slaughter (darker color associated with an older age), stress prior to slaughter, and storage time (Jeremiah et al., 1972; Wyle et al., 2003). Stressful harvest environments can result in reduced meat quality due to biochemical changes resulting from the animal's physiological response to trauma. As meat is stored in a retail display case, it will gradually become darker in color. The fat should be very firm and white or creamy in color (Hilton et al., 1998). Yellow fat may indicate inferior breeding or advanced age in the animal at time of slaughter. Certain finishing rations such as those high in grain (Strachan et al., 1993) may also cause yellowing of fat, but fat is often yellow and very soft due to substandard breeding or old age.

The characteristics of the bone must be taken into consideration when quality is evaluated because as the animal progresses in age, the quality of meat is gradually reduced (Wulf et al., 1996). Bone characteristics convey a fairly good approximation of age of the animal at time of slaughter. A young animal is characterized by bones which are very porous and dark-red with a large amount of cartilage on the feather bone. Bones of older cattle are grayer in color with little or no cartilage present (Lawrence et al., 2001). USDA beef carcass maturity classification is based on assessing the degree of cartilage ossification in the sacrum, the lumbar, and thoracic vertebra and the ribs to identify the age and physical maturity of the animal.

Lastly, the meat must be aged properly under controlled conditions of temperature and humidity for high quality factors mentioned previously to be achieved. Aging of the meat from 10 to 14 d is needed for to develop desirable palatability attributes. Aging of meat allows the naturally occurring enzymes within the tissues to break down some of the components of the tissue and improve overall eating quality.

The second definition of meat quality takes into consideration tenderness and palatability attributes including color, aroma, flavor, and juiciness that hold relative importance by the consumer. Several intrinsic (marbling, color, tenderness) and extrinsic (price, product presentation, origin, brand) meat quality attributes have been the focus of many research investigations attempting to better understand the mind of the average consumer (Grunert et al., 2004; Peri, 2006; Korzen and Lassen, 2010). Consumers make judgments on beef where cut type, color, and marbling heavily impact their quality expectations.

The first quality attribute seen by the consumer, beef color, is a direct indicator of fresh red meat quality (Renerre and Labas, 1987). While the color of fresh meat is not closely related to eating quality, consumers find a bright cherry-red color desirable and will not purchase discolored beef products. Meat color is dependent on the concentration and chemical state of meat pigments (myoglobin and hemoglobin) and on physical characteristics of meat (light scattering and absorbing properties) (Kropf, 1993). Environmental factors such as age, exercise, diet of the animal, and genetics can influence myoglobin concentration (Livingston and Brown, 1981). Deoxymyoglobin, oxymyoglobin, and metmyoglobin are different iron (Fe) states of myoglobin that result in distinct colors of red meat. Immediately after cutting into a deep muscle, a deep purplish-red color is characteristic of deoxymyoglobin in the ferrous (Fe^{2+}) state. The desirable cherry-red form of myoglobin results from deoxymyoglobin being exposed to oxygen, and is short-lived as further oxidation results in the ferric ($Fe3^{+}$) metmyoglobin state synonymous with an undesirable brown meat color (Hood and Riordan, 1973). Discoloration of meat can also be influenced by innate factors such as muscle pH and fiber type, as well as breed, sex, and plane of nutrition. Pre- and post-slaughter factors including hot-boning, electrical stimulation, and chilling of carcasses can also impact meat color.

Another important visual quality determinant to consumers is the presence and extent of marbling. Also known as intramuscular fat, marbling has been shown to enhance flavor, juiciness, and tenderness of meat with increased marbling being highly correlated with increased palatability (Miller, 2002). However, consumers also see greater amounts of intramuscular fat as unhealthy, resulting in challenges to meet a complex consumer demand for palatability and meat quality attributes. Flavor and juiciness, along with tenderness, are additional important factors in meat palatability. Alterations to these attributes pre- and post-slaughter influence meat quality in the context of the consumer. For example, muscle restraint, or hot-boning techniques are being used to increase sarcomere lengths, resulting in decreased shear force and increased tenderness (Sørheim and Hildrum, 2002). Additionally, post mortem aging is being utilized in the development of beef flavor quality. In a study by Warren and Kastner (1992) beefy flavor and brown/roasted flavor increases in intensity in dry aged beef.

Myostatin Genetic Mutation and Double-Muscling in Beef Cattle

Myostatin or growth and differentiation factor-8 (GDF-8) is first produced in skeletal muscle as a 375-amino acid protein before undergoing proteolysis, resulting in a mature carboxy-terminal myostatin. The effects of the protein were first observable in mice (McPherron et al., 1997) when increased skeletal muscle mass, due to increases in both fiber number (hyperplasia) and myofiber size (hypertrophy), was seen in myostatin-null mice when compared with their wild type littermates. Due to these findings, several groups discovered that the ‘double-muscling’ condition seen in Belgian Blue and Piedmontese cattle breeds were the result of 2 distinct mutations in the coding sequence of the myostatin gene (Grobet et al., 1997; Kambadur et al., 1997; McPherron et al., 1997). Since then, several other mutations have been identified in the

coding region of the myostatin gene corresponding to heavy muscling in different breeds of cattle.

The expression of the GDF-8 gene results in the production of myostatin. A study conducted by Jeanplong and others in 2001 showed through molecular analysis that the bovine myostatin gene consists of 3 exons and 2 introns. The sizes of the first and second exons are 506 and 374 base pairs (bp) respectively. The size of the third exon was found to be dependent upon the polyadenylation site and found to vary (1701, 1812, or 1887 nucleotides). The size of the 2 introns are 1840 and 2033 bps. Using RT-PCR to analyze samples from the *biceps femoris* and *semitendinosus* collected at d 1, 8, and 14 of development, their results indicate that the highest level of myostatin expression appears on d 1 and gradually reduces out to d 14.

Additional studies support the idea that myostatin gene expression is developmentally regulated (Kambadur et al., 1997; Ji et al., 1998). Investigation of myostatin mRNA levels from d 21 of gestation to 7 wk old piglets showed that myostatin mRNA abundance significantly increases by d 49 of gestation and begins to decrease from d 105 of gestation to its lowest level 2 weeks postnatally (Ji et al., 1998). In this study researchers also observed low-birth-weight piglets having 65% higher mRNA expression levels than those piglets with normal birth weight, suggesting that increased abundance is associated with lower birth weight. Increasing myostatin during gestation followed by a reduction at birth is a developmental pattern similar to that of primary and secondary muscle fiber formation, and its reduction may be explained by a decrease in myoblast activity (Beerman et al., 1978).

The relative increase in fiber number observed in early pregnancy leads to a calf having almost double the number of muscle fibers at the time of birth. Commonly known for this condition, Belgian Blue and Piedmontese animals exhibiting double muscling will have less

bone, less fat, and 20% more muscle on average (Hanset 1986, 1991; Shahin and Berg 1985). However, before discussing the physiological influence of myostatin, it is important to understand how mutations in the coding region of the GDF-8 affect the functionality of the gene product. Myostatin is a member of the transforming growth factor beta (TGF-β) superfamily of cytokines, which has been shown to have significant effects on cellular proliferation, differentiation, and growth. Members of this family typically have several highly conserved features including N-linked glycosylation sites, 9 cysteine residues, hydrophobic dimer interface, and protein backbone (McPherron and Lee, 1996). TGF-β signaling is initiated through highly specific binding and intricate formation between an active ligand molecule and its corresponding cell surface receptor (serine/threonine kinases). Once the ligand binds to its type II receptor, the type II receptor associates with its corresponding type I receptor, forming a receptor complex. Kinase activity of the receptor complex is then activated upon transphosphorylation of the type I receptor. Now activated, the receptor complex phosphorylates receptor-bound Smad proteins, allowing them to interact with the Smad4 complex which moves into the nucleus. Inside the nucleus, the Smad complex works with transcription factors and co-factors to regulate target gene transcriptions (Kollias and McDermott, 2008).

The role of TGF-β and myostatin in skeletal muscle development is a subject of continuing research as it is not yet clear what is responsible for the sharp increase in skeletal muscle mass. Through an vitro study in the 1980s, scientists were able to locate the origin of TGF-β in the ectoderm and observed its influence on adjacent mesenchymal cells. In vitro experiments in mice revealed that TGF-β regulates proper muscle formation during embryonic development by inhibiting premature differentiation in migrating myoblasts (Yanagisawa et al., 2001). While searching for mammalian members of the TGF-β superfamily, McPherron and Lee

(1996) identified myostatin before sequencing mutations of the gene in Belgian Blue and Piedmontese cattle.

A myostatin mutation in Piedmontese and Belgian Blue cattle results in a reduction in size of internal organs, a reduction in fertility, lower viability of offspring, and a delay in sexual maturation (Ménissier, 1982). Despite these production limitations, the superiority of the carcasses of double-muscled cattle increases their utilization in beef production systems. The Piedmontese breed, particular to the experiment discussed in Chapter 2 of this dissertation, originated in Italy. Sartore and Chiappone (1982) reported, of the purebred Piedmontese cattle in Italy, almost 100% of the sires used in natural service are artificial insemination were double-muscled. While there is a wealth of information available on double-muscled cattle, few studies have investigated the potential of utilizing the Piedmontese breed in double-muscled crossbred cattle.

Past studies have crossed double-muscled males with non-double-muscled females (Cundiff et al., 1993, 1994). In these studies, the Piedmontese-Angus crossbred, among Charolais, Gelbvieh, and Salers, had the highest percentage of retail product and the lowest marbling score (select quality grade). Piedmontese crosses excelled in carcass composition, and while comparable to Hereford-Angus crosses in final weight, they ranked second to Charolais crosses in weight of totally trimmed retail product due to a higher dressing percentage and a significantly higher retail product percentage when compared with other breeds. The Piedmontese crossbred cattle ranked higher among breeds for marbling score than they did for shear force tenderness with reported low shear force values and relatively tender meat.

More recent studies (Casas et al., 1998, 1999; Short et al., 2002) have investigated the Piedmontese-Angus cross exhibiting 1 or 2 copies of myostatin alleles, and the resulting

comparative effects on growth and carcass traits. In a study evaluating traits of progeny from Piedmontese sires (n = 209), cattle inheriting 1 copy of a myostatin allele, Casas and others (1998) observed increased ribeye area (REA), retail production yield, and body weight, along with decreased marbling, yield grade, fat, and kidney, pelvic, and heat fat (KPH). It was determined from the results of their study that a single mutation produced a leaner, more heavily muscled carcass, while avoiding the calving problems associated with animals having 2 copies of the myostatin allele. This information was further confirmed by a study carried out by Casa and collaborators in 1999 investigating calving data of Piedmontese-cross animals homozygous and heterozygous for myostatin C313Y copies. Birth weight and calving difficulty increased linearly with number of myostatin copies introduced, suggesting that a single functional copy of myostatin, resulting in heavier weaning and yearling weights and higher production yield, is best for beef production systems.

The use of Piedmontese genotypes in breeding systems to capture benefits of myostatin were also investigated (Short et al., 2002). In agreement with the work of Casas, Short reported linear increases in birth weight and dystocia scores as 1 or 2 copies of myostatin alleles were introduced to the calf. Addition of the myostatin allele in this study increased muscle area of the calf but did not influence *longissimus* muscle (LM) area. All traits of fat deposition measured (marbling score, fat depth over the loin, yield grade, and KPH) decreased linearly with addition of number of copies of myostatin alleles, further confirming the increase in muscularity achieved by using the myostatin allele associated with the Piedmontese breed.

Post Mortem Environment and Processing Techniques

Ultimate meat quality and value is heavily influenced by the muscle-to-meat conversion after slaughter, as well as various processing techniques utilized to improve highly valued consumer traits (tenderness, juiciness, and flavor). The following discussion will address environmental SM skeletal muscle changes that occur during the transitioning of muscle to meat, and how they are influenced by a hot-boning processing technique.

Post Mortem Muscle

Post mortem conditions during the muscle-to-meat conversion can heavily influence meat quality attributes, and variability in quality traits including color and tenderness have been observed within the muscle. Due to the considerable size, thickness, and variation seen in the SM, many investigators have distinguished outer and inner SM locations (Sammel et al., 2002; Seyfert et al., 2005; Kim et al., 2010). The outer, or surface SM chills slower than the inner, or deep SM location, resulting in faster glycolysis and pH decline post mortem. When pH was measured from normally-chilled carcasses at 1.5, 5, and 8 cm from the surface of the SM, Tarrant (1977) found pH declines of 0.07, 0.16, and 0.25 units, respectively. While the surface SM (1.5 cm) reached ultimate pH values 24 to 48 h postmortem, the deeper location (8 cm) only took 6 h to reach ultimate pH, while its temperature was still above 30°C.

When the pH of the deep portion of the SM drops that quickly, denaturing of proteins and subsequent detrimental effects on meat quality are likely (MacDougall, 1982; Hector et al., 1992; Den Hertog-Meischke, 1997). Kim and others (2010) observed increased protein denaturation in the deep portion of the SM that altered the progression of proteolysis by negatively affecting calpain 1 activation, resulting in negative impacts on associated meat tenderness. However, when Tarrant utilized the hot-boning technique, he saw a major effect on the rate of metabolism in the

muscle post mortem, with the hot-boned muscle having a relatively uniform rate of glycolysis compared with the carcass muscle chilled normally in which the rate of glycolysis increased with depth. It is important to note that Tarrant also observed a 50% decrease in ATP content in the hot-boned muscle at 8 h post mortem with completion of rigor within 24 h.

Hot-Processing on Meat pH, Color, and Texture

The pH of fully hot-boned bovine SM chilled at 2 or 10°C is not different at 24 and 48 h post mortem, and the authors observed no significant effect of chilling on the rate of pH drop, suggesting a more efficient chilling method, such as direct immersion in a water-bath, should be applied to muscles to more efficiently demonstrate the effect of temperature on post mortem glycolysis (White et al., 2006). Conversely, full removal of LL and SM muscles 45 to 60 min post mortem from cull beef cows reduces pH in hot-boned muscle when compared with conventionally chilled beef. Conflicting results from studies using similar methods found no differences in pH between hot- and cold-boned beef product (Penney et al., 1998), while hot-boning increases pH when compared with cold-boned beef (Claus et al., 1998; Jerez et al., 2003; Stephens et al., 2006).

Hot-boning was first developed to reduce energy use and operating costs in beef processing facilities (Cuthbertson, 1980). It has been estimated that hot-boning could save beef packers 50% in refrigeration energy and 80% in cooler space requirements. In addition to potential economic advantages, hot-processing has also been shown to provide more uniform and stable product color (Cross and Tennet, 1980; Sammel et al., 2002). Using a modified hot-boning technique that involved cutting the SM at its insertion at the medial surface of the distal femur and separating it anteriorly from the surrounding muscle but leaving it intact at its origin, similar to the experiment discussed in Chapter 3 of this dissertation, Sammel and others (2002) observed

slower chill rates, faster pH declines, less metmyoglobin reducing ability, less oxygen consumption, water holding capacity, and color stability in the cold-boned inner SM when compared with the hot-boned inner SM. Furthermore, both locations of the hot-boned SM (inner and outer) had similar chilling rates and pH declines, which resulted in a more uniform stable color measured in a 5-d display study. Although the partial hot-boning technique used in this study enhanced meat color of the inner SM, no measurements of detrimental impacts on meat tenderness due to hot-processed muscle excision were evaluated.

Partially hot-boning the beef knuckle (quadriceps muscles) within 1.5 h post mortem did affect tenderness of the *vastus lateralis*, *rectus femoris*, SM, or *biceps femoris* (BF) muscles (Seyfert et al., 2005). Increased tenderness of the BF from hot-boning has been observed (Schmidt and Gilbert, 1970; Kastner et al., 1973), but only when the whole muscle was excised from the carcass as opposed to the modified chilling of the SM remaining attached to the skeleton used in the experiment examined in this dissertation. Full excision of muscles early postmortem can also increase risk of cold-shortening when muscles are chilled more rapidly, and this can lead to the development of a tougher product (Marsh and Leet, 1966). However, even when hot-boned sides are exposed to temperatures of 0 ± 2°C, no cold shortening has been observed (Schmidt and Kenman, 1974), indicating that a hot-boning technique can be used effectively without negatively influencing product quality. A more recent study (Neto et al., 2013) investigating the effects of full hot-boning on shear force and tenderness of the LL from Nelore steers chilled at -20°C and 0°C supports the above mentioned results. Shear force and tenderness values were not different between cold- and hot-boned treatments, and aging of LL for 4 and 14 d post mortem did not reduce shear force values of the hot-boned muscles. The removal of specific muscles from the carcass during the pre-rigor phase allows the muscle to

more easily contract than muscles that are chilled while still attached to the skeleton, and different muscles connected to the skeleton will exhibit different degrees of contraction when entering a state of rigor (Locker, 1960).

Effects of β-Adrenergic Receptor Agonists and Anabolic Implants on Animal Growth

β-Adrenergic Receptor Agonist

The physiological β-adrenergic receptor agonists are norepinephrine and epinephrine, the 2 major chemical signaling molecules that make up the sympathetic nervous system. Epinephrine (adrenaline) is a hormone produced by the adrenal medulla and released to the plasma. It circulates at lower concentrations than norepinephrine in most mammalian species, but during stress it usually responds to a greater extent than norepinephrine. Epinephrine is biosynthesized from norepinephrine and is the methylation product of norepinephrine. Biosynthesized from tyrosine, norepinephrine (nonadrenaline) is a neurotransmitter substance produced and released from nerve axons, or central nervous system nerve endings, following stimulation with acetylcholine. Binding of 1 of these chemicals to an adrenergic receptor will elicit a response, depending on the receptor type bound.

In 1948 adrenergic receptors were divided into 2 subgroups of agonists: alpha (α), which elicited an excitatory response, and beta (β), which resulted in mostly inhibitory responses (Ahlquist, 1948). Since then, the adrenergic system has been found to be even more complex, with 6 α- and 3 β-subtypes now determined, with skeletal muscle predominantly utilizing the β-adrenoreceptor family.

Adrenoreceptors belong to the quinine nucleotide-binding G protein-coupled receptor (GPCR) family, comprising the largest group of receptors located on the cell surface in mammals. Beta adrenergic receptors belong to the rhodopsin receptor family, in addition to

dopaminergic, adenosine, and histamine receptors (Strosberg, 1990; Fredriksson et al., 2003). Unique to the GPCR family, the receptors bind to heterotrimeric (3 subunit composition of "αβγ") guanine nucleotide-binding regulatory proteins (G proteins) (Fig. 1.1). The β-adrenergic receptor structure consists of 7 transmembrane α-helices that form 3 extracellular loops, an NH_2, or amino terminus, and three intracellular loops with a carboxylic acid (COO^-) terminus (Morris and Malbon, 1999; Johnson, 2006).

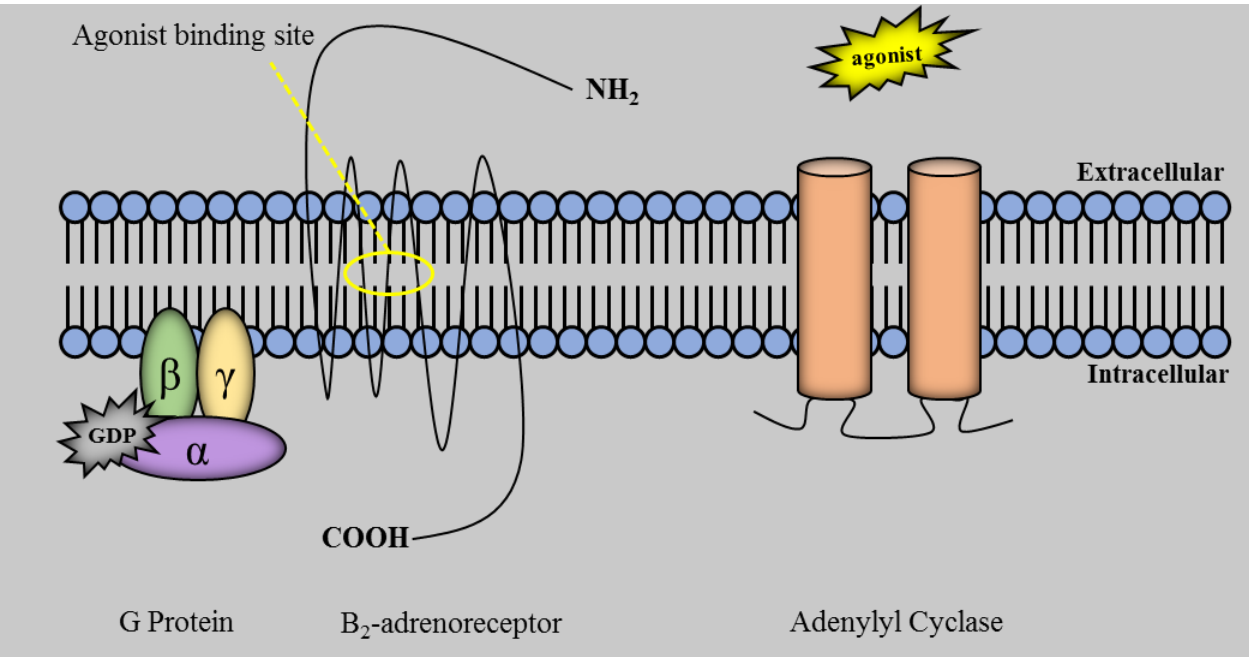

Figure 1.1. The general β_2-adrenoreceptor signaling pathway including the receptor, heterotrimeric G protein, and the membrane-bound adenylyl cyclase. Adapted from Lynch and Ryall, 2008. Physiol. Rev. 88:729-767.

The G proteins interact with an intracellular loop of the GPCR in the cytoplasmic space, specifically, the active G protein βγ subunits form a tightly interacting dimer that is bound to the intracellular plasma membrane through an isoprenyl component located on the COOH terminus of the γ-subunit (Bockaert and Pin, 1999) (Fig. 1.2). Activation of the GPCR causes a conformational change in the intracellular loops, exposing a previously covered G protein binding site (Meng and Bourne, 2001; Filipek et al., 2004; Klco et al., 2005). Binding of the

ligand to the third loop of the GPCR stimulates the Gα subunit to release GDP and bind GTP, thus, activating the Gα subunit and opening up effector-interaction sites in the Gβγ dimer (Rodbell et al., 1971; Gilman, 1995; Bockaert and Pin, 1999; Hampoelz and Knoblich, 2004). The expression and/or activity of the GPCR determines the level of exogenous G protein expression and subsequent GPCR-G protein-effector signaling.

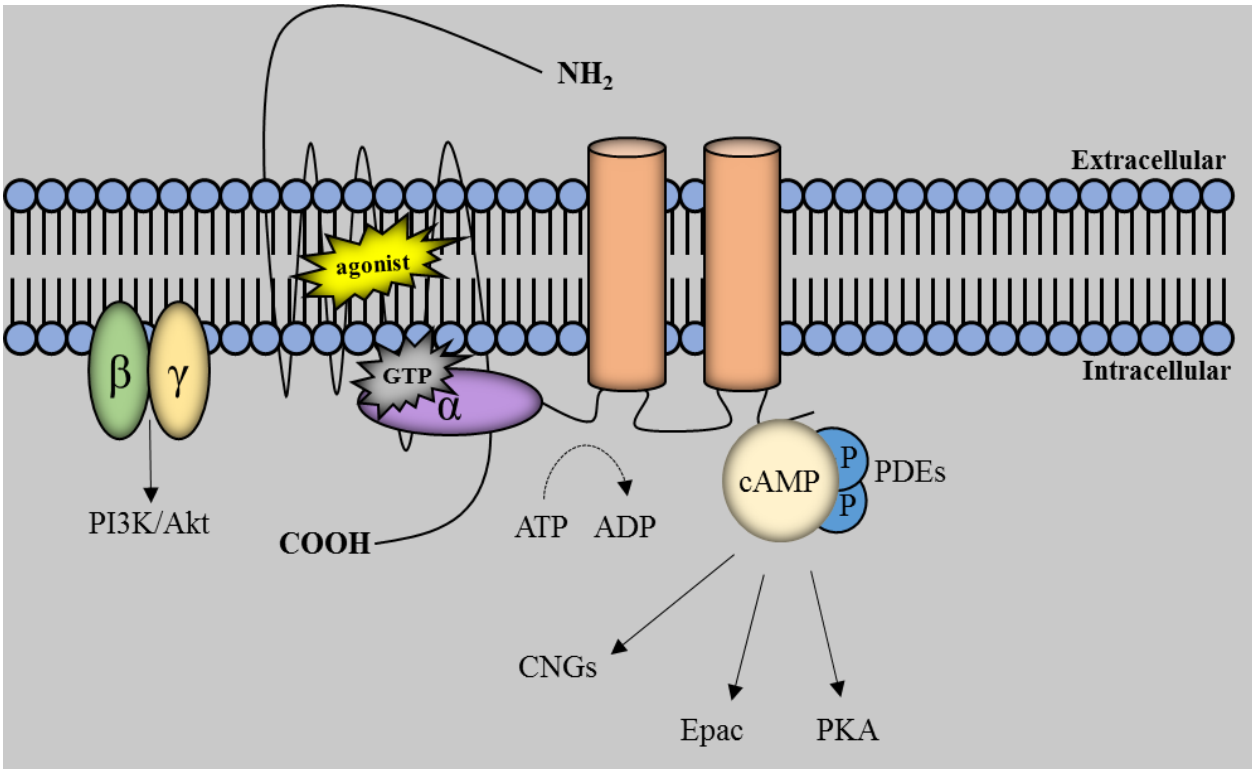

Figure 1.2. Binding of the agonist activates the β_2-adrenoreceptor and associates the heterotrimeric G protein with the third intracellular loop of the β_2-adrenoreceptor. This association results in GTP movement of GDP from the α-subunit of the G-protein, resulting in a conformational change in the protein which allows both the α- and βγ-subunits to activate downstream signaling targets including PI3K/Akt, protein kinase A (PKA), Epac, and cyclic nucleotide-gated (CNG) signaling pathways. The cAMP signal in stopped through its hydrolization to 5'-AMP by phosphodiesterases (PDEs). Adapted from Lynch and Ryall, 2008. Physiol. Rev. 88:729-767.

Traditionally, β-adrenoreceptor agonists (β-agonist) have been used for the treatment of bronchial conditions including asthma and other pulmonary disorders through their capability to dilate tubes and air passages (Fernandes et al., 2004). However, work by Emery and others

(1984) promptly demonstrated the ability of some β-agonists to increase skeletal muscle mass and decrease body fat. These findings sparked the study of β-agonist administration in both animal and human models in an attempt to develop new therapeutic strategies for muscle wasting disorders (Carter et al., 1991; Maltin et al., 1993; Kissel et al., 1998; Lynch et al., 2001; Ryall et al., 2007). Competitive bodybuilders and athletes were also using β-agonists to decrease body fat and build muscle mass for strength-related sports. In addition to its functional application in humans, the use of β-agonists proved advantageous in the livestock industry as well, with administration resulting in alterations to feed efficiency and meat quality.

The potential effects of β-agonists on skeletal muscle have been the object of extensive research in the field of animal science over many years showing significant impact on growth of striated muscle. Past investigators determined that β-agonists operate through β-adrenergic receptors to decrease lipogenesis and increase lipolysis (Liu and Mills, 1989; Dunshea, 1993; Mersmann, 1998). While results are dependent upon species, dosage amount, and length of treatment, ruminants receiving β-agonists typically have leaner muscle due to reduced fat accumulation and adipose tissue growth (Moody et al., 2002; Sissom et al., 2007).

The β-agonist ractopoamine hydrochloride (RAC), which will be the focus of this discussion, has been approved for use in the United States and several other countries as an in-feed ingredient to increase lean muscle growth and improve production efficiency in pigs (Paylean®) and cattle (Optaflexx®) (Dunshea et al., 2005; Dikeman, 2007). Findings suggest RAC supplementation 30-50 d before slaughter improves growth rate, time on feed, dressing percentage, and carcass composition, with little to no detrimental influence on meat quality and sensory traits including marbling, color, and firmness (McKeith et al., 1994; Dunshea and Gannon, 1995). These results are attained by way of β-adrenergic receptors, allowing RAC to

influence cellular metabolism through signaling cascades. Fat deposition, for example, is altered relative to the density of available β-adrenoreceptors present in tissues. While an increase in RAC administration has been shown to decrease the density of receptors present in adipose tissue in ruminants, this number remains unchanged in pigs (Spurlock et al., 1994; Mills, 2002). Muscle cells also express β-adrenergic receptors that transfer signals from β-agonists to muscle metabolic enzymes. Bovine satellite cells isolated from the SM have decreased mRNA expression and protein content of β-adrenergic receptors after β-agonist treatment, demonstrating the possibility for altered muscle cell response time (Miller et al., 2012).

There exists conflicting results on how treatment of RAC impacts protein synthesis and degradation in beef muscle. Different β-agonists have been shown to increase the activity of the major skeletal muscle protease inhibitor calpastatin. Steers receiving ractopamine (30 ppm) for the last 30 d on feed have increased calpastatin activity when compared with control animals. (Strydom et al., 2009). However, calpain 1 and calpain 2 are not influenced by β-agonist treatment in their study. Conversely, Weber and others (2013) observed no effect of RAC treatments (8.33 mg/kg of feed) for 25 d in the LM from cull cows on desmin degradation. Differential effects observed on post mortem proteolysis following RAC supplementation may be due to disparities between animal populations, dosage, and/or length of treatments among studies. It is not surprising then, that subsequent effects of RAC on meat quality are equivocal.

Although RAC supplementation may improve conformation and yield traits, it has been shown to cause variable and undesirable changes in meat quality traits (Scramlin et al., 2010; Van Donkersgoed et al., 2011). Several studies have supplemented RAC at 200 mg/d/steer or heifer for 28 to 33 d (Quinn et al., 2008; Gonzalez et al., 2009; Strydom et al., 2009; Scramlin et al., 2010; Arp et al., 2014). If treated for no more than 28 d, Arp and Quinn reported no

difference in LM Warner-Bratzlet shear force (WBSF) values were observed when compared to control animals. Gonzalez and others (2009) reported a large (21%) fiber type switch in the *vastus lateralis* (VL) from type I to type IIA fibers, as well as a detrimental effect on lean color and surface discoloration of VL steaks (aged 13 d) during the last 3 d of a 5-d retail display study. However, if kept at the same RAC dosage level (200 mg/d) and treated for a longer period (30 to 33 d), Scramlin and Strydom observed increased WBSF values in the LM and *semitendinosus* (respectively) from beef from feedlot cattle aged 7 d. However, after 14 d of aging, no tenderness differences remained when compared to WBSF values from control animals in both studies. Arp and others (2014) also compared RAC treatments at increased dosages of 300 and 400 mg/d/steer for 30 d of feeding, and found that steaks aged 14 d have higher WBSF values than those steaks from the LM of steers receiving only 200 mg/d. Taken together, these findings demonstrate how many different factors influence tenderness including RAC dosage level, length of RAC administration, aging time, muscle fiber type, and muscle location.

Anabolic Implants

Anabolic steroid implants are used in the feedlot industry to increase growth rate and improve feed efficiency by increasing protein synthesis and/or decreasing protein degradation. Early on, the mechanisms responsible for increased muscle growth were uncertain, but they were found to have direct effects on protein accretion and degradation in the skeletal muscle cell of mice, as well as to influence hormone concentrations that had consequent anabolic effects in muscle (Buttery et al., 1978; Flaim et al., 1978; Roader et al., 1986). Presently, several mechanisms have been hypothesized by which implants increase skeletal muscle growth including stimulation of insulin-like growth factor-I (IGF-I), activation of quiescent satellite cells, regulation of cell function through GPCR binding, and phosphatidylinositol 3-kinase

(PI3K/Akt) intracellular signaling pathways (Johnson et al., 1998b; Dunn et al., 2003; Revankar et al., 2005; Cheskis et al., 2007; Prossnitz et al., 2007; Pampusch et al., 2008; Dubois et al, 2012). Although these mechanisms in anabolic steroid stimulated muscle growth in beef cattle are not known with certainty, recent studies utilizing anabolic implants have observed significant improvement in performance characteristics and carcass quality attributes including average daily gain (ADG), feed efficiency, hot carcass weight (HCW), REA, marbling, and satellite cell number in the LM.

Cattle producers continue to face high feed costs, and decreasing cost of gain by improving feed efficiency through the use of technological advancements in anabolic implant growth-promoting programs is extremely valuable during these times. Depending on the anabolic implant strategy chosen, implanting cattle during the finishing period has been shown to increase ADG from 16 to 20% and decrease the feed-to-gain ratio from 6.2 to 13.5% (Duckett and Pratt, 2014). Different implant regimens are utilized in beef production systems. One common strategy is to implant at d 0 followed by a second implant within 70 to 80 d containing a greater dose of anabolic steroid. Another program option involves a single implant with delayed release technology to gain the same effect as the double implant. Estrogenic, androgenic, and combination anabolic implants approved for use in feedlot heifers and steers for increased rate of weight gain and improved feed efficiency are presented in Table 1.1. Two main hormones, estradiol (E_2), trenbolone acetate (TBA) are administered alone or in combination to achieve the effects observed in beef cattle. Estrogen, progesterone, and testosterone are natural hormones already present in beef cattle regardless of whether or not they have been treated. Trenbolone acetate is a steroid compound with a binding affinity for the androgen receptor 5 times as high as that of testosterone (Bauer et al., 2000; Wilson et al., 2002; Ankley et al., 2003; Durham et al.,

2006). The receptor-mediated action of TBA has recently been shown to increase cell proliferation through muscle IGF-I (Kamanga-Sollo et al., 2008; Pampusch et al., 2008).

Estrogenic implants, administered once or twice during the finishing period, increase HCW 3 to 5% over that of nonimplanted controls (Duckett and Pratt, 2014). However, a common type of anabolic implant, often referred to as a combination implant, used by the beef industry containing both E_2 and TBA has been reported to increase HCW by 6 to 7.5%. Combination implants also influence REA (7.4 to 9% increase) when compared with implanting with estrogen once or twice (2.8 to 4.8%). Taken together, these findings show how growth promoting implants provide considerable improvements in production efficiencies to the beef cattle industry (Webb et al., 2002; Folmer et al., 2009; Prouty and Larson, 2010).

The use of anabolic implants in growth promoting programs has also been shown to influence marbling, tenderness, and consumer ratings of beef palatability. A single estrogenic implant lowers marbling scores on average 3.7% when compared with nonimplanted animals (Duckett and Pratt, 2014). Combination and re-implanting treatments (1 or 2) result in even greater reductions in mean marbling scores from commercially fed cattle (Platter et al., 2003; Schneider et al., 2007; Boles et al., 2009). Research conducted by Duckett and others in 1999 indicates a dilution effect, with intramuscular lipid amount and composition altered through increased REA as a result of implanting treatment. However, the influence of anabolic implants on marbling remains unclear as several studies have found similar marbling scores between implanted and non-implanted cattle (Gerken et al., 1995; Johnson et al., 1996).

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.

The timing of implant administration has been shown to result in different degrees of marbling modification. Delaying a single combination implant may reduce marbling score to a greater extent than those administered earlier and throughout the finishing phase (Scheffler et al., 2003). Re-implantation halfway through the finishing phase has also been shown to decrease marbling when compared with single initial implant programs (Duckett et al., 1996; Roeber et al., 2000). It is important to note the findings regarding marbling scores in these studies were also highly correlated with their respective LM growth. The muscle area growth occurs through cell functional changes; however, studies show anabolic compounds do not alter lipolysis rates, intramuscular adipocyte content, or mRNA levels of key lipogenic enzymes (Green et al., 1992; Waylan et al., 2004; Smith et al., 2007.) In fact, implanting has actually been shown to down-regulate certain lipogenic genes in a recent investigation of the adipocyte transcriptome identifying 36 genes differentially expressed due to implant treatment (Duckett et al., 2012). Combination of implant strategies with enhanced marble deposition periods during the finishing period is a potential way to mitigate effects of implants on marbling score.

Results evaluating the influence of implanting on muscle tenderness, trained sensory, and/or consumer sensory panels appear to be different across implant regimen, as well as management and processing strategy. Some studies report decreases in tenderness in implanted cattle when compared with non-implanted cattle (Samber et al., 1996; Duckett et al., 1997; Morgan, 1997), while others observed no effects of implantation on tenderness (Gerken et al., 1995; Pruneda et al., 1999; Kerth et al., 2003; Schneider et al., 2007). Generally, when cattle were implanted once, authors observed little to no detrimental effects on tenderness, but WBSF values increased linearly as dosage increased. Similarly, combination implants and re-implantation negatively influenced tenderness to a greater extent when compared with cattle

receiving only 1 implant. Palatability traits including sustained juiciness, flavor intensity, and overall mouth feel were influenced in a similar manner. The decreased tenderness observed may be due to decreased protein degradation in animals with greater muscle area growth, causing a reduction in the effect of aging on post mortem proteolysis and subsequent tenderization. However, aging of meat has been able to alleviate many of the detrimental effects on meat tenderness and palatability attributes (Schneider et al., 2007; Igo et al., 2011). Together, these findings suggest marbling and associated palatability traits are differentially influenced depending on implant treatment strategy. Different animals also have different responses to implant programs, indicating their individual contribution to global meat quality variability.

The Use of Proteomics in Meat Science

Proteomics, or the study of proteomes and their functions, has recently taken a major role in the advancement of animal science research. Specifically, the application of proteomics in meat science has revealed an unparalleled ability to identify and quantify thousands of proteins important to the improvement of meat quality attributes in our livestock species. This section will discuss the process of 2 dimensional in-gel electrophoresis (2D DIGE) coupled with mass spectrometry (MS) in the execution of a proteomic experiment, as well as the application of proteomics in meat science research.

Proteomic Technologies

Proteomic experiments can focus on a certain known protein of interest, or an indirect approach can be taken. When searching for unidentified proteins in a shotgun approach, large complex proteomes are analyzed for changes in protein presence, expression, and modification. There are several types of gel electrophoresis procedures utilized in proteomic experiments, with the most common being 2D DIGE. This type of gel electrophoresis separates proteins by 2

different physical attributes (Stasyk and Huber, 2004; Elrick et al., 2006). First, proteins are separated by their isoelectric points (pI) in an isoelectric focusing (IEF) gel. Different IEF gels offer several pH ranges in order to provide different levels of resolution. Proteins are typically pipetted onto IEF gels, commonly found in the form of immobilized pH gradient (IPG) strips. The pI is the pH at which a protein has no net charge and cannot migrate further in an electric field.

Before protein samples are pipetted onto IEF gels, the lysine groups on proteins can be labeled with 1 of 3 spectrally-resolvable fluorescent cyanine dyes (CyDye) for further comparative analysis of multiple samples on the same gel (Figure 1.3). The CyDye DIGE fluor minimal dye has an *N*-hydroxysuccinimidyl ester reactive group. CyDyes are designed to form a covalent bond with the epsilon amino group of lysine in proteins via an amide linkage. The single positive charge of the CyDye replaces the single positive charge of the lysine at neutral and acidic pH, keeping the pI of the protein relatively unchanged. The labeling reaction is dye limiting and the ratio of CyDye to sample protein (100 to 300 pmol: 50 µg) ensures that the dyes label approximately 1 to 2% of lysine residues. Therefore, each labeled protein carries only 1 dye label and is visualized as a single protein spot. Labeling of 2 to 3 samples with different CyDyes on the same 2-dimensional gel reduces spot-to-spot variability and the number of gels in an experiment, while allowing more accurate spot matching (Tannu and Hemby, 2006). Typically, a pooled internal standard is labeled with cyanine 2 yellow dye (Cy2) and the treatment or control groups are labeled with either cyanine 3 red dye (Cy3) or cyanine 5 blue dye (Cy5). The individual protein data from control and/or treatment samples are then normalized against the Cy2-labeled standard. The internal standard serves the purpose of decreasing biological and

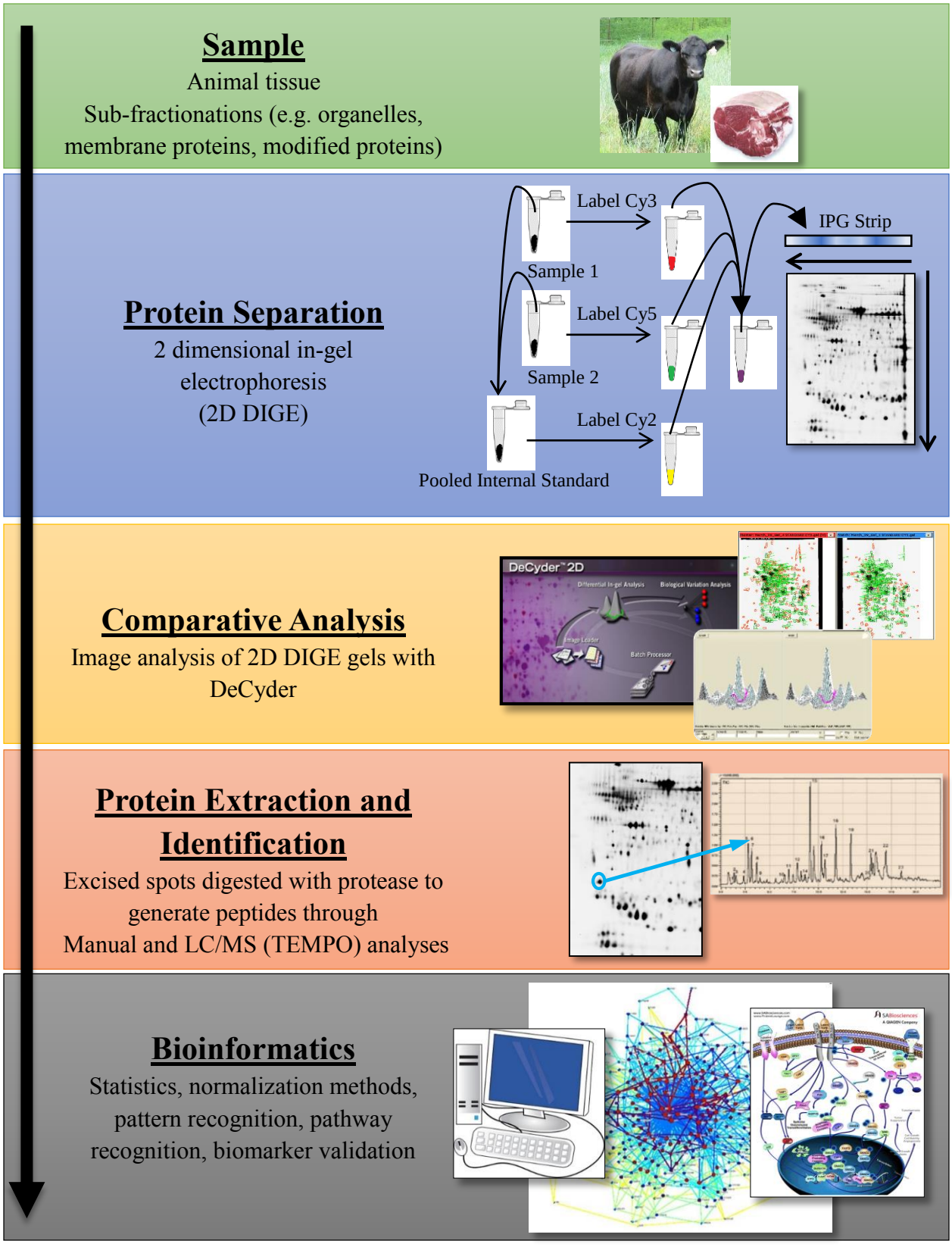

Figure 1.3. Schematic of the different steps in the proteomic work flow using 2 dimensional in-gel electrophoresis (2D DIGE) and mass spectrometry.

experimental between-gel variation and improves statistical analysis. Once sample proteins are labeled they are pipetted onto IPG strips and an electrical field is applied. The sample containing a mixture of CyDye-labeled proteins migrates through the pH gradient, whereby individual proteins become immobilized in the pH gradient as they approach their specific pI.

After proteins have migrated in the gel to their pI, the gel strips are placed directly on top of a denaturing polyacrylamide gel, and proteins are run into the gel and separated on the basis of molecular weight (Figure 1.3). Running of proteins in their second dimension results in hundreds of protein spots on a gel where IEF separation is left to right and up and down separation is molecular weight (Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis). Three scanned images of each 2D DIGE gel are taken corresponding to each of the Cy2, Cy3, and Cy5-labeled samples, respectively. DeCyder software developed for the DIGE system (DeCyder™ Amersham Biosciences, Piscataway, NJ) is typically used to analyze the images. Spot intensities from the Cy3 and Cy5 images are normalized using the corresponding Cy2 spot intensities for each gel. The standardized abundances can then be compared across groups to detect changes in protein expression, independent of whether the samples to be compared have been resolved on the same 2D DIGE gel. Running of repetitive gels with swapping of CyDyes can help to minimize dye-specific effects that may result from labeling technique or different fluorescent characteristics of acrylamide at the different wavelengths of excitation for Cy2, Cy3, and Cy5, particularly at low protein spot volumes. The number of duplicate gels in an experiment must take into account the variation typically seen in the sample group being investigated. This approach allows the measurement of more subtle protein expressional differences with increased statistical confidence (Lilley and Friedman, 2004).

Utilizing the DeCyder software, spots of interest may be collected based on differential spot abundance between groups compared. Spots of interest are typically chosen by looking for significant change (less than 0.05%) across groups using a Student's t-test or ANOVA (Fodor et al., 2005). Once proteins of interest are determined, duplicate 2D DIGE gels are run with an unlabeled protein load representing all samples investigated in the experiment (often an increased protein load of the pooled standard). After running the duplicate gels in the first and second dimension similar to the methods described above, they are typically stained with a colloidal Coomassie blue staining method (Candiano et al., 2004) intended for high-sensitivity protein visualization on 2D DIGE gels. From Coomassie-stained gels, proteins of interest are manually picked for further identification by MS.

Mass Spectrometry

The idirect experimental approach often leads to a large data set of protein expression with the capacity to produce unexpected results and fuel hypothesis-driven research. The sensitivity of the mass spectrometer and the number of proteins it can identify are crucial to strength of proteomic research. Manually excised proteins are first subjected to proteolytic digestion, followed by individual peptide mass determination via MS. Weights of individual peptides coupled with database searches of known protein sequences and enzyme cleavage locations are then used to establish protein identity. Studies in this dissertation utilized both manual and liquid chromatography/MS (TEMPO) analyses. However, there are many types of mass spectrometers that can be used for proteomic studies, and each has the ability to identify peptides in a slightly different ways. (Moyer et al., 2003; Lippolis and Reinhardt, 2008).

Protein identification is an essential step to understanding the functional roles of proteins in the skeletal muscle cells and their corresponding relevance in muscle growth and

development. However, MS methods for the identification of proteins in the field of animal/meat science is met with numerous challenges. The majority of the protein identification software [e.g., Universal Protein resource (UniProt), MASCOT] performs under the assumption that the peptides being sequenced exist in the protein database, and when this is not the case, the software will locate the closest matching peptide in the database. Unfortunately, current agricultural animal protein databases are considerably incomplete, resulting in mismatched proteins and insufficient protein identification. As current and future sequencing projects for livestock species are completed and annotated, the quality and quantity of protein identifications will increase (Lippolis and Reinhardt, 2008).

The complexity of bovine skeletal muscle tissue also proves challenging, particularly when trying to identify low-molecular weight peptides. A large number of membrane-associated proteins, combined with many muscle components demonstrating very high molecular mass, make it increasingly difficult to identify more minor protein changes during physiological adaptations or cellular processes. These changes, while diminutive, may hold valuable information with regard to biochemical pathways governing skeletal muscle cell functions and corresponding protein interactions. Further isolation and separation of proteins may facilitate protein identification with the aim of identifying entire muscle biology proteomes.

Applying Proteomics in Meat Quality Research

Growth and development

Proteomic studies of bovine and porcine myogenesis (Chaze et al., 2008; Rajesh et al., 2011; Xu et al., 2013) have assembled a proteome profile including metabolic enzymes (e.g. enolase, aldehyde dehydrogenase, lactate dehydrogenase, malate dehydrogenase), contractile and structural proteins (e.g. myosin, actin, tubulin, desmin), stress proteins (e.g. peroxiredoxin,

superoxide dismutase, heat shock proteins) associated with protein synthesis and myogenic differentiation of satellite cells. The process of myogenesis is well known, but many of the mechanisms involved in the regulation of skeletal muscle cell proliferation still remain unclear. The findings from these proteomic studies highlight proteins that control cell cycle activity and apoptosis, as well as cellular metabolism and cell organization. Proteomic analysis of bovine skeletal muscle hypertrophy by Bouley and others (2005) studied myostatin deletions in Belgian Blue bulls to better understand the role of myostatin in muscle growth. After analysis of bulls with a myostatin deletion, the authors were able to identify 13 proteins, corresponding to 28 protein spots that were significantly altered in response to the loss of myostatin function. The proteomic approaches utilized in these animal investigations help build a foundation of myogenic proteins that stimulate hypothesis-driven research to enhance our understanding of muscle development in meat producing animals.

The proteomic approach has also been applied to examine changes in protein expression influenced by pre-slaughter conditions. One such area of recent interest is adverse effects on meat quality resulting from pre-slaughter stress (Franco et al., 2015). The authors from this study were able to identify 10 differentially expressed structural-contractile skeletal muscle proteins and metabolic proteins associated with pre-slaughter stress. Identifying protein patterns and/or individual proteins at the level of the proteome that can influence the quality of the final product, as carried out in this study, shows the value of proteomics-based methodologies in livestock species.

Post mortem changes

Proteomic approaches are being used extensively to study bovine post mortem changes to improve our current understanding of biochemical mechanisms controlling muscle changes after

slaughter that influence meat quality attributes (Jia et al., 2007; Laville et al., 2009; Zapata et al., 2009; Bjarnadóttir et al., 2010; Zhang et al., 2010). Investigations have identified structural proteins, metabolic enzymes, and cellular defense/stress proteins having a role in post mortem beef tenderness. For example, the investigation of proteome changes of the insoluble and soluble protein fraction in bovine LM during post mortem storage revealed a connection between the stability of myofibrillar proteins and the solubility of metabolic enzymes (2,3-bisphosphoglycerate mutase and NADH dehydrogenase) and cellular defense/stress proteins (heat shock protein 70 kDa) (Bjarnadóttir et al., 2010).

While the identification of single proteins is important to our understanding of the conversion of muscle to meat, there has recently been investigative focus into the influence of protein isoforms on meat quality traits (Choi et al., 2010; D'Alessandro et al., 2012). Post-translational regulation is a key process which influences protein functionality. Proteomic studies have identified different heat shock (Hsp) and troponin-T isoforms for their corresponding proteins which could relate to post-translational modifications (Chaze et al., 2009) in LM beef muscle. Among post-translational modifications, phosphorylation (Shen et al, 2006; Muroya et al., 2007; Underwood et al., 2008; Li et al., 2012), glycosylation (Iqbal et al., 1999), oxidation (Bernevic et al., 2011; Lund et al., 2011), and ubiquitination/sumoylation (Sekikawa et al., 1998) have been shown to play major roles in post mortem metabolism of skeletal muscle. The findings from these proteomics studies demonstrate the post-translational modifications of proteins may be as important as total protein in determining mechanisms governing post mortem tenderization. Taken together, these investigations will improve slaughter processing procedures that have the ability to produce higher quality beef for consumers.

Sensory quality

Color

Meat color is a quality attribute considered by consumers as an indicator of spoilage or bad hygienic quality; thus, it is considered one of the most economically important traits to be controlled for fresh meat. Myoglobin is the pigment primarily responsible for meat color, and recent studies utilizing proteomic technologies have investigated the redox stability of myoglobin and color of fresh meat (Sayd et al., 2006; Guillemin et al., 2011; Pearce et al., 2011; Joseph et al., 2012). Darker meat has been positively correlated with increased expression of oxidative mitochondrial enzymes of the respiratory chain, hemoglobin, and chaperone or regulatory proteins (Hsp 27, αβ crystalline, and glucose related protein), while lighter meat has been associated with overabundant glycolytic enzymes (glutathione-S transferase). The sarcoplasmic proteome from beef LL showed greater expression in 3 glycolytic enzymes (phosphoglucomutase-1, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase) positively correlated to redness. In this study, phosphorylation or possibly other post-translational modifications of myoglobin were suggested to be regulators of beef color stability due to the discovery of different isoforms of phosphoglucomutase-1 and glyceraldehyde-3-phosphate dehydrogenase identified in the LL sarcoplasmic proteome (Canto et al., 2015).

Several proteomic studies have focused on identifying molecular properties of unstable and color-stable beef muscle. Findings from these studies highlight the major role of oxidation processes in meat color and its stability during storage. Oxidation of meat is caused by the production of reactive oxygen species (ROS) resulting from environmental stress, such as handling conditions. Skeletal muscle antioxidant enzymes and vitamins resist oxidation through the development of cellular mechanisms for detoxification of free radicals produced by ROS.

Findings by Rowe and others (2004) indicate increased oxidation of muscle proteins early post mortem may have negative effects on fresh meat color. Antioxidant enzymes observed in their study were suggested to possibly protect proteases from oxidation and inactivation preventing darker meat color. Sarcoplasmic proteome analysis of beef LM revealed higher abundance of anti-oxidant and chaperone proteins including peroxiredoxin-2, peptide methione sulfoxide reductase, and Hsp 27 in color-stable meat from 7 beef carcasses collected 24 h post mortem (Joseph et al., 2012). Collectively, 2D DIGE and MS methods are discovering variations in the beef proteome that contribute to the necessity of further development of animal production and meat processing strategies to improve beef color.

Intramuscular fat

Proteomic approaches have also been applied to improve knowledge of intramuscular fat (IMF) content which plays an important role in eating quality and acceptability of beef. More than 80% of IMF is stored in adipocytes found throughout the perimysium and less than 20% of IMF is located within the cytoplasm of myofibers (Grobert et al., 2014). 2D DIGE has been utilized to characterize adipocytes according to their location (intramuscular, intermuscular, subcutaneous, and perirenal) and 149 spots have been identified as differentially expressed between intramuscular adipocytes and the fat cells obtained from the 3 other adipose locations (Gondret et al., 2008). Proteins downregulated in IMF cells including malate dehydrogenase, isocitrate dehydrogenase, enolase, aldolase, perilipin, long chain fatty-acyl CoA dehydrogenase, and electron flavoprotein indicate a decreased glycolytic metabolism when compared with adipocytes from the 3 other adipose locations.

Early adipogenesis and the regulation of IMF development was investigated with 2D DIGE and MS in bovine skeletal muscle demonstrating higher IMF scores (Liu et al., 2009;

Zhang et al., 2010). Carbonic anhydrase 2 and myosin light chain 3 were found to be down regulated during adipogenic differentiation in the *longissimus dorsi* from Korean beef steers, revealing their potential as regulators of IMF development. Lipid deposition has been shown to have inter-individual variability, and many environmental factors (i.e., reproduction, nutrition, breed, sex) certainly influence IMF development. However, these studies and several others (Kim et al., 2008; Katsumata, 2011) increase our knowledge of the expression patterns and cellular processes behind bovine lipid deposition and contribute to current development of biomarkers for meat quality.

Tenderness

Of all meat quality traits investigated through proteome analysis, tenderness has been investigated the most, likely due to it being the primary and most important quality attribute to consumers. The biochemistry behind bovine meat tenderness, despite decades of research, remains a topic of interest. Proteomics investigations on bovine meat tenderness have recently focused on the role of structural proteins, glycolytic enzymes, Hsp/chaperones, and proteins related to apoptosis in post mortem meat tenderization (Kim et al., 2008; Morzel et al., 2008; Jia et al., 2009; Laville et al., 2009; Zapata et al., 2009; Bjarnadóttir et al., 2010; Polati et al., 2012; Longo et al., 2015).

Monitoring post mortem changes at 0, 1, 10, 17, and 44 d of bovine LM through 2D DIGE techniques coupled with MS spot identification, Longo and others (2015) analyzed biochemical changes in the aging of meat and their direct connection to apoptotic events that have been shown to heavily effect the ultimate meat product, particularly tenderness. The authors propose pathways linking their proteomics results to apoptosis as a principle mechanisms involved in the aging of meat.

Many proteomic approaches may be taken to isolate and identify proteins using MS. Particularly, the use of 2D DIGE has become a powerful tool for the analysis of protein expression resulting in the identification of livestock factors that contribute to the ultimate goal of improving the quality of production.

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CHAPTER 2. THE CONTRIBUTION OF DISRUPTIVE VARIANTS OF THE PIEDMONTESE MYOSTATIN ALLELE ON CARCASS AND MEAT QUALITY TRAITS IN BEEF CATTLE

Abstract

This study examined the effects of myostatin sequence variants, C313Y and Q204X, which cause muscle hypertrophy (double-muscling) and their influence on carcass characteristics and meat quality in beef cattle. One hundred twenty-six crossbred (Angus x Piedmontese) heifers were weighed, randomly allotted to 1 of 16 pens, and fed for approximately 120 d on corn-based total mixed rations containing either no field pea, dry-rolled field pea included at 15% of diet dry matter (DM), pea hulls fed at an equivalent portion to 15% whole peas in the ration, or pea chips fed at the equivalent portion to 15% whole peas in the ration. Heifers were fed in 16 identical pens with 4 pens (replicates) in each treatment. Feed intake was recorded daily, and heifers were weighed individually every 28 d to monitor performance during the feeding period. During finishing, samples were taken from the ear of each heifer, and a genetic profile was determined by Igenity®. Due to crossbreeding, heifers were also evaluated for different sequence variants of the myostatin gene, referred to as having 0, 1, or 2 copies of the myostatin variants present. When heifers reached 14 to 16 mo of age (580 ± 43 kg), they were transported to a commercial slaughter facility. The *longissimus thoracis* (LT), *semimembranosus* (SM), *biceps femoris* (BF), and *supraspinatus* (SS) were sampled, vacuum sealed, and aged for 14 d at 4°C. Carcass data were collected after a 48-h chill. Traits analyzed were live weight; hot carcass weight (HCW); 12th rib fat depth (FT); rib-eye area (REA); estimated kidney, pelvic, and heart fat (KPH); USDA yield grade (YG); and marbling score (MARB). Minolta colorimeter measurements were taken, and meat tenderness was measured as Warner-Bratzler shear force (WBSF) at 14 d post

mortem. Data were analyzed using Proc Mixed in SAS with block and slaughter date in the model. Diet was not significant for all triats investigated and was removed from the model. The presence of myostatin allelic variants had no effect on HCW or MARB. FT was significantly lower ($P = 0.03$) in heifers with 1 and 2 disruptive myostatin variants compared with those animals having zero alterations. REA was significantly higher in those inheriting either 1 or 2 myostatin variants compared with heifers having no myostatin mutations (94.06 ± 4.95 cm^2 and 92.83 ± 4.78 cm^2 vs. 77.67 ± 11.99 cm^2, respectively). Heifers with zero disruptive myostatin variants had higher KPH ($P < 0.01$) compared with those inheriting 1 or 2 alterations to the myostatin gene. Heifers having 1 or 2 sequence variants present had higher ($P < 0.001$) YG when compared with animals with zero mutations (2.63 ± 0.12 and 2.72 ± 0.12 vs. 3.93 ± 0.28, respectively). The LT from heifers with a single disruptive myostatin variant were more tender ($P = 0.02$), as measured by WBSF, when compared with animals with zero or two copies of myostatin sequence variants. The LT of heifers inheriting 1 myostatin variant tended to have a higher a* (27.22 ± 0.32 vs. 26.15 ± 0.30) compared with animals with 2 copies ($P < 0.06$). The LT from heifers having 2 myostatin variants had lower b* (7.18 ± 0.23 vs. 8.04 ± 0.25) compared with those having only 1 copy of myostatin variants present ($P = 0.04$). The L* value of the SM was lower in heifers having 1 or 2 disruptive myostatin variants (37.43 ± 0.27 vs. 39.04 ± 0.71) when compared with animals possessing zero myostatin variants ($P < 0.03$). Our data indicate that the presence of 1 or 2 disruptive myostatin variants influences carcass and meat quality in Angus x Piedmontese crossbred heifers. This study suggests a relationship between myostatin gene alterations and beef quality.

Introduction

The growth differentiation factor-8 (GDF-8) gene is a member of the transforming growth factor beta (TGF-β) superfamily, and its expression results in the production of myostatin. First produced in skeletal muscle as a 375 amino acid protein, myostatin then undergoes proteolysis resulting in a functional myostatin. This mature form of myostatin has been shown to regulate both prenatal and postnatal myogenesis through the controlling of myoblast proliferation and differentiation during early myogenesis (muscle fiber hyperplasia), while functioning postnatally by regulating satellite cell activation (Ríos et al., 2002; McCroskery et al., 2003). Loss of functional myostatin in various cattle breeds results in a condition referred to as double-muscling, categorized by generalized hyperplasia and hypertrophy of most of the muscle.

Known to be controlled by mutations in the myostatin gene, this dramatically enhanced muscle development has been shown in homozygous cattle with 2 copies of the same mutation (Ménissier, 1982). The muscular hypertrophy causing visible heavy muscling is a heritable condition with the mutation in the myostatin coding sequence having a frequency greater than 98% in the Piedmontese cattle breed (Grobet et al., 1998). In Piedmontese, C313Y is a guanidine-to-adenine transition causing a substitution of a cysteine with a tyrosine residue (Kambadur et al., 1997; Grobet et al., 1998). This population is characteristic for having a significantly higher number of muscle fibers at birth and at slaughter, a greater proportion of muscle, and lower proportions of bone and fat. Q204X is an isolated cytosine-to-thymine substitution in the second exon of the myostatin gene (Grobet et al., 1998; Dunner et al., 2003). The presence of the myostatin variant Q204X has been shown to increase muscling, decrease fat, and decrease collagen content in meat from European beef breeds including Charolais,

Limousine, and Blonde d'Aquitaine (Phocas, 2009; Allais et al., 2010), but no studies have investigated its segregation in crossbred Angus x Piedmontese heifers.

Generally, past investigations of double-muscling in cattle indicate that beef quality traits are both negatively [tenderness, color, water holding capacity (WHC)] and positively (tenderness, feed efficiency, muscle mass, fat deposits) influenced when compared with that from normal cattle, although many of these studies exhibit differences in myostatin genotype, muscle location, and animal breed. The number of copies of mutated alleles has been reported to influence postnatal growth and associated carcass traits (Casas et al., 1999). Expressed by number of C313Y copies, Casas reported heterozygous Piedmontese x Angus and Piedmontese x Hereford dams had decreased calving difficulty, as well as heavier birth weights (3.2 ± 8 kg) and yearling weights (20.8 ± 8 and 24.5 ± 8 kg, respectively) when compared with homozygous animals.

In addition to postnatal growth, Casas and others (1998) showed that a single copy of the myostatin mutation increased hot carcass weight (HCW) and rib-eye area (REA) (+1.60 and +1. 35 relative standard deviation, respectively), while decreasing yield grade (YG), 12th rib back fat (FT), and kidney, pelvic, and heart fat (KPH) (-1.42, -0.84, and -0.86 RSD, respectively) of Belgian Blue and Piedmontese animals when compared with homozygous cattle. These findings demonstrate the variable impact the frequency of myostatin variants can have on growth and performance traits. However, no studies have investigated the effect of the Q204X myostatin variant on Angus-Piedmontese cattle already having a C313Y mutation. Therefore, the objective of this study was to determine the relative contribution of 2 disruptive myostatin variants (C313Y and Q204X) of the myostatin allele to carcass characteristics and meat quality traits of

longissimus thoracis (LT), semimembranosus (SM), biceps femoris (BF), and supraspinatus (SS) in crossbred Angus x Peidmontese cattle.

Materials and Methods

This study was conducted at the North Dakota State University Carrington Research Extension Center and North Dakota State University Main Campus, Fargo. All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee.

Animals

One hundred twenty-six crossbred (Angus x Piedmontese) yearling heifers were allotted by initial weight (427. 28 ± 6.52 kg) in a randomized complete block design and sorted into 16 identical pens. Heifers were fed for 120 d on corn-based mixed rations (Table 2.1) containing either no field pea (CON), dry-rolled field pea included at 15% of diet DM (WRL), pea hulls fed at an equivalent portion to 15% whole peas in the ration (HULL), pea chips fed at the equivalent portion to 15% peas in the ration (CHIP). Ear punch samples were taken during the finishing period for genetic profiling by IGENITY®.

Igenity Scores

Cattle were sampled using the Igenity tagging system for obtaining ear tissue samples. Samples sent to Igenity in Lincoln, NE, for analysis. Scores were determined in the fall of 2010 and reported on a scale of 1 to 10, excluding 12th-rib fat thickness and YG, whose greater score indicated a more desirable phenotype. Traits analyzed by Igenity included average daily gain (ADG), tenderness (TEND), marbling score (MARB), YG, percent choice (%CH), 12th-rib fat thickness (FT), and rib-eye area REA. Although some may not be found in all breeds of cattle, Igenity also analyzed for 9 different variants of the myostatin gene. The 3 variants of myostatin that were present in cattle from this population were C313Y and Q204X, both of which cause

muscle hypertrophy (double-muscling), larger birth weights, increased dystocia, and enhanced tenderness. Descriptive statistics of Igenity scores are presented in Table 2.2.

Table 2.1. Finishing rations for beef cattle formulated with field pea and pea components.

	Diet Treatments[1]			
Item	CON	HULL	CHIP	WRL
Feeds	(%, DM basis)			
Straw, chopped	16	16	16	16
Corn grain, dry rolled	58	56	55.25	54
Field peas, dry rolled	0	0	0	15
Pea chips	0	0	12.75	0
Pea hulls	0	1.67	0	0
Dry distillers grains	18	18.33	8	7
Calcium carbonate	1	1	1	1
Rumatec Supplement- 1/3 lb	2	2	2	2
Condensed distillers solubles	5	5	5	5
Total, percent %	100	100	100	100
Nutrients				
DM, %	84.88	84.98	84.98	84.89
Net Energy Gain, Mcal/lb	60.61	60.26	60.87	60.61
CP[2], %	12.56	12.63	12.60	12.62
Calcium, %	0.62	0.63	0.63	0.64
Phosphorus, %	0.33	0.33	0.34	0.34
Potassium, %	0.61	0.61	0.65	0.65

[1]Dietary treatments included the replacement of corn with pea hulls (HULL), pea chips (CHIP), or field peas (WRL).
[2]CP = crude protein.

Table 2.2. Descriptive statistics of Igenity scores for cattle.

Igenity Score	n	Mean	SD	Range
ADG[1]	125	5.3	1.0	2 to 7
Tenderness	125	5.5	1.7	3 to 10
Marbling	125	5.9	1.2	3 to 9
Percent Choice	125	5.9	1.2	3 to 9
Yield grade	125	6.0	0.9	3 to 8
12th-rib fat thickness	125	6.0	0.9	3 to 8
Rib-eye area	125	4.8	1.0	3 to 8

[1] ADG = average daily gain.

Carcass Data

At approximately 14 to 16 mo of age cattle were delivered to a commercial packing facility in two groups on September 13, 2010 and September 20, 2010 (North Dakota Natural

Beef, New Rockford, ND). Carcass measurements including HCW, REA, YG, FT, and MARB were obtained at 48 h post mortem and 7-cm thick LT, SM, BF, and SS samples were placed in labeled re-sealable zipper storage bags, placed in a cooler with wet ice, and transported to the North Dakota State University meat laboratory. Meat samples were unpacked upon arrival, and 2.54-cm thick steaks were cut from collected samples, vacuum sealed, aged for 14 d at 4°C, and then frozen until Warner-Bratzler shear force (WBSF) analysis.

Instrumental Color Measurement

Instrumental readings of d 14 steak portions from the LT (n = 88), SM (n = 94), BF (n = 90), and SS (n = 71) were measured upon arrival to North Dakota State University meat laboratory using a Minolta colorimeter calibrated against a standard white tile immediately before data collection (Konica Minolta, NJ, USA). The L^*, a^*, and b^* values of each steak were determined from the average of three readings on the cut surface using illuminant D65, a 5-cm aperture, and a 2° standard observer after a minimum 15 min bloom time.

Warner-Bratzler Shear Force

Steaks from the LT, SM, BF, and SS of each animal were allowed to thaw for 16 h in a 4° commercial refrigerator before removal from vacuum packages and then allowed to come to room temperature (approximately 18°C) before being weighed in preparation for cooking and shear force measurements. A copper-constantan thermocouple (Omega Engineering Inc., Stamford, CT) was inserted into the geometric center of the steak. Steaks were cooked on clamshell-style grills (George Foreman grill Model No. GRP99, Columbia, MO) to an internal temperature of 71°C and then removed from the grill and allowed to cool to room temperature (approximately 21°C). Steaks were weighed again, and cook loss was calculated using the equation [(final cooked weight – raw weight)/(raw weight)] x 100. Six, 1.27-cm cores were taken

from each steak parallel to the muscle fibers and sheared once perpendicular to the muscle fibers for measurement of tenderness using a WBSF machine (G-R Manufacturing, Manhattan, KS) (AMSA, 1995). The WBSF values from the 6 cores within each steak were averaged before statistical analyses.

Statistical Analysis

Data were analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) with slaughter date and pen included in the model upon analysis. Diet had no effect and was dropped from the model. Means were separated using the LSMEANS option of SAS and were considered significant when $P \leq 0.05$.

Results and Discussion

Carcass Characteristics

Least squares means and standard errors calculated for carcass characteristics of beef heifers having the occurrence of disruptive myostatin variants C313Y and Q204X are presented in Table 2.3. Live weight, HCW, and MARB did not differ between heifers with 0, 1, or 2 copies of myostatin variants ($P > 0.09$). In disagreement with our study, Wiener and others (2009) saw an increased HCW (14.1 ± 6.45 kg per allele copy) in South Devon cattle with an 11-bp (nt821) deletion in the myostatin gene when compared to non-carriers. In contradiction to these data are past investigations on Angus cattle demonstrating, that, although double-muscled calves are born heavier, there is a significant reduction in growth rates leading to a reduced feed intake compared with control animals (Nott and Rollins, 1979). Consistent with our results, studies of Belgian Blue cattle have not revealed a significant difference in growth rates between double-muscled and non-double-muscled individuals (Hanset et al., 1987; Hocquette et al., 1999). Differences in myostatin alleles investigated appear to result in variable effects on beef carcass characteristics.

Conflicting data could also be due to breed and/or management differences. Further investigation into the interaction between myostatin sequence variants, genetic background, diet, and production strategies is needed to explain these differences.

Table 2.3. Carcass characteristics of heifers having the occurrence of disruptive myostatin gene variants C313Y and Q204X.

Item	No variant	C313Y	C313Y and Q204X	*P*-value
Live weight, kg[A]	589.77 ± 11.5	578.71 ± 5.33	568.27 ± 5.2	0.09
Hot carcass weight, kg	359.10 ± 8.39	358.30 ± 3.58	356.30 ± 3.46	0.09
Adj. fat thickness, cm	1.73^{a} ± 0.13	1.37^{b} ± 0.06	1.38^{b} ± 0.06	0.03
Marbling score[B]	435.90 ± 21.12	403.50 ± 8.74	399.30 ± 8.40	0.28
REA[C], cm^2	77.68^{b} ± 11.99	94.06^{a} ± 4.98	92.84^{a} ± 4.77	< 0.01
KPH[D], %	2.90^{a} ± 0.18	2.30^{b} ± 0.07	2.48^{b} ± 0.07	< 0.01
USDA yield grade[E]	3.93^{a} ± 0.29	2.63^{b} ± 0.13	2.73^{b} ± 0.12	< 0.001

Within a row least squares means lacking a common superscript letter differ ($P < 0.05$).
[A] No variant (n = 9), C313Y (n = 50), C313Y and Q204X (n = 55). Hiefers fed for 120 d with corn-based total mixed rations containing the inclusion of field peas, pea hulls, or pea chips.
[B] 300 to 399 = slight; 400 to 499 = small; 500 to 599 = modest, 600 to 699 = moderate.
[C] REA = rib-eye area.
[D] KPH = kidney, pelvic, and heart fat.
[E] Calculated yield grade = 2.5 + (2.5 x adjusted fat thickness, 12^{th} rib, cm) + (0.0038 x hot carcass weight, kg) + (0.2 x KPH) – (0.32 x REA area, cm^2).

Concerning muscle composition, we observed no effect of myostatin alleles on MARB. However, findings by several groups have established an effect of myostatin genotype on carcass fat (Shahin and Berg, 1985; Clinquart et al., 1998; Wiener et al., 2002; Casas et al., 2004). Intermuscular fat increased ($P < 0.05$) in Hereford and Beef Synthetic populations when compared with double-muscled animals in a study conducted by Shahin and Berg in 1985. Unique to their study, researchers observed how the maturity of the animal affected fattening patterns, fat partitioning, and overall fat deposition through the progression of growth and development. Both genetic differences and age influence animals' ability to produce and store fat. Some breeds deposit fat at lighter and younger ages (Hereford, Angus), whereas others tend to store fat at older, heavier weights (Charolais). Similarly, Casas et al. (2004) examined the

effect of inactive myostatin alleles within sire breeds (Charolais and Belgian Blue x British Breed), and found that all animals with 1 or 2 copies had lower MARB ($P < 0.001$) when compared with animals having zero inactive myostatin alleles. Our contrasting results could be explained by the frequency of myostatin mutations in breeds from other studies when compared with those from the present study. The populations used in the Casas (2004) study were not directly tested to determine the myostatin genotype, and, in the present study, there was a low number of animals having zero myostatin variants. In addition, other studies of double-muscling in crossbred populations using the Piedmontese breed as a source of inactive myostatin alleles have reported lower-than-predicted representation of homozygous individuals (Casas et al., 1999; Short et al., 2002).

In the present study, FT and kidney, pelvic, and heart fat were higher ($P < 0.03$ and $P < 0.01$, respectively) in heifers with no myostatin variants present when compared with groups carrying 1 or 2 copies. Wiener et al. (2002) reported an additive effect on muscle score (1-15, 1.096 ± 0.39, $P < 0.005$), muscle depth (1.14 ± 0.91 mm), and fat depth (-4.057 ± 1.02 mm, $P < 0.0005$) in 321 UK South Devon cattle, both male and female, with an 11-bp mutation within the GDF-8 gene. In agreement with these data, Casas and others (1999) observed animals from a Charolais sire breed population that inherited zero copies of the inactive (11-base pair deletion) myostatin allele displayed more fat thickness, fat yield, fat weight, and KPH fat ($P < 0.05$).These data support the view that disruptive sequence variants in the myostatin gene are associated with overall reduced carcass fat levels.

Characteristic of the Piedmontese breed, the predominant mechanism in enhanced muscle growth causing double-muscling is increased protein synthesis. In the present study, REA was larger ($P < 0.01$) in heifers with 1 or 2 copies of myostatin variants when compared with animals

with no alterations. Prior studies in agreement with our results have shown that a myostatin mutation in cattle causes a reduction in sizes of internal organs and an increase in muscle mass (Kambadur et al., 1997; McPherron and Lee, 1997; Grobet et al., 1998; O'Rourke et al., 2008; Wiener et al., 2009; Allais et al., 2010). When investigating the inactive allele, Q204X, Allais et al. (2010) found that the heterozygous cattle in their Charolais population to have a larger REA and a greater carcass yield percentage. In agreement with these findings, a study investigating the 11-bp deletion in the South Devon breed, Wiener et al. (2009) observed an association with increased muscle and carcass confirmation scores and the myostatin allele. It is important to note that differences between the present study and others may result from the fact that the allele found in the Piedmontese breed is not the same as that found in Belgian Blue and South Devon Breeds.

Interaction between carcass traits and the myostatin gene has recently been shown to exist beyond the myostatin allele. Two studies (Sellick et al., 2007; Esmailizadeh et al., 2008) found that a leucine-phenylalanine substitution (F94L) in the myostatin gene causes an increase in REA and a decrease in fat deposition in Limousin x Jersey cattle. The results from this crossbred population suggest that myostatin variants not only act differently, but F94L may positively influence carcass traits without some of the negative effects (dystocia, stress susceptibility, fertility) of the cysteine-to-tyrosine substitution associated with Piedmontese cattle. Due to their double-muscle phenotype, the Piedmontese breed have an increased ability to convert feed into lean muscle and produce a higher percentage of top-quality meat cuts. Cattle in the current study having genetic mutations for myostatin had a lower YG ($P < 0.001$) than their Piedmontese x Angus crossbred heifer counterparts possessing no myostatin variants. This is in agreement with studies (Ménissier, 1982b; Casas et al. 1998; Arthur, 1995; Allais et al., 2010)

who found an increased retail yield in animals inheriting a single copy of the mutant myostatin allele from crossbred Charolais, Limousin, Belgian Blue, and Piedmontese populations. The presence of 1 copy of a disruptive myostatin allele was shown to increase the beef value of the animals in all of these studies drastically, while disregarding the frequency myostatin variants in study populations.

Meat Quality

Our study indicates that the contribution of disruptive myostatin variants minimally influenced tenderness and color in the 4 muscles investigated. Least squares means of WBSF measurements on cooked meat and colorimeter estimates on raw meat are presented in Table 2.5. WBSF was not affected by genotype in the SM, BF, and SS muscles ($P > 0.22$). The LT from heifers having 1 sequence variant present were tougher ($P < 0.02$) when compared with animals having no alterations or both C313Y and Q204X variants present. With regards to Igenity results (data not shown) relative to the frequency of myostatin alleles in the present study (with 1 being the least tender and 10 being the most tender), there was no difference in average tenderness scores from heifers having 1 or 2 copies present. However, it is important to note heifers with no myostatin mutation present had an average tenderness score of 6.7 (data not shown), associating the lack of myostatin alleles with more tender meat. Decreased tenderness may be a consequence of less intermuscular fat and larger muscle fiber sections as both characteristics have been shown to influence muscle tenderness (Renand et al., 2001). Muscle composition plays a large role in tenderness as several authors have discussed how the myostatin gene modifies muscle fiber type at the cost of meat quality (Hocquette et al., 1998; Klont et al., 1998). An increase in glycolytic fibers results in a faster decline of post mortem pH, accelerated glucose depletion, and consequently, tougher meat. Conversely, fast glycolytic fibers are also associated with a lower

collagen content, implying decreased toughness in meat from double-muscled animals. Taken together, these observations on muscle conformation contribute to a large variation in tenderness from animals with mutations in the myostatin gene.

There are inconsistent conclusions regarding the contribution of myostatin on tenderness in results from other studies (Table 2.4). Differences in findings may be the consequence of alterations in breed in addition to difficulty in correctly genotyping animals. The results of the present study may differ from other authors due to the fact that the allele found in the Piedmontese breed is not the same as that found in other breeds including the Belgian Blue, Charolais, and Limousin. Also, it is important to recall that carcass traits enhanced through myostatin alleles do not rely on only that allele, as research has shown the capacity to select for improvement (Casas et al., 2000). Clusters of genes affecting growth and carcass traits reside in similar chromosome regions in beef cattle. For example, Moody (1996) found an association between insulin-like growth factor (IGF) I gene (located near the quantitative trait loci for carcass traits) and growth in Hereford cattle, suggesting the possibility for a neighboring gene to be associated with skeletal muscle growth. Further research is needed to elucidate the extent of surrounding genes' usefulness and potential mechanistic action on meat quality attributes, in addition to their response to alternate forms of myostatin.

Indeed, tenderness is a beef quality trait that is not fixed at slaughter and can be significantly affected by the post-slaughter environment. Processing conditions during the first 24 h after slaughter and throughout aging may influence the meat tenderness phenotype. Shackelford et al. (1995, 1997) concluded that it was not appropriate to use WBSF to compare differences in tenderness among muscles and that trained sensory panel evaluation should be used for muscle comparisons.

Table 2.4. Effects of myostatin gene on beef tenderness.

Reference	Breed	Genotype	Muscle (s)	Method	Results
Bailey et al., 1982	Charolais	based on double-muscling conformation	LT	Compression force	↑ tenderness in double-muscled animals compared with non-double muscled animals
Ménissier, 1982	Charolais	" "	LT	WBSF	no difference
Tatum et al., 1990	Piedmontese	" "	LM	Sensory panel	↑ tenderness in double-muscled animals compared with non-double-muscled animals
Tatum et al., 1990	Piedmontese	" "	LM	WBSF	no difference
Uytterhaegen et al., 1994	Belgian Blue	" "	LD	WBSF	↓ tenderness in double-muscled animals compared with non-double muscled animals
Casas et al., 1998	Belgian Blue Piedmontese	single copy of mh allele	LM	WBSF	no difference
Wheeler et al., 2001	Piedmontese	0 (+/+), 1 (mh/+), or 2 (mh/mh) copies of inactive myostatin alleles	LD, GM, SM, and BF	Sensory panel	↑ tenderness in all muscles in (mh/+) than (+/+) and no difference between (mh/+) and (mh/mh) except in BF
Ngapo et al., 2002	Belgian Blue	nt821 (del. 11) with 0 (+/+), 1 (mh/+), or 2 (mh/mh)	ST and GB	WBSF	no difference in ST ↑ tenderness of GB in (mh/mh) when compared with (+/+) and (mh/+)
Short et al., 2002	Piedmontese	0 (+/+),1 (mh/+), or 2 (mh/mh)	LT	WBSF	no difference
Levéziel et al., 2006	Charolais	Q204X with 0 (+/+) or 1 (mh/+)	LT	WBSF	no difference
Esmailizadeh et al., 2008	Limousin	F94L allele	LM	WBSF	no difference
Lines et al., 2009	Limousin	F94L allele	ST	WBSF and compression force	↑ tenderness in animals with two copies allele when compared with 0 or 1

Wheeler et al. (2001) found that Piedmontese x Angus steers and heifers having 2 copies of the same inactive myostatin allele had more tender meat when compared with animals having zero copies in a trained sensory panel evaluation of four muscles. Tenderness ratings from this study were different ($P < 0.05$) with LT > SS > SM > BF. Similar to our study, Short et al. (2002) found no differences in WBSF of the *longissimus* from Piedmontese (0, 1, or 2 inactive myostatin alleles), Hereford, or Limousin cross progeny.

Table 2.5. Color scores and WBSF estimates of steaks aged 14 d from heifers having the occurrence of disruptive myostatin gene variants C313Y and Q204X.

Item	No mutation			C313Y			Q204X and C313Y			*P* - value
Longissimus thoracis (LT), n = 88										
L*[A]	42.63	±	1.10	41.47	±	0.45	40.85	±	0.43	0.23
a*[B]	26.97[ab]	±	0.85	27.22[a]	±	0.32	26.16[b]	±	0.31	0.06
b*[C]	8.04[a]	±	0.66	8.04[a]	±	0.25	7.18[b]	±	0.24	0.04
WBSF, kg[D]	3.08[b]	±	0.29	3.58[a]	±	0.11	3.16[b]	±	0.11	0.02
Semimembranosus (SM), n =94										
L*	39.05[a]	±	0.72	38.31[ab]	±	0.27	37.44[b]	±	0.27	0.03
a*	25.75	±	0.69	26.73	±	0.25	26.08	±	0.26	0.14
b*	7.04	±	0.51	7.60	±	0.19	7.06	±	0.19	0.13
WBSF, kg	3.41	±	0.44	4.23	±	0.17	4.18	±	0.18	0.22
Biceps femoris (BF), n = 90										
L*	38.11	±	0.81	38.11	±	0.23	37.79	±	0.23	0.54
a*	24.47	±	1.60	25.00	±	0.41	25.14	±	0.42	0.99
b*	5.99[ab]	±	0.66	6.06[a]	±	0.17	5.47[b]	±	0.17	0.06
WBSF, kg	3.58	±	0.35	3.53	±	0.93	3.40	±	0.10	0.40
Supraspinatus (SS), n = 71										
L*				39.12	±	0.36	39.14	±	0.38	0.94
a*				25.04	±	0.30	25.46	±	0.32	0.25
b*				6.41	±	0.21	6.61	±	0.22	0.57
WBSF, kg				3.86	±	0.15	3.64	±	0.16	0.28

Within a row, least squares means lacking a common superscript letter differ ($P < 0.05$).

[A] L* is a measure of lightness where a higher value indicates a lighter color.

[B] a* is a measure of redness where a higher value indicates a redder color.

[C] b* is a measure of yellowness where a higher value indicates a more yellow color.

[D] WBSF = Warner-Bratzler shear force.

While the lightness of the LT was not influenced by genotype, animals with zero or 2 myostatin variant copies tended to be less red (lower a* value; $P < 0.06$) than heifers with only

the C313Y variant present. These results could be related in part with the pigment content of meat. Several authors have reported a significantly lower pigment content in animals having 2 copies of myostatin alleles (Bailey et al., 1982; Boccard, 1982; De Smet et al., 2000). Meat from animals with zero or 1 copy of a disruptive myostatin variant were more yellow (greater b^* value; $P < 0.04$) than those from animals having both Q204X and C313Y variants. Steaks from the SM of heifers with no alterations were lighter (higher L^* value; $P < 0.03$) when compared with steaks from animals having disruptive myostatin variants. In a study investigating muscle color of 3,641 carcasses from diverse biological types, Shackelford (1994) saw the second highest frequency of unacceptably dark lean color scores in the LM from Piedmontese crosses. The BF of heifers with a C313Y variant tended to be more yellow (greater b^* value; $P < 0.06$) than BF from animals with both Q204X and C313Y variants, but did not differ from those with zero sequence variants of the myostatin gene present. In regards to muscle location, authors have suggested the presence of a two-toned color in deeper parts of the hindquarter (De Smet et al., 2008; De Boever et al., 2009). The temperature and pH will drop differently in the outer and inner BF, and chilling method of these large beef muscles is vital to color stability.

Meat color, irrespective of muscle location, is affected by characteristics of the live animal and carcass, in addition to events taking place during growth and development. Traits having an influence on meat color including pH, muscle structure, fiber composition, post-slaughter conditions, and many immeasurable live animal characteristics. A great majority of the literature states that meat of double-muscled animals is leaner and lighter in color than those of non-double-muscled animals (Bailey et al., 1982; Boccard, 1982; Arthur, 1995; De Smet et al., 2000). With regards to muscle fiber composition, several authors have associated a paler meat

color in double-muscled animals with a higher proportion of white fibers (Batjoens et al., 1991; Fiems et al., 2000) and a lower myoglobin content in meat (Boccard, 1982).

The C313Y mutation with or without the presence of the Q204X variant resulted in similar effects on carcass characteristics of Angus x Piedmontese crossbred heifers including decreased FT, KPH, and YG, as well as increased REA, indicating improved cutability of beef carcasses. Additionally, meat quality attributes (tenderness, color) measured in the present study were minimally influenced by the presence of 1 or both disruptive myostatin variants. While 1 copy of the myostatin allele had a detrimental effect on tenderness of the LT muscle, C313Y with or without Q204X had no negative influence on tenderness in the other 3 muscles investigated. How the presence of 1 or 2 copies of the same mutation, or different mutations, impact beef muscle remains unclear. The effect of 2 copies of the mutated allele, nt821, were generally threefold the effects of a single copy in a crossed Belgian Blue-British breed (Angus and Hereford) population (Casas et al., 2004). However, studies also show that 2 copies of a myostatin allele are associated with heavier birth weights and increased calving difficulty (Short et al., 2002; Weiner et al., 2009). Important to the present study, Short and others (2002) observed increased calving difficulty in Piedmontese heifers as 1 and 2 copies of the inactive allele were added to an individual calf, but they found no effect with cows. Mitigating negative effects on carcass and associated meat quality (dystocia, calving difficulty, and palatability traits) appears to be possible through both knowledge of the myostatin genotype of the mating bulls and crossbreeding programs that will allow production of animals with 1 copy of an inactive myostatin allele, resulting in improved animal performance, carcass yield, and meat quality.

Final Considerations

The results presented here show that disruptive myostatin gene variants both positively and negatively influence global meat quality. The substantial benefits including heavier HCW, larger REA, lower YG, larger retail product yield, and larger bone yield observed in these animals should be of high regard when considering the limited undesirable effects on meat quality. Recent technological advances in genetic selection parameters provide producers the ability to design genetically controlled breeding programs that will limit the frequency of myostatin alleles in order to minimize the detrimental effects on meat tenderness observed in double-muscle cattle. The present study demonstrated the encouraging potential of Angus as a cross to improve compositional quality while maintaining palatability factors important to consumers. Furthermore, it is now possible to identify and differentiate, through direct genotyping, between heterozygous animals carrying 1 copy of the inactivated myostatin gene and those homozygous animals carrying 2 inactivated myostatin gene copies. Production systems that produce calves with 1 copy of an inactive myostatin allele will benefit economically from heavier weaning weights and higher production yield when compared with animals having no copies of myostatin alleles. Future investigations may also elucidate ways to control these adverse effects on meat tenderness through specific post-slaughter carcass management and/or processing strategies.

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CHAPTER 3. THE INFLUENCE OF BEEF QUADRICEPS HOT-BONING ON CARCASS CHILLING AND BEEF ROUND MUSCLES

Abstract

The objective of the present study was to determine the effects of partial hot-boning on meat palatability attributes and myofibrillar protein degradation in deep and superficial portions of beef *semimembranosus* (SM). The beef knuckle (quadriceps muscles) was partially hot-boned within 1.5 h post mortem for 1 randomly selected side of each beef carcass (n = 15), whereas the opposite side remained intact throughout the 48-h chilling period. Randomized treatments include deep SM hot-boned (DH) or cold-boned (DC), and superficial SM hot-boned (SH) or cold-boned (SC). Post mortem temperature decline was monitored every 10 min for 24 h and post mortem pH decline recorded at 45 min, 3 h, and 24 h in superficial and deep portions of the SM. Samples from the superficial and deep portions of the SM from all treatment sides were collected after 24 h (protein degradation and protease activity measurements) and 10 d (tenderness and display life evaluation) of aging in an air-chilled cooler. The deep portion of the SM had a slower chill rate and a sharper pH decline when compared with the superficial SM in all sides investigated, regardless of hot-boning treatment. No treatment differences were observed for L* and b* values ($P > 0.05$). By d 4 of display life, both deep muscle treatments were less red ($P < 0.001$) when compared with superficial SM locations. However, by d 10 of display life, only SC-treated SM had a greater a* value ($P < 0.01$) compared with all other treatments. Modified hot-boning did not ($P > 0.49$) influence tenderness in deep or superficial portions of the SM. At 24 h post mortem calpain 1 activity and autolyzed calpain 1 activity were different between deep and superficial SM treatments, regardless of partial hot-boning treatment. Superficial SM from SC and SH-treated sides had increased ($P < 0.01$) calpain 1 activity when

compared with both deep SM treatments. These results coincided with those of the autolyzed form, which was decreased ($P < 0.01$) in both superficial treatments compared with DH and DC treatments. These data coincided with less troponin-T degradation products in DC-treated beef sides. Taken together, shifting the progression of proteolysis and altering meat palatability is contingent upon the ability of the hot-boning technique to significantly alter temperature and pH decline in the deep portion of the SM.

Introduction

Compared with traditional boning of refrigerated carcasses, partial hot-boning (limited separation of meat from the skeleton pre-rigor) is a well-established technique used to optimize meat processing parameters including processing time, chilling costs, and contamination reduction (Kastner, 1977; Cuthbertson, 1984; Sørheim and Hildrum, 2002; Røtterud et al., 2006). While this method can optimize processing procedures, it has also been shown to influence underutilized beef round muscles, which are known to be less tender and display a two-toned color defect. Inconsistent meat quality attributes of the SM are possibly due to variations in pH and temperature decline between the deep and superficial muscle locations (Tarrant and Mothersil, 1977; Sammel et., al., 2002; Seyfert et al., 2004; Sawyer et al., 2007). Differences in fiber type have also been observed between the inner (11% β-red, 27.8% α-red, and 61.3% α-white) and outer (15.5% β-red, 32.9% α-red, and 51.1% α-white) portions of the SM (Hunt and Hedrick, 1977). However, the variability in shape of the muscle proves challenging in distinguishing between inner and outer muscles when attempting to determine fiber types differences (Kirchofer et al., 2002). Generally, the deep portion of the SM is a larger, thicker muscle, exhibiting a slower chill rate and more rapid pH decline than its superficial counterpart. As a result, meat from the inner SM tends to have damaged color stability and protein

functionality. Tenderness differences within the beef round muscle are well documented (Johnson et al., 1988; Reuter et al., 2002; Kim et al., 2010). The pH/temperature conditions in the deep portion of the SM cause decreased calpain 1 activity and autolysis, resulting in adverse effects on eventual meat tenderization.

Limited studies have investigated how partial hot-boning of the SM might affect post mortem determinants of meat quality in view of differences in chilling and pH conditions of the superficial and deep SM. Therefore, the objective of this study was to examine the effects of partial hot-boning on pre-rigor temperature and pH decline on the proteolysis of the superficial and deep portions of the SM in relation to beef palatability traits.

Materials and Methods

Project Description

Fifteen market weight beef heifers were purchased from area commercial feedlots and harvested at the USDA-inspected meat laboratory at North Dakota State University. The carcasses were split and one half of each carcass was randomly assigned to a hot-boning technique conducted 60 to 90 min after stunning, whereas the other half of each carcass remained as a control. Following the hot-boning procedure, temperature monitoring probes were inserted into the deep and superficial portions of the SM from both treatment and control sides of carcasses for 24 h. Post mortem pH decline was also monitored in superficial and deep portions of the SM from all carcass sides examined in the experiment. At 24 h post mortem, muscle biopsies were taken within the SM from both the treatment and control sides of the carcass. Biopsies were taken from the deep portion (the medial inner 1/3 closest to the femur) and the superficial portion (the lateral 1/3 closest to the surface of the carcass) (Kim et al., 2010). Superficial and deep samples were treated as separate muscles for each treatment. Samples

collected at 24 h post mortem, placed on dry ice, and frozen at -80°C for measurement of protein degradation, as well as calpain 1 and calpain 2 activity. Hanging carcasses were air-chilled in a dark cooler for 10 d at 4°C upon which steaks were cut from deep and superficial portions of the SM from all carcass sides for immediate display life assessment and tenderness evaluation.

Hot-Boning Technique

The process of hot-boning consisted of separating the quadriceps complex (knuckle) along with the patella from their insertion at the distal end of the femur near their origin at the proximal end of the femur (Figure 3.1). The quadriceps was left attached to origin of attachment so that the quadriceps hung away from the femur (Seyfert et al., 2004). Then, carcasses were air chilled in the cooler at 2.2°C ± 2°C for 24 h. Removal of quadriceps muscles attempted to permit an accelerated chill of the *semimembranosus* muscle (SM), *biceps femoris* (BF), and quadriceps muscles (Seyfert et al., 2004).

Figure 3.1. Hot-boning technique used on the inside round of beef cattle.

Temperature and pH

Post mortem temperature decline was monitored every ten min for 24 h using temperature loggers (Omega; Model HH506RA, Stamford, CT, USA) with probes inserted into superficial and deep portions of the round muscles. Post mortem pH was recorded at 45 min, 3 h, and 24 h in superficial and deep portions of the round muscles using an IQ150 pH meter with a PH57-SS non-glass probe (IQ Scientific Instruments, Carlsbad, CA, USA) calibrated to pH range 4-7 between each time point at carcass temperature.

Warner-Bratzler Shear Force

Steaks were cut from the superficial and deep portion of the SM of hanging carcasses (aged 10 d at 4°C) and analyzed for tenderness by WBSF. Prior to cooking and shear force measurements, the 2.54-cm steaks were allowed to come to room temperature (approximately 18°C) before being weighed. A copper-constantan thermocouple was inserted (Omega Engineering Inc., Stamford, CT) into the geometric center of the steak. Steaks were cooked on clamshell-style grills (George Foreman grill Model No. GRP99, Columbia, MO) to an internal temperature of 71°C and then removed from the grill and allowed to cool to room temperature (approximately 21°C). Cook loss steaks was calculated using the equation [(final cooked weight – raw weight)/ (raw weight)] × 100. Six, 1.27-cm cores were taken from each steak parallel to the muscle fibers and sheared once perpendicular to the muscle fibers for measurement of tenderness using a WBSF machine (G-R Manufacturing, Manhattan, KS) (AMSA, 1995). The WBSF values from the 6 cores within each steak were averaged before statistical analyses.

Display Life

Cut from suspended carcasses aged for 10 d in darkness at 4°C, 1.25-cm steaks were individually overwrapped with clear cellophane and placed on flat shelving in a 0 to 2°C cooler

under continuous fluorescent light for 10 d (Sylvania, 32-watt, T-8 Cool White, Sylvania, Danvers, MA). Overwrap treatments were balanced and steak shelf positions were rotated daily. Objective color (L*, a*, and b*) values of the SM beef steaks were determined from the average of two readings on the cut surface measured through the packaging film with a portable colorimeter (CR-410 Chroma Meter, Konica Minolta, NJ, USA) using illuminant D65, a 5-cm aperture, and a 2° standard observer. The instrument was calibrated against a standard white tile wrapped in cellophane immediately before data collection.

Statistical Analysis

Data for protein degradation and protease activity was analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). Analysis of variance for WBSF values was performed using PROC GLM of SAS. Means for both procedures were separated using the LSMEANS option of SAS and were considered significant when $P \leq 0.05$. Color, temperature, and pH measurements were analyzed as repeated measures using treatment as the source of variation and day (color) or time (temperature and pH) as the repeated measure, and significance was declared at $P \leq 0.05$.

Protein Extraction

Sarcoplasmic and myofibrillar protein fractions were extracted from samples collected and frozen at 24 h and 10 d of aging (Huff-Lonergan et al., 1996b; Rowe et al., 2004; Melody et al., 2004). Briefly, 5 g of muscle tissue was minced and then homogenized in 20 mL of extraction buffer [10 m*M* EDTA; 100 m*M* Tris; 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 loosely 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. The pellet that remained in the 50-mL tubes was saved on wet ice until extraction of myofibrillar protein fraction. Briefly, 15 mL of extraction buffer [10 m*M* Sodium phosphate buffer, pH 7.0; 2% (wt/vol) SDS] was added to each pellet, broken up with a plastic spatula, vortexed, and homogenized with a 10-sec burst of a Polytron Kinematica. The homogenate was then centrifuged at 2000 x *g* for 15 min at 20°C (Allegra 25 R Centrifuge, Beckman Coulter, Fullerton, CA). The clear supernatant was then transferred to microfuge tubes and stored at -80°C until protein determination.

The protein concentration of each cleared extract was determined using the Quick Start Bradford Dye Reagents (BioRad 500-0205; Hercules, CA). Protein extracts were diluted with water for a final concentration of 0.64 µg/µL. One volume of each extract was combined with 0.5 volume sample/buffer tracking dye solution [3 mM ethylenediamine tetraacetic acid (EDTA), 3% (wt/vol) SDS, 30 mM Tris, pH 8.0; 30% (vol/vol) glycerol, 0.003% (wt/vol) pyronine Y] (Wang, 1982) and 0.1 volumes of 2-mercaptoethanol to produce gel samples that had a final concentration of 4 µg/µL. Gel samples were heated to 60°C for 15 min and then frozen at -80°C until further analysis by Western blotting.

SDS-PAGE Electrophoresis

Samples in sample buffer/tracking dye for troponin-T (TnT) were run on 1.5 mm thick, 15% acrylamide separating gels (acrylamide: *N*, *N*'-bis-methylene acrylamide = 37.5:1 [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 acylamide = 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 m*M* Tris, 192 m*M* glycine, 1.7 m*M* EDTA, 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 µg of protein per lane and run at a constant voltage of 120 V for 3 h.

Transfer Conditions

Proteins were transferred onto Millipore Immobilon-P polyvinylidene diflouride transfer membrane (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, 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 m*M* disodium hydrogen orthophosphate, 100 m*M* 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, 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. 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. 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.).

Western blotting statistics

TnT Western blot data were analyzed using the GLM procedure in SAS (v.9.2; SAS Inst., Inc., Cary, NC) to determine differences between treatments. Least squares means were separated using the PDIFF option. The ratio of the intensity of the sample band to the intensity of the same band in the control sample was used to analyze differences in TnT isoforms in the myofibrillar fraction of SM between treatments. The control sample ran on all SDS-PAGE gels was a pooled sample representative of all samples compared for TnT degradation collected at 24 h post mortem.

Casein Zymography

To prepare protein gel samples, sarcoplasmic protein samples (stored at -80°C) and Sample Tracking Dye [150 m*M* Tris, pH 6.8; 20% (vol/vol) glycerol; 0.1 mg/mL bromophenol blue] containing 0.75% (vol/vol) MCE were calculated to allow for loading of 150 µg of protein into each lane using a ratio of 60:40 sample to tracking dye. Individual volumes of protein sample and Sample Tracking Dye were aliquotted into 0.5 mL microcentrifuge tubes, vortexed, and stored on ice until ready to load. 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 11% acrylamide separating gels [405 m*M* 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 m*M* Tris, pH 8.3; 192 m*M* glycine; 1 m*M* EDTA; 0.1% (vol/vol) MCE] (Melody et al., 2004; Raser, et al., 1995). Electrophoresis was carried out on a Hoefer SE 245 Dual Gel Caster system (Hoefer, Inc., Hollison, MA). Gels were pre-run at 100 V, loaded with 150 µg of protein per lane, and run overnight at 40 V until sample tracking dye ran off (10 to 15 h total electrophoresis time). Gels were then removed from glass sandwiches and incubated in 100 mL of Tris-Casein Gel Incubation Buffer [50 m*M* Tris; 5 m*M* $CaCl_2$, 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 [40% (vol/vol) methanol, 7.5% (vol/vol) acetic acid] for 30 min. This destain step was repeated 2 additional times. 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.). Densitometry measurements were done using the AlphaEaseFC software (Alpha Innotech Corp.).

Casein zymography statistics

Data were analyzed using the GLM procedure in SAS (v.9.2; SAS Inst., Inc., Cary, NC) to determine differences between treatments. Means were separated using the LSMEANS option

of SAS and were considered significant when $P \leq 0.05$. The ratio of the intensity of the sample band to the intensity of the same band in the control sample was used to analyze differences in SM native calpain 1 activity, autolyzed calpain 1activity, and native calpain 2 activity between treatments. Briefly, autolysis of calpain occurs in the presence of Ca^{2+} and reduces the mass of 80 kDa subunit of calpain 1 to a 76 kDa product through a 78 kDa intermediate and that of calpain 2 to a 78 kDa product. For small 28 kDa subunits, both calpain 1 and calpain 2 have the same autolysis pattern and both are reduced to 18 kDa fragments.

Results and Discussion

Hot-Boning Effects

Changes in pH during rigor development

Immediately following slaughter, oxygen depletion occurs and the animal shifts from aerobic to anaerobic metabolism. Due to the abrupt need for energy reserves, glycogen is quickly converted to lactic acid, lowering muscle pH from ~7 to 5.6. Figure 3.2 represents the average pH fall for the 4 muscle locations and their corresponding treatments (n = 15 per treatment), which declined from an average value of 6.66 at 1 h post mortem to 5.68 at 24 h post mortem. DH and DC had similar ($P > 0.05$) pH decline indicating that hot-boning was not enough to slow pH decline in the deep portion of the SM. SC and SH pH decline values were similar ($P > 0.05$). Differences between superficial and deep for either boning treatment were significant ($P < 0.05$) at 45 min and 3 h post mortem, however, all treatments had similar pH values 24 h post mortem.

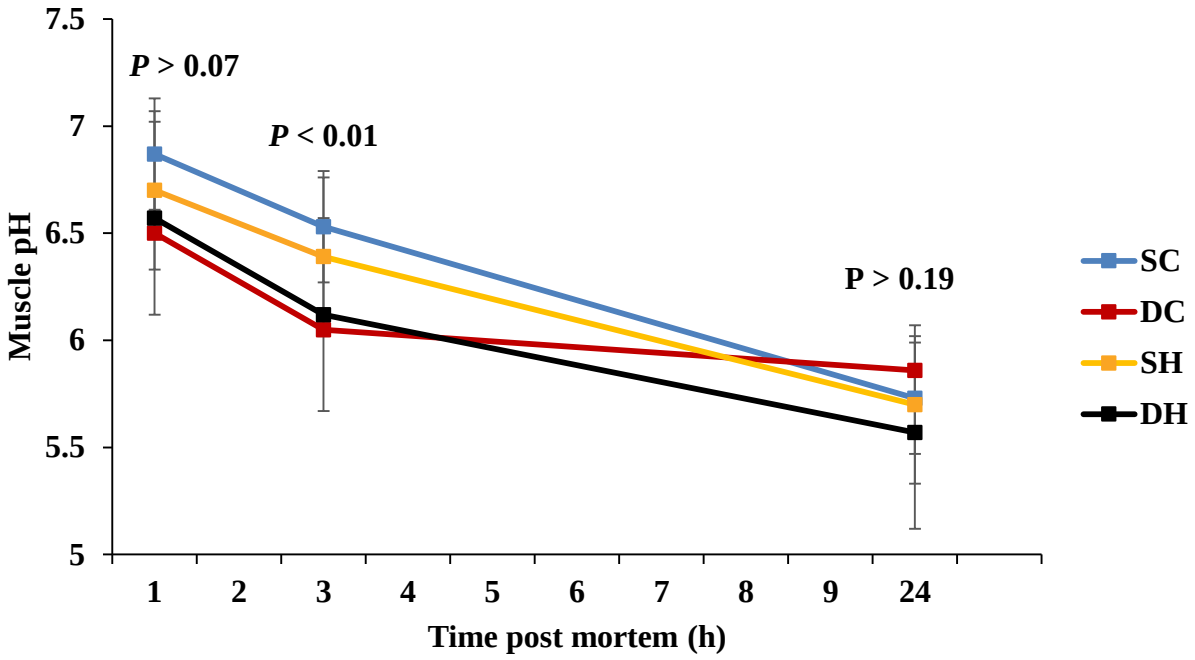

Figure. 3.2. Mean pH decline [± standard deviation] of deep and superficial portions of bovine *semimembranosus* with modified processing conditions during the first 24 h post mortem (n = 15 per treatment). Treatments include deep SM hot-boned (DH) or cold-boned (DC), and superficial SM hot-boned (SH) or cold-boned (SC).

Changes in temperature during rigor development

In addition to pH decline, chill rate has been shown to influence beef round quality development. Optimizing the slaughter process for palatability traits originated from the results of Locker and Hagyard (1963) who showed that myofibrillar shortening occurred when pre-rigor muscle was held at either low or high temperatures. Figure 3.3 shows the mean temperature profile of the muscles and corresponding muscle locations monitored in this study. Our results show similar temperature decline among all muscle locations monitored. As expected, the superficial muscles investigated (both SC and SH) had a faster rate of decline than those locations from the deep SM. HD and CD SM had similar temperature decline rates indicating that partial hot-boning was not enough to hasten temperature decline in the deep portion of the SM.

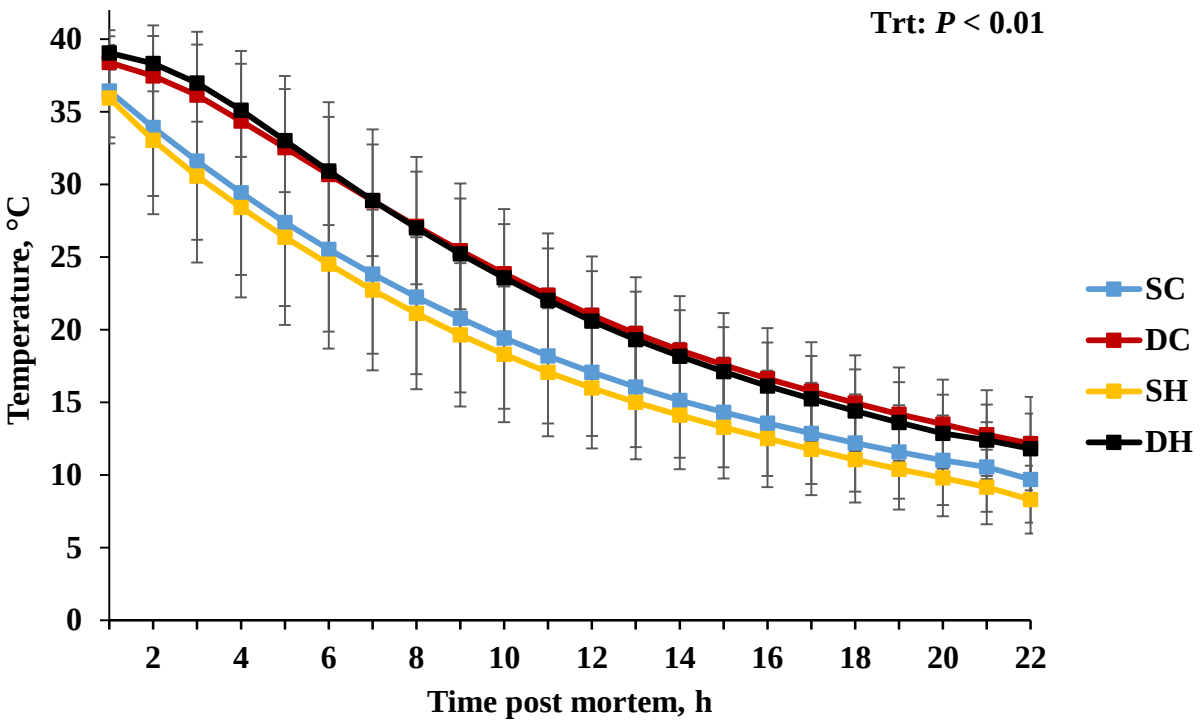

Figure. 3.3. Mean temperature fall [± standard deviation] of bovine *semimembranosus* with modified processing conditions up to 22 h post mortem (n = 15 per treatment). Treatments include deep SM hot-boned (DH) or cold-boned (DC), and superficial SM hot-boned (SH) or cold-boned (SC).

A higher muscle temperature post mortem has been shown in some cases to cause enhanced muscle shortening of the sarcomere leading to increased toughness (Simmons et al., 1997; Devine et al., 2002). The onset of rigor can begin at various pH values and ATP concentrations, while the completion of rigor is dependent upon the ATP concentration in the muscle, which is usually reached between 10°C and 38°C at pH 5.5 to 5.6 and less than 0.5 µ*M*ol ATP/g of muscle (Honikel et al., 1983). At adequate ATP concentration and temperature (below 15°C), pre-rigor beef muscles contract in an event known as cold shortening. . Cold shortening can began at a pH of around 7 and at maximum ATP concentration (4 µ*M*ol ATP/g). However,

higher temperatures imposed just prior to rigor onset cause rigor shortening, a process occurring at a pH around 6.0 and half ATP concentration (2 µ*M*ol ATP/g).

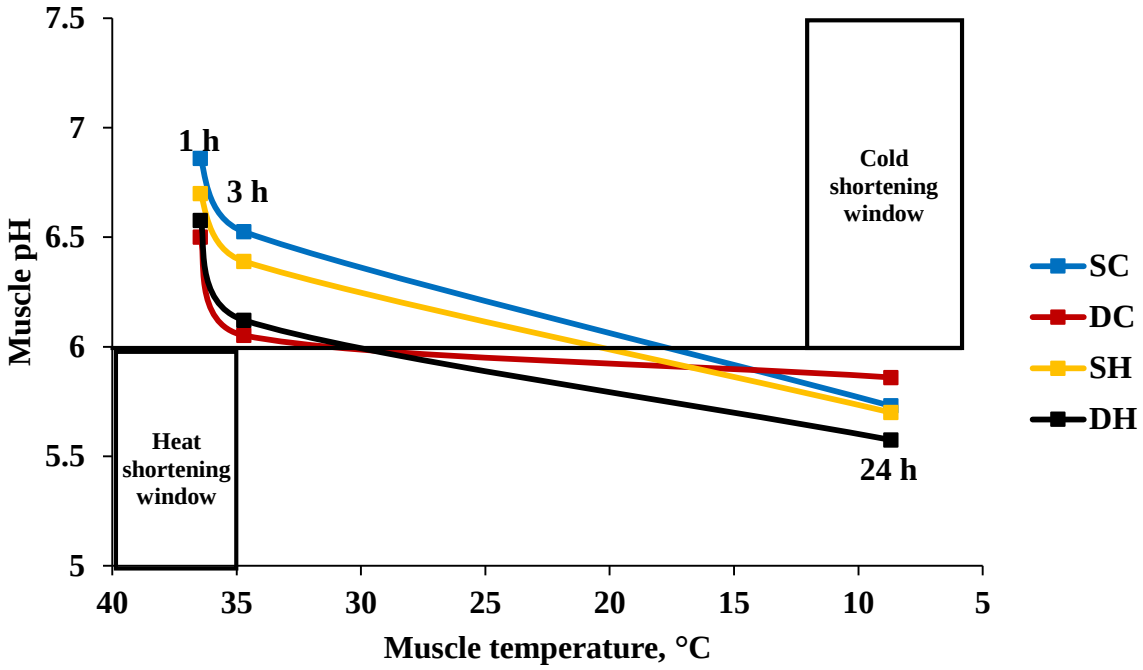

Figure 3.4. Means of decline in pH at 1, 3, and 24 h post mortem relative to the temperature of the *semimembranosus* muscle location. Heat and cold shortening windows adapted from Thompson, J. 2002. Meat Sci. 62:295-308. Treatments include deep SM hot-boned (DH) or cold-boned (DC), and superficial SM hot-boned (SH) or cold-boned (SC).

Rigor shortening, also known as heat shortening, was determined to be the result of a combination of high temperature and low pH in the muscle causing decreased calpain activity and increased calpastatin activity (Dransfield, 1993; Simmons et al., 1996). The goal of the present study was to expose the deep portion of the SM to cooler air quicker by application of the hot-boning technique. However, DH-treated SM showed no alterations in temperature post mortem, suggesting that possible additional processing conditions did not permit the hot-boned deep portion of the SM to cool at a faster rate than their control cold-boned counterparts. The temperature of the cooling chamber during the SM treatment oscillated around 5°C during the first 12 h and reached 2.5°C at the end of chilling. While 2.5°C is consistent with similar studies

investigating beef hot-boning treatments (Seyfert et al., 2005; White et al., 2006; Jenschke et al., 2008; Pivotto et al., 2014), the warmer temperature observed in the present study during the first 12 h may have adversely influenced the speed at which SM muscle temperature dropped.

Times that the carcasses are placed into the cooler and temperatures at which they are held could be a way to affect muscle shortening after slaughter. Bendall (1973) determined that muscles with temperatures less than 10°C are susceptible to cold shortening until a muscle pH of less than 6.2 is reached and warned against cooling beef carcasses below 10°C internally in less than 15 h or before completion of rigor mortis. All muscle locations monitored in the present study were not chilled to below 10°C within the first 20 h post mortem (Fig. 3.3) following Bendall's rule of thumb (1972) to avoid cold shortening. Additionally, pH followed the optimal rate of decline (Figure 3.4), suggesting that no shortening of the muscles investigated occurred.

Display Life

Hot-boning the SM has been shown to improve the color and color stability of beef round muscles by accelerating post mortem chill rate and decreasing the rate of pH decline (Nicholas and Cross, 1980; Seyfert et al., 2004, 2005). However, in our study, modified hot-boning had minimal effects on objective color. No treatment differences ($P > 0.05$) were observed for L* and b* values (Table 3.1; Fig. 3.5 and 3.7, respectively). By d 4 of display life, both deep muscle treatments were less red ($P < 0.001$ and Fig. 3.6) when compared with superficial SM locations. However, by d 10 of display life, only SC-treated SM had a greater a* value ($P < 0.01$) compared with all other treatments.

There are conflicting results on the potential of hot-boning to positively influence color. When chilled at the same rate, the inner and outer portion of the SM have been reported to have similar color, independent of hot-boning treatment (Sammel et al., 2002). Others (Meade et al.,

1992) have observed darker muscle due to whole muscle boning of the beef round muscle. In a study using pre-rigor excision to separate the top and bottom round at their natural seams, Jenschke and others (2008) found when moving from the point of attachment to the end of the *rectus femoris* and *vastus lateralis* in beef muscle, the proximal aspect was darker, less red, and less yellow in color. Consistent with the results from our study, no differences in lightness between deep and superficial SM occurred when muscles were hot-boned 1 to 2 h after slaughter (Taylor et al., 1980). These data suggest that the activity of metmyoglobin reducing enzymes in the deep muscle locations post mortem was greater than that of the superficial SM locations due to a slower temperature drop in the deep SM. Because of this higher activity, the enzymes' capacity to reduce metmyoglobin was decreased more rapidly in superficial muscle locations entering rigor at lower temperatures.

Differences in previous findings on boning treatment and evaluation of color from that of the present study may result from differences in pre-rigor excision modifications, slaughter environment, and/or cooler conditions. Few studies differentiate between superficial and deep SM; thus, comparing results is difficult. Because color is a key factor in selection criterion of consumers, it is important to map color differences between the superficial and deep SM. In the present study, retail display of steaks from all muscle locations investigated, regardless of hot-boning treatment, indicates a clear difference in redness between the deep and superficial SM, suggesting further research is needed to improve retail appearance in the round muscle.

Table 3.1. Colorimeter estimates of *semimembranosus* from heifers with modified processing conditions.

Item[A]	Treatment[B]	Display Day											
		0			4			7			10			Significance[C]
L*	SH	44.72	±	0.72	44.35	±	0.58	43.33	±	0.74	45.16	±	0.56	n.s.
	SC	44.98	±	0.72	44.49	±	0.58	44.45	±	0.74	45.43	±	0.56	n.s.
	DH	45.76	±	0.72	44.61	±	0.58	44.62	±	0.74	45.55	±	0.56	n.s.
	DC	45.28	±	0.72	44.60	±	0.58	44.47	±	0.74	45.23	±	0.56	n.s.
	Significance													
a*	SH	20.59^{a}	±	0.46	20.01^{av}	±	0.43	17.38^{bv}	±	0.57	14.95^{cvx}	±	0.77	***
	SC	20.85^{a}	±	0.46	20.62^{av}	±	0.43	17.98^{bv}	±	0.57	16.12^{bv}	±	0.77	***
	DH	22.16^{a}	±	0.46	18.16^{bx}	±	0.43	15.10^{cx}	±	0.57	12.95^{dx}	±	0.77	***
	DC	22.09^{a}	±	0.46	18.09^{bx}	±	0.43	15.00^{cx}	±	0.57	12.83^{dx}	±	0.77	***
	Significance				***			***			**			
b*	SH	7.69^{ab}	±	0.30	8.06^{a}	±	0.22	7.43^{ab}	±	0.24	7.15^{b}	±	0.24	*
	SC	7.90^{ab}	±	0.30	8.48^{a}	±	0.22	7.78^{ab}	±	0.24	7.58^{b}	±	0.24	*
	DH	8.72^{a}	±	0.30	7.89^{b}	±	0.22	7.24^{c}	±	0.24	7.19^{c}	±	0.24	***
	DC	8.88^{a}	±	0.30	8.11^{b}	±	0.22	7.55^{bc}	±	0.24	7.38^{bd}	±	0.24	***
	Significance													

[A] L* is a measure of lightness where a higher value indicates a lighter color. a* is a measure of redness where a higher value indicates a redder color. b* is a measure of yellowness where a higher value indicates a more yellow color.

[B] Treatment abbreviations: SH = superficial hot-boned, DH = deep hot-boned, SC = superficial cold-boned, and DC = deep cold-boned.

[a,b,c,d]: Means within a row with different letters are significantly different ($P < 0.05$).

[v,x,y,z]: For each treatment, means within a column with different letters are significantly different ($P < 0.05$).

[C] Significance for day (row) and treatment (column): n.s. = not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

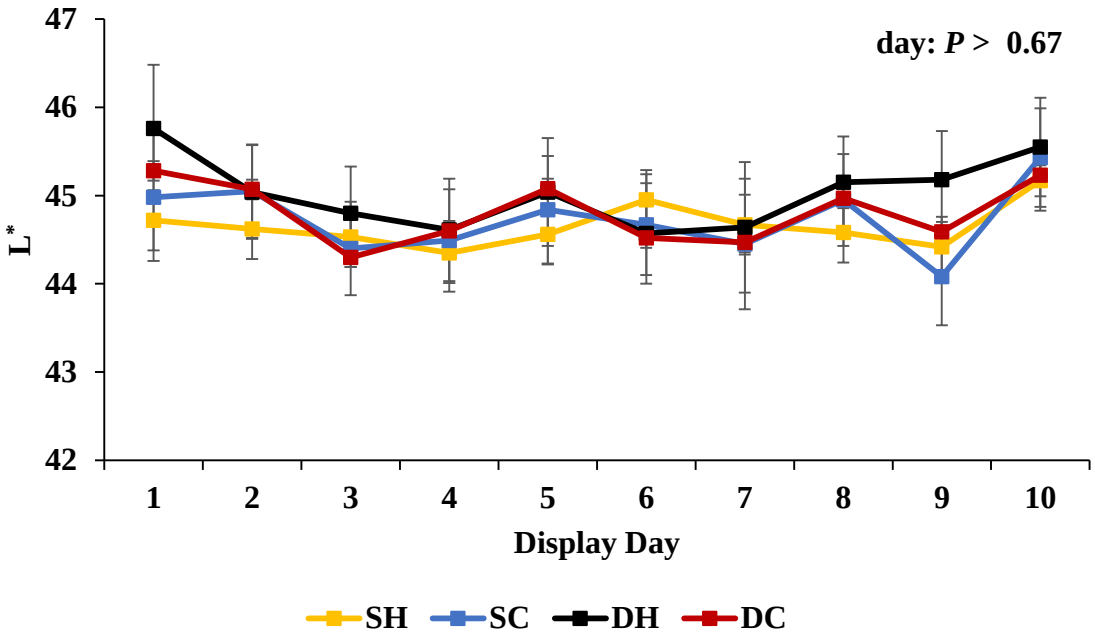

Figure 3.5. Minolta L* score values of beef *semimembranosus* from heifers with modified processing conditions (SH = superficial SM hot-boned, SC = superficial SM cold-boned, DH = deep SM hot-boned, DC = deep SM cold-boned).

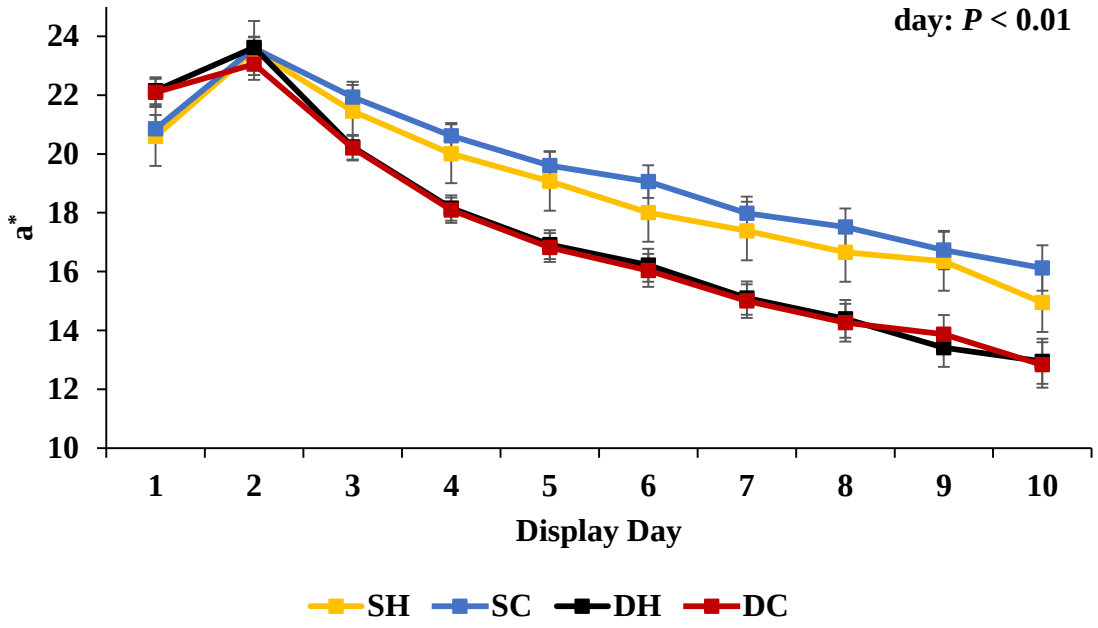

Figure 3.6. Minolta a* score values of beef *semimembranosus* from heifers with modified processing conditions (SH= superficial SM hot-boned, SC = superficial SM cold-boned, DH = deep SM hot-boned, DC = deep SM cold-boned).

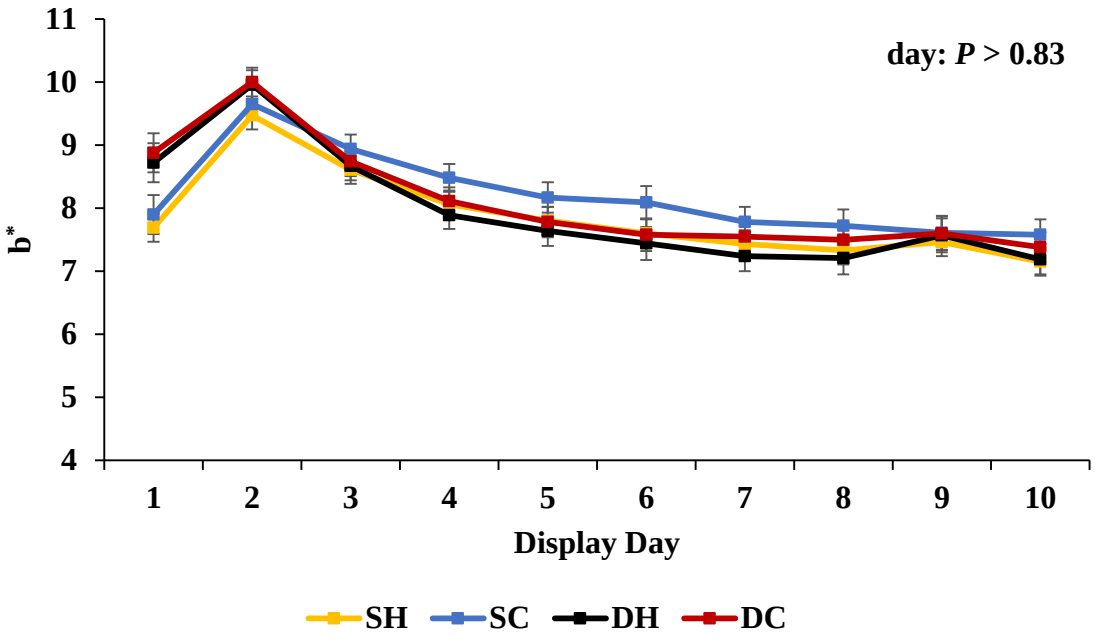

Figure 3.7. Minolta b* score values of beef *semimembranosus* from heifers with modified processing conditions (SH = superficial SM hot-boned, SC = superficial SM cold-boned, DH = deep SM hot-boned, DC = deep SM cold-boned).

WBSF, Western Blotting, and Casein Zymography

Shear force values did not differ ($P > 0.49$) between hot- and cold-boned SM muscle locations (Table 3.2). No differences in tenderness may be due to the fact that the SM was still intact on the carcass with skeletal limitations. The complete removal of the SM from the carcass during the pre-rigor phase would have allowed the muscle to contract more than muscles still being held in place by the skeleton. Wheeler and others (1991) reported no difference ($P > 0.05$) in shear force values from SM muscles partially hot-boned within 30 min post mortem from the carcasses of 9 *Bos indicus* steers. Previously, tenderness has been found to be unaffected and sometimes decreased, by full hot-boning treatments (Seideman and Cross, 1982; Sammel et al., 2002; Pivotto et al., 2014). Hot-boning alone resulted in tougher beef regardless of chilling rate when compared to cold-boned bovine SM muscles (White et al., 2006). However, it is important to note, similar to our study, White did not observe a difference between pH of hot-boned and

cold-boned muscles. Using a modified hot-boning technique very similar to that of the present study, Sammel and others (2002) reported no effect of boning method on shear force values from deep and superficial beef SM, despite seeing altered pH and temperature decline in hot-boned samples. The results of the present study support previous reports acknowledging hot-boning has no detrimental effects to meat tenderness and resulting product quality.

Table 3.2. Least squares means and standard errors for tenderness measurements from *semimembranosus* of beef heifers with modified processing conditions.

Variable[A]	SH	DH	SC	DC	SEM	*P*-value
WBSF[B], kg	4.13	4.13	3.87	4.12	0.26	> 0.49
calpain 1 activity[C]	0.33[a]	0.10[b]	0.29[a]	0.15[b]	0.04	< 0.01
Autolyzed calpain 1 activity[C]	0.01[a]	0.07[b]	0.02[a]	0.06[b]	0.01	< 0.01
calpain 2 activity[C]	0.40	0.40	0.42	0.43	0.03	> 0.80
Troponin-T, 30-kDa band[D]	1.16[a]	0.82[ac]	0.83[ac]	0.50[bc]	0.15	< 0.01

[A] Treatment abbreviations: SH = superficial hot-boned, DH = deep hot-boned, SC = superficial cold-boned, and DC = deep cold-boned.
[B] Warner-Bratzler shear force.
[C] Calpain activity was assessed in SM collected at 24 h post mortem by casein zymography. Values are relative to the activity of a control of partially purified calpain 2 which was set to 1.0.
[D] Troponin-T degradation in SM collected at 24 h post mortem was assessed by Western immunoblotting and values are relative to the 30-kDa band of a pooled-control loin sample.
[abc] Within rows, mean values without a common superscript differ ($P < 0.05$).

The residual activity of calpain 1 and calpain 2 were determined with casein zymography (Table 3.2 and Fig. 3.8). At 24 h post mortem calpain 1 activity and autolyzed calpain 1 activity were different between deep and superficial SM treatments, regardless of partial hot-boning treatment. SC- and SH-treated sides had greater ($P < 0.01$) calpain 1 activity when compared with both deep SM treatments. These results coincided with those of the autolyzed form, which was less ($P < 0.01$) in both superficial treatments compared with the 2 deep treatments. These results show that the calpain 1 activity is directly related to the rate of pH decrease; a rapid pH decline gave rise to an earlier loss of enzyme activity. Previous studies in beef have reported a

similar relationship of calpain 1 activity and pH decline (Hwang and Thompson, 2001; Rhee et al., 2006).

The activity of native calpain 2 was similar for all treatments at 24 h post mortem (Table 3.2 and Fig. 3.8). The absence of autolyzed calpain 2 in our study suggests calpain 2 has little to no involvement in post mortem tenderization in the first 24 h. Calpain 2, a member of the calpain system, undergoes autolysis in the presence of calcium and has been found to be active in d 3 post-mortem porcine muscles detected on casein zymography gels (Pomponio et al., 2008). While the variation in post mortem activity of calpain 1 is reported, studies of bovine and ovine muscle reveal the activity of calpain 2 remains remarkably stable (Ducastaing et al., 1985; Veiseth et al., 2001). The role of calpain 2 in meat tenderization is not fully understood, and results from the present study indicate the activity was constant during the 24 h conditioning period, independent of muscle location, temperature, and pH decline.

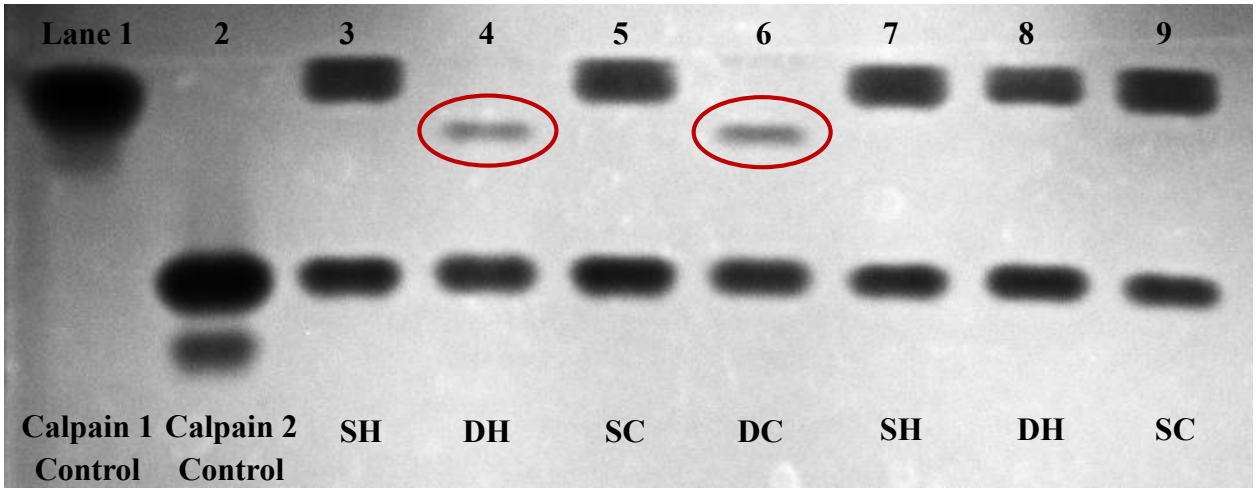

Figure 3.8. Casein zymography gel showing calpain autolysis (circled in red) when compared with a partly purified calpain 1 (lane 1, far left).

A faster pH decline accompanied by decreased myofibrillar degradation has been previously reported in beef studies (Soares and Aréas, 1995; Wu et al., 2014). In agreement with

these findings, the present study found the least TnT degradation products in the DC ($P < 0.01$; Table 3.2 and Fig. 3.9) compared with SH-treated sides, with DH and SC treatments being intermediate. The degradation of myofibrillar proteins, such as TnT, have been studied in order to determine the effect of calpain enzyme activity during post mortem aging on meat tenderness (Huff-Lonergan et al., 1996a; Koohmaraie, 1996). In the present study, while we did see differences in troponin-T degradation, we did not observe differences in tenderness. However, as expected, we do see more degradation in treatments having increased calpain 1 activity (Table 3.2 and Fig. 3.8).

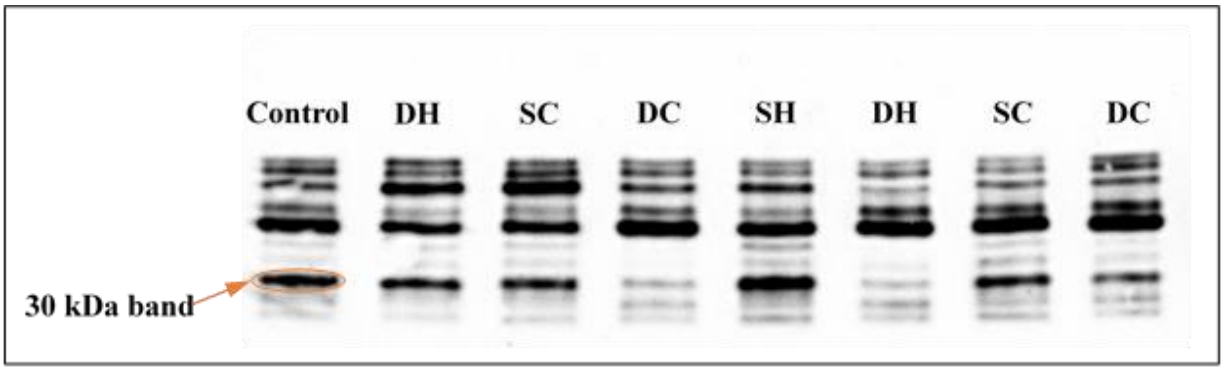

Figure 3.9. Representative Western blot of whole muscle protein extracted from deep and superficial portions of beef *semimembranosus* collected 24 h post mortem. The troponin-T degradation product (30 kDa) from all treatments was run on 15% polyacrylamide denaturing gels. All lanes were loaded with 3 µg of protein.

Troponin-T degradation was assessed in this study in an attempt to investigate possible tenderness differences in the deep and superficial portions of the SM. Partial hot-boning did not influence temperature and pH decline in DH-treated carcasses. Our modified processing technique had little to no impact on proteolysis of myofibrillar proteins in the hot-boned deep SM.

Conclusion

Results indicate that differences in meat quality across deep and superficial locations of the SM were not altered by partial hot-boning. Establishing different declines in temperature and pH between DH and all other treatments through hot-processing was essential to this experiment. Without altering temperature decline in the DH-treated carcasses, we did not affect pH decline or associated post mortem glycolysis. The implementation of a more efficient chilling technique was needed to effectively improve cooling of the deep round muscle. Results from this study did reveal location differences within the muscle regarding meat quality and protease activity, demonstrating the need for future investigations to consider variation between the inner and outer SM when developing strategies to improve overall quality of the beef round muscle.

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CHAPTER 4. IMPACT OF GROWTH PROMOTANT TECHNOLOGIES ON THE PROTEIN PROFILE OF THE LONGISSIMUS FROM BEEF CATTLE

Abstract

Dietary ractopamine and anabolic implants improve beef leanness, whereas their effect on sarcoplasmic and myofibrillar proteomes has not been characterized. Therefore, the objective of this study was to identify proteins in bovine *longissimus lumborum* (LL) muscle in beef cattle as influenced by growth promotant technologies during the finishing period. Two-dimensional difference in-gel electrophoresis (2D DIGE) coupled with mass spectrometry (MS) was used to investigate the sarcoplasmic and myofibrillar protein fractions from LL samples of beef heifers randomly assigned to 1 of 3 treatment including cattle not exposed to growth promotant technologies (CON, n = 11), cattle exposed to growth promoting implants (IMP, n = 11), and cattle exposed to growth promoting implants and beta-agonists (OPT, n = 11). Cattle in the IMP treatment group received two steroidal implants (Component E-S followed by T-S, Elanco Animal Health) over the course of the finishing period. Cattle in the OPT treatment group received the same implant protocol as the IMP group and were also supplemented with ractopamine hydrochloride (RAC; Optaflexx, Elanco Animal Health) during the final 28 d of feeding. At 1 h post mortem, a section of the LL was removed from beef heifers (n = 33) and frozen in liquid nitrogen until analysis by 2D DIGE. Skeletal muscle samples were run using an immobilized pH 4-7 gradient (IPG) in the first dimension, followed by running proteins in their second dimension by SDS-PAGE. Mass spectrometry analyses with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF/TOF) was performed on in-gel spots of interest to identify proteins. Peptide identifications with > 95% probability with at least 2 identified unique peptides were accepted. Twenty spots from the sarcoplasmic fraction and 7

spots from the myofibrillar fraction were selected for further identification by MS. Of these, there were 25 spots in the sarcoplasmic fraction corresponding to 21 proteins that differed in relative abundance among growth promoting programs. Nine spots from the myofibrillar fraction corresponding to 6 proteins were also identified to be different among treatments. Overabundance ($P < 0.05$) of identified proteins in sarcoplasmic and myofibrillar fractions of the LL muscle from heifers subjected to exogenous growth promoting programs included metabolic enzymes (creatine kinase M-type, triosephosphate isomerase, beta-enolase), oxidative resistant proteins (peroxiredoxin-6, peroxiredoxin-2, protein deglycase DJ-1), muscle recovery proteins (myosin binding protein H, eukaryotic translation initiation factor 5A-1), and chaperone proteins (heat shock 70 kDa protein 1A). These results suggest exogenous growth promoting programs altered the protein profile of beef LL muscle, influencing the abundance of glycolytic enzymes and proteins possessing oxidative resistant, protective, regenerative, recovery, and anti-apoptotic properties that may potentially effect resulting meat quality.

Introduction

Growth promoting technologies, in particular, anabolic implants and β-adrenergic agonists, increase animal performance during the beef cattle finishing period. Anabolic implants have been shown to increase final body weight (BW) while maintaining a similar body composition to non-implanted cattle (Samber et al., 1996; Guiroy et al., 2002; Bryant et al., 2010), while β-adrenergic agonists fed for the last 20 to 40 d of finishing improves weight gain and increases muscle leanness (Avendano-Reyes et al., 2006; Boler et al., 2012). Producers often apply a combination implant consisting of trenbolone acetate (TBA) and estradiol (E_2), two compounds used to stimulate protein synthesis and satellite cell proliferation and differentiation, resulting in muscle fiber hypertrophy (Dayton and White, 2014). Ractopamine hydrochloride is a

β_1-adrenergic agonist that works as a repartitioning agent to redirect nutrients toward lean muscle accretion. These technologies are often used in combination to maximize animal productivity.

While several studies have documented the positive effects of growth promoting technologies on animal efficiency and carcass yields, authors have also reported their ability to negatively impact meat tenderness and quality traits (Garber et al., 1990; Foutz et al., 1997; Culp et al., 2013; Arp et al., 2014; Bohrer et al., 2014; Martin et al., 2014). Major changes in muscle protein architecture are associated with the conversion of muscle to meat (Huff-Lonergan et al., 2010). These authors have demonstrated varied expression levels of major structural proteins found in the myofibrillar protein fraction of skeletal muscle including myosin, actin, titin, troponin-T (TnT), and desmin to directly influence meat tenderization. Similarly, the sarcoplasmic protein fraction, consisting of soluble proteins and enzymes making up one-third of the total proteins in skeletal muscles, governs the biochemical processes influencing muscle metabolism (Scopes, 1974). Essential to the conversion of muscle to meat, sarcoplasmic proteins play a critical role in metabolic changes occurring during the transitional period. However, no inquiry has been made to study the individual and additive effects on growth technologies on the sarcoplasmic and myofibrillar proteome of *longissimus lumborum* (LL) beef muscle. Therefore, the objective of this study was to identify differential proteins in bovine LL muscle from beef cattle fed RAC with or without anabolic implant treatment during the finishing period.

Materials and Methods

Experimental Design

Crossbred feedlot heifers (n = 33, initial BW 430 ± 7 kg) were blocked by weight and assigned to 1 of 3 treatments consisting of: no implant or Optaflexx (CON; n = 11); implant, no Optaflexx (IMP; n = 11); Component T-200 implant and Optaflexx (OPT; n = 11). Cattle were

housed in individual pens located in an enclosed barn at Kansas State University Beef Cattle Research Center 10 d prior to the start of treatment. Each pen was 4.64 m^2 and contained metal pipe sidewalls, slatted floors for waste removal, an individual waterer, and a 75 x 51 cm feed bunk. Animals were fed a similar diet (Table 4.1), 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 of the study, animals assigned to be implanted were administered a Component TE-200 (Elanco Animal Health, Greenfield, IN) implant. Ractopamine hydrochloride (RAC; Optaflexx, Elanco Animal Health) was included in the total mixed ration of OPT animals to provide approximately dietary 400 mg·d^{-1}·$heifer^{-1}$ for 28 d before harvest. Upon completion of the treatment period, heifers were shipped 275 km to Creekstone Farms in Arkansas City, KS, for harvest. Animals were slaughtered and then allowed to chill for 48 h before carcass grading measurements were recorded. Strip loins were collected and transported back to the Kansas State University Meat Laboratory for further fabrication.

Collection of Muscle Sample

Approximately 1 h post-exsanguination, an approximate 10-g section of muscle was removed from the anterior portion of the LL, beginning at the 13^{th} rib of each loin, immediately placed on dry ice, and then stored at -80°C until analysis. Sample collection was performed in a chilling cooler, and muscle sections were immediately placed on dry ice until transfer to Kansas State Meat Laboratory. Samples were shipped on dry ice overnight from Kansas State University (Manhattan, KS) to the North Dakota State University muscle biology laboratory (Fargo, ND) for 2D DIGE analysis.

Table 4.1. Diet percentages (DM basis) for crossbred heifers[1] subjected to three exogenous growth promoting programs

	Treatment		
Ingredient, %	CON	IMP	OPT
Steam-flaked corn	57.91	57.91	57.12
Corn gluten feed	30.00	30.00	31.08
Ground alfalfa hay	8.00	8.00	7.76
Feed additive premix[2]	2.16	2.16	-
Vitamin/mineral supplement[3]	1.93	1.93	1.90
Ractopamine supplement[4]	-	-	2.14

[1] Crossbred heifers (n = 33) were subjected to one of three treatments: no implant or ractopamine hydrochloride (CON); Component TE-200 implant on d 0 of study with no ractopamine hydrochloride (IMP); Component TE-200 implant on d 0 of study and ractopamine hydrochloride at 400 mg•d^{-1}•$heifer^{-1}$ 28 d (OPT).
[2] Formulated to provide 300 mg/d monensin and 90 mg/d tylosin per animal in a ground corn carrier.
[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 400 mg•d^{-1}•$heifer^{-1}$ of ractopamine hydrochloride for 28 d.

Extraction of Sarcoplasmic Fraction

The highly soluble sarcoplasmic fraction was extracted from the LL samples. Approximately 1.5 g of muscle was homogenized in low ionic strength sarcoplasmic extraction buffer [50 m*M* Tris, 1 m*M* ethylenediaminetetraacetic acid (EDTA); pH 8.5; Anderson et al., 2012] with a Polytron (Brinkmann, Westbury, NY). Samples were centrifuged at 21,100 × *g* for 30 min at 4˚C. The supernatant (sarcoplasmic protein fraction) was decanted (using a transfer pipet) into 15-mL polypropylene conical tubes and the protein concentration was determined using the Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA; Bradford et al., 1976). The pellet (myofibrillar protein fraction) was saved on wet ice until ready to process. The protein concentration of the sarcoplasmic fraction was adjusted to 6 mg/mL using cold LISS, separated into 0.5 mL aliquots, and stored at -80˚C until analysis.

Extraction of Myofibrillar Fraction

To each myofibrillar protein pellet, 5.0 mL of cold LISS was added (washing step) and samples were centrifuged at 21,000 × *g* for 30 min at 4˚C. The supernatant was then discarded using a transfer pipet and the LISS washing step was repeated. The supernatant was discarded, and 10 mL of warm (≤ 30˚C) myofibrillar solubilizing buffer [8.3 *M* urea, 2 *M* thiourea, 2% (wt/vol) 3-[(3-cholamidopropyl) dimethyl-ammonia]-1-propanesulfonate (CHAPS), pH 8.5; Bjarnadóttir et al., 2011] was added to the myofibrillar protein fraction. The tubes were then vortexed and incubated in a warm water bath (≤ 30˚C) for 30 min. Each pellet was homogenized with a Polytron (Brinkmann, Westbury, NY) and centrifuged at 21,000 × *g* for 30 min at 25˚C. The supernatant was then transferred (using a transfer pipet) to a 15-mL polypropylene conical tube and stored at room temperature until same-day protein concentration determination with the Quick Start Bradford Protein Assay (Bio-Rad Laboratories). The protein concentration of the myofibrillar fraction was adjusted to 5 mg/mL using cold extraction buffer, separated into 0.5 mL aliquots, and stored at -80 ˚C until analysis.

Two-Dimensional Difference In-Gel Electrophoresis (2D DIGE) and Data Analysis

The sarcoplasmic and myofibrillar fractions of bovine LL from 6 heifers from each treatment (CON; n = 6, IMP; n = 6, OPT; n = 6) were chosen at random to be further analyzed by 2D DIGE in order to observe the difference in protein expression among animals receiving growth promoting technologies. First, an internal standard for each (sarcoplasmic and myofibrillar) fraction was created using a pooled sample representative of the samples used in the study and ran as a standard labeled with cyanine 2 yellow dye (Cy2) for all gels (Rozanas and Loyland, 2008; Westermeier and Scheibe, 2008). Then, samples from both fractions were labeled

with cyanine dyes (CyDye) according to the trials designed for the experiment (Table 4.2) and the manufacturer's directions (GE Healthcare, Piscataway, NJ).

Combinations of 15 µg of protein from the CON, IMP, and/or OPT and 15 µg from the internal standard were loaded on each immobilized pH gradient (IPG) strip (pH 4-7) for a total of 45 µg of protein per strip (Table 4.2; Fig. 4.1). Samples from three treatments (CON, IMP, and OPT) were labeled alternately with cyanine red dye (Cy3) and cyanine blue dye (Cy5) to avoid a labeling bias. Three trials were created among the 3 treatments (Table 4.2) that allowed 2D DIGE comparison between all exogenous growth promoting programs. Trials 1-3 compared IMP (n = 6) to CON (n = 6), OPT (n = 6) to CON (n = 6), and IMP (n = 6) to OPT (n = 6). Trials 1, 2, and 3 were run for both sarcoplasmic and myofibrillar fractions, and duplicate strips were run to minimize variation (36 sarcoplasmic + 36 myofibrillar = 72 total IPG strips in the experiment).

Separation of proteins in the first dimension by isoelectric point (pI) was accomplished using Immobiline DryStrips (11 cm, pH 4-7, GE Healthcare, Piscataway, NJ) containing 2.5 m*M* DL-dithiothreitol (DTT). Focusing of proteins on IPG strips was performed on an Ettan IPGphor 3 isoelectric focusing system (GE Healthcare, Piscataway, NJ). Initially, a low voltage (500 V) was applied, followed by a stepwise increase to 8000 V to reach a total of 18,500 V h. After isoelectric focusing, strips were equilibrated for 15 min in 10 mL of equilibration buffer (50 m*M* Tris pH 8.8, 6 *M* urea, 30% (vol/vol) glycerol, 2% (wt/vol) SDS, and a trace of bromophenol blue) containing 65 m*M* DTT. This was followed by an equilibration for 15 min in 10 mL of equilibration buffer containing 135 m*M* iodoacetamide (Rozanas and Loyland, 2008).

Second dimension electrophoresis (separation by molecular weight using SDS-PAGE) was run on 12.5% acrylamide gels (acrylamide: *N,N'*-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% SDS [wt/vol], 0.05% *N,N,N',N*-tetramethylethylenediamine (TEMED), 0.05% [wt/vol]-

Table 4.2. CyDye labeling[1] of 2 dimensional difference in-gel electrophoresis comparison of CON, IMP, and OPT d 1 bovine LL for crossbred heifers[2] subjected to three exogenous growth promoting programs.

IPG Strip	Cy2	Cy3	Cy5
		Trial 1	
1	Standard[3]	IMP	CON
2	Standard	OPT	IMP
3	Standard	CON	OPT
4	Standard	CON	IMP
5	Standard	IMP	OPT
6	Standard	OPT	CON
		Trial 2	
1	Standard	IMP	CON
2	Standard	OPT	IMP
3	Standard	CON	OPT
4	Standard	CON	IMP
5	Standard	IMP	OPT
6	Standard	OPT	CON
		Trial 3	
1	Standard	IMP	CON
2	Standard	OPT	IMP
3	Standard	CON	OPT
4	Standard	CON	IMP
5	Standard	IMP	OPT
6	Standard	OPT	CON

[1]Trials 1-3 were run for both myofibrillar and sarcoplasmic protein fractions. Trials were run in duplicate to minimize gel-to-gel variation.
[2] Crossbred heifers (n = 18) were subjected to one of three treatments: no implant or ractopamine hydrochloride (CON); Component TE-200 implant (Elanco Animal Health) on d 0 of study, no ractopamine hydrochloride (IMP); Component TE-200 implant on d 0 of study and ractopamine hydrochloride at 400 mg•d^{-1}•heifer^{-1} for last 28 d of finishing (OPT).
[3]Standard = pooled sample created for each protein fraction representative of all samples used in the study.

ammonium persulfate, and 0.5 *M* Tris, pH 8.8) with a Ettan DALTsix vertical slab gel unit (GE Healthcare, Piscataway, NJ). Two strips were placed side-by-side on the top of each gel, ran at 100 V for the first hour, and then run at 150 V until the tracking dye was 1/2 in from the bottom of the gel. Gels were then imaged on a Typhoon FLA 9500 biomolecular imager (GE Healthcare,

Piscataway, NJ) with 3 images for each strip gel area (Cy2, Cy3, and Cy5, respectively). All images were uploaded into DeCyder 2D software (v. 6.5; GE Healthcare, Piscataway, NJ) and analyzed to identify differences between treatments in relative abundance of individual spots.

After analysis of all spots was completed using DeCyder, a last set of gels (i.e., pick gels) was poured in order to manually pick gel spots for further identification and analysis by mass spectrometry (MS). Pick gels were 12.5% acrylamide gels (acrylamide: *N,N'*-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% SDS [wt/vol], 0.05% TEMED, 0.05% [wt/vol] ammonium persulfate, and 0.5 *M* Tris, pH 8.8) run on a Hoefer SE 600 Chroma unit (Hoefer Scientific Instruments, Hollison, MA). Four Immobiline IPG strips for pick gels were prepared as previously described, but with a load of 1000 µg of pooled unlabeled protein from all 3 treatment samples (CON, IMP, OPT) combined.

IPG strips were focused in the first dimension as previously described. For second dimension electrophoresis, two duplicate pick gels were run on each fraction (2 sarcoplasmic and 2 myofibrillar, respectively) at 150 V until tracking dye had just run off. After second dimension electrophoresis, pick gels (total of 4) were stained with a sensitive colloidal coomassie blue solution (10% [wt/vol] ammonium sulfate, 20% [vol/vol] methanol, 10% [vol/vol] phosphoric acid, and 0.12% [wt/vol] Coomassie G-250 for 24 h, followed by destaining for up to 4 h. Proteins of interest and those previously determined by differences detected in comparison studies using DeCyder 2D software were manually excised from the gel, placed into individual snap cap microcentrifuge tubes, and stored at -80°C until digestion and identification by MS.

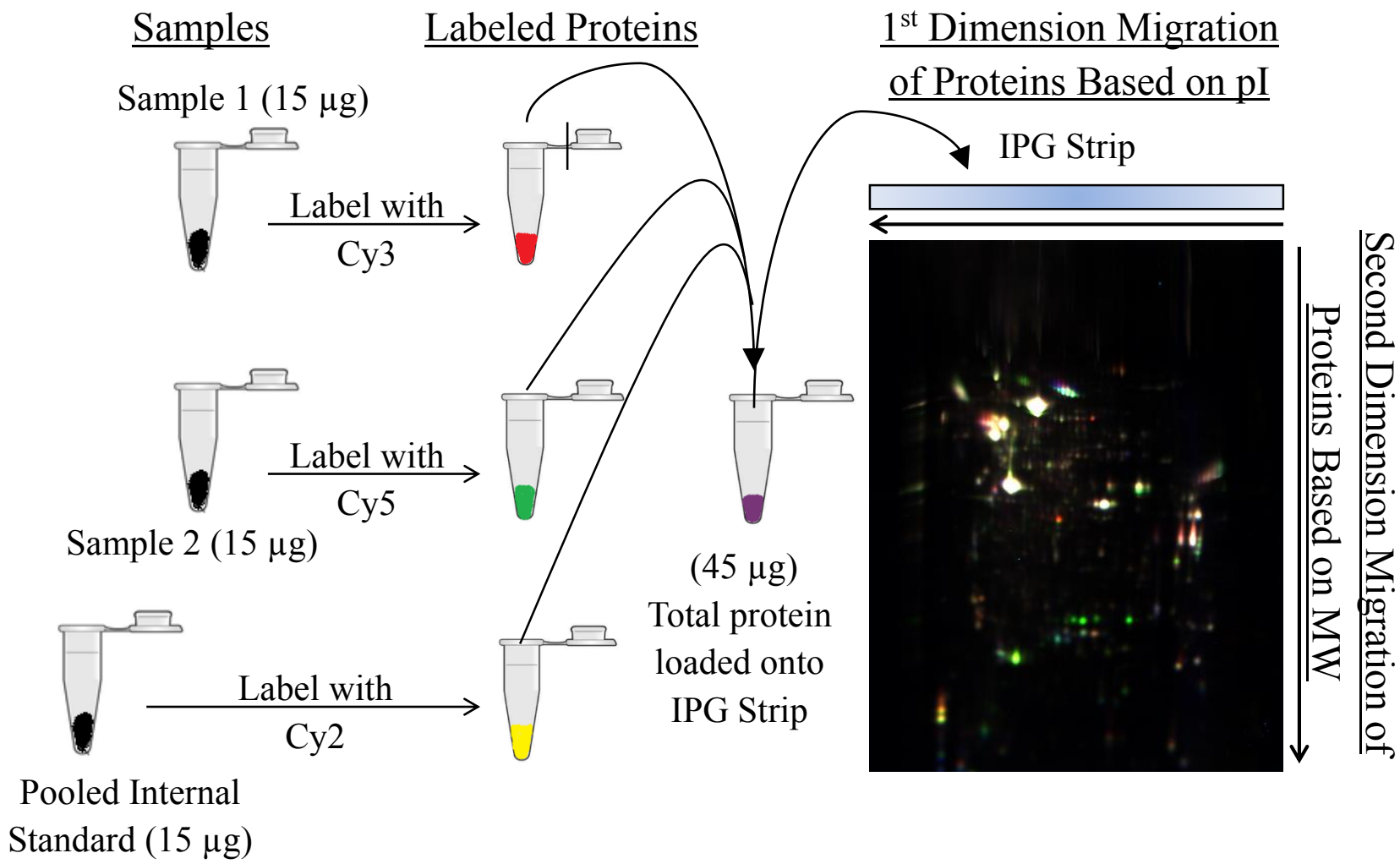

Figure 4.1. Two Dimensional In-Gel Electrophoresis (2D DIGE). Sample preparation for 2D PAGE. Adapted from Corbett, J. 1994.

In-Gel Protein Digestion

Spots were destained and in-gel trypsin digested by standard laboratory protocol (Shevchenko et al., 1996). Gel spots were destained and proteins were reduced in-gel with 4 m*M* DTT in 50 m*M* ammonium bicarbonate for 15 min at 60˚C. Iodoacetamide was added to make 16 m*M*, and alkylation was allowed to proceed for 30 min at room temperature in the dark. The reaction was quenched with an additional 3 m*M* DTT. The gel spot was then equilibrated with 55 m*M* ammonium bicarbonate, dehydrated with 100% acetonitrile, and rehydrated in 0.02 µg Trypsin Gold (Promega, Madison, WI) in 40 m*M* ammonium bicarbonate. Digestion was allowed to proceed overnight at 37˚C. Approximately 50 µL of peptides were extracted. The samples were acidified with formic acid to make 0.1% final concentration.

Manual Spot Analysis

A subset of spots was initially analyzed by manual spotting. Peptides from these samples were captured by C18 solid-phase extraction using Zip-Tips by passing 13 µL through the tip. The remaining partially depleted sample was retained and stored at -80˚C. The recovered peptides were eluted and 0.5 µL was mixed with an equal volume of 10 mg/mL of α-cyanohydroxy cinammic acid (CHCA) in 75% [vol/vol] acetonitrile/0.1% formic acid and spotted onto a MALDI target. Samples were analyzed using an AB 4800 MALDI TOF/TOF. Top 20 MS precursors were selected and analyzed by MS/MS from weakest to strongest. The remaining spots were analyzed by manual spotting.

LC-TEMPO Analyses

Samples were analyzed by reverse phase high performance liquid chromatography (rpHPLC) and spotted onto a matrix-assisted laser desorption/ionization (MALDI) target plate using a TEMPO-LC integrated nanoflow HPLC/spotter. A total of 8.8 µL of sample was injected

onto a Proteocol C18 0.3 c 10 mm trap (3 µm, 300A pore size). The samples were desalted with 2% acetonitrile/2% formic acid (vol/vol) for 10 min at a flow rate of 10 µL/min. Peptides were eluted in-line through a 0.1 x 100 mm Magic AQ C18 (5 µm) column using a 30 m gradient from 100% Buffer A to 60% Buffer A, 40% Buffer B (98% [vol/vol] acetonitrile, 0.1% [vol/vol] formic acid) at a flow rate of 1 µL/min. Eluate was mixed post-column with an equal volume of 10 mg/ml CHCA in 75% [vol/vol] acetonitrile and 0.1% [vol/vol] formic acid. The matrix/eluent mix was spotted at 18 sec/spot. The column was regenerated at 70% (vol/vol) acetonitrile/0.1% formic acid.

Protein Isolation and Identification by Mass Spectrometry

Spot sets were analyzed on the AB4800 MALDI TOF/TOF in reflector positive ion mode. For MS the *m/z* range was 800 to 4000. A total of 400 subspectra were accumulated per spectrum. For MS/MS, the top 10 precursors were selected per spot with the weakest precursor first. A total of 600 subspectra were accumulated per MS/MS spectrum.

Raw spectra were converted to T2D files using 4000 series explorer software (ABI) version 3.5.28193. The file extension T2D stands for technology transfer database; a binary format data file that is exported from an Oracle database. These files were converted to Mascot generic format (mgf) files using Peaks to Mascot. All spectra were searched with Mascot (v. 2.3.02, Matrix Science) against the Universal Protein resource (UniProt) database restricted to the bovine complete proteome, human complete proteome, or the sheep proteome. The latter was incomplete and contained many sequence fragments. The search parameters were trypsin with up to one missed cleavage, carbamidomethyl (N-term C), Gln>pyro-Glu (N-term Q), Oxidation (M). Mass values were monoisotopic, peptide mass tolerance was 100 ppm, and fragment ion tolerance was 0.5 Da. Mascot searches were combined and analyzed in Scaffold (v. 3.00.08) to

validate peptide and protein identifications. Each spot was treated as a biological replicate. This permitted spot-to-spot comparison which helped to flag some identifications as carry-over contaminants from proceeding runs. Peptide identifications with > 95% probability and protein identifications with > 99% probability and at least 2 identified peptides were accepted.

Results

Animal Performance and Carcass Characteristics

Body weight (BW) was not different across treatments before and after RAC supplementation ($P > 0.14$). Consistent with these findings, average daily gain (ADG), dry matter intake, and gain to feed did not differ ($P > 0.11$) across growth promoting programs. These results differ from other studies (Scramlin et al., 2010; Bohrer et al., 2014) who reported increased ADG and feed efficiency in beef cattle supplemented with RAC during the finishing period. While Scramlin saw increased final BW, Bohrer observed no differences in hot carcass weight (HCW) of steers fed RAC. However, dosage amounts (200 to 400 mg/d) and duration of treatment (28 to 33 d) differs between studies. Upon completion of RAC supplementation, cattle were transported to a commercial abattoir for harvest. HCW was measured at time of harvest, while other carcass characteristics were taken following a 48-h chill period. Although HCW tended ($P < 0.09$) to differ across treatments, dressing percentage and yield grade did not differ ($P > 0.12$) across treatment groups. Regarding the amount of lean muscle deposition, there was an increase in ribeye area (REA). Heifers subjected to OPT and IMP treatments had increased ($P > 0.01$) REA when compared with those from CON, but they did not differ ($P > 0.53$) from one another. In relation to REA, loin weight was also increased ($P < 0.05$) for OPT and IMP strip loins when compared with CON loins. Adipose measurements of back fat and marbling were unaffected ($P > 0.45$) by growth promoting treatment. These results are in agreement with past

findings in beef cattle supplement with RAC with or without anabolic implants (Foutz et al., 1997; Scramlin et al., 2010; Martin et al., 2014).

Loin pH, Cook Loss, and Warner-Bratzler Shear Force

Strip loins were fabricated and aged 2 d prior to Warner-Bratzler shear force tenderness analysis to investigate negative tenderness attributes associated with exogenous growth promotants. Loin pH and cook loss were not different ($P > 0.23$) across treatments. Growth promoting treatments increased ($P < 0.01$) the shear force value of steaks aged 2 d. The OPT steaks has increased ($P < 0.01$) shear force values when compared with CON-treated steaks, whereas the IMP steaks shear force value tended to decrease ($P < 0.07$) when compared to the OPT steaks and tended to increase ($P < 0.10$) when compared to the CON steaks.

Protein Profile

The proteins identified by MS are presented in Tables 4.4 and 4.5 (sarcoplasmic and myofibrillar fractions, respectively) with their UniProt name, sequence coverage, isoelectric pH (p*I*), molecular weight (MW), and location. The functional roles of the differentially abundant proteins are grouped by their overabundant treatment expression in Tables 4.6, 4.7, and 4.8 (CON, OPT, and IMP, respectively).

Sarcoplasmic Fraction

Image analyses of the 2D DIGE gels identified 25 differentially abundant protein spots (Table 4.4 and Fig. 4.2) among CON, OPT, and IMP comparisons. Eight protein spots over-abundant ($P < 0.05$) in CON-treated LL (Table 4.6) were identified as phosphoglucomutase, adenosine kinase, actin (alpha cardiac muscle 1), actin (cytoplasmic 1 or 2), phosphoglycolate phosphatase (in 2 different spots), heat shock protein beta-1, and apolipoprotein A-1. Seventeen

protein spots overabundant ($P < 0.05$) in OPT-treated LL (Table 4.7) were identified as serum albumin, serotransferrin, aldehyde dehydrogenase, adenosylhomocysteinase, alpha-enolase,

Table 4.3. Live performance, carcass characteristics, boneless strip loin characteristics, and objective strip loin measurements from the *Longissimus lumborum* of crossbred heifers[1] subjected to three exogenous growth promoting programs.

	Treatment				
	CON[1]	IMP[1]	OPT[1]	SEM	*P*-value
Live Performance					
Initial body weight, kg	472.4	486.1	483.2	7.4	0.39
Final body weight, kg	512.9	530.2	531.2	7.2	0.14
Average daily gain, kg	1.48	1.62	1.76	0.10	0.18
Dry matter intake, kg	9.25	9.18	9.00	0.30	0.83
Gain to feed, kg	0.16	0.17	0.21	0.08	0.11
Carcass Characteristics					
Hot carcass weight, kg	327.1	340.7	341.9	5.2	0.09
Dressing percentage, %	63.6	63.8	64.3	0.4	0.41
Ribeye area, cm^2	84.2[a]	91.2[b]	92.6[b]	0.3	< 0.01
12^{th} rib back fat, cm	1.3	1.1	1.2	0.1	0.45
Yield grade	2.8	2.3	2.4	0.2	0.12
Marbling[2]	519	503	519	20	0.80
Initial loin weight, kg	5.71[a]	6.16[b]	6.14[b]	0.14	0.05
Objective Measures					
pH[3]	5.49	5.49	5.50	0.01	0.98
Cook loss[4], %	20.80	22.78	23.58	0.62	0.23
WBSF[5], kg	5.00[a]	5.47[ab]	6.05[b]	0.27	< 0.01

[a,b] Means within a row with a different superscript are different ($P < 0.05$).
[1]Crossbred heifers (n = 33) were subjected to one of three treatments: 1) no implant and no Optaflexx supplementation (CON); 2) implanted with Component TE-200 on d-0 of feeding, no Optaflexx supplementation (IMP); and 3) implanted with Component TE-200 on d-0 of feeding, and supplemented with 400 $mg \bullet d^{-1} \bullet heifer^{-1}$ Optaflexx for 28 d (OPT).
[2]USDA Marbling scores: 400-499 = small; 500-599 = modest.
[3] pH was measured using a calibrated pH meter (model HI 99163; Hanna Instruments, Smithfield, RI) from the geometric center of the strip loin on d 2 of post mortem aging.
[4] Cook loss steaks aged 2 days calculated using the equation [(raw weight – final cooked weight)/ (raw weight)] × 100.
[5] WBSF = Warner-Bratzler shear force. Steaks aged 2 d and then cooked to an internal temperature of 66.7°C.
Note. Adapted from unpublished data collected at Kansas State University by J. Gonzalez and S. Ebarb, 2014.

beta-enolase, creatine kinase M-type (2 different spots), malate dehydrogenase (cytoplasmic), carbonic anhydrase, peroxiredoxin-6, triosephosphate isomerase (2 different spots), protein deglycase DJ-1 (2 different spots), peroxiredoxin-2, and eukaryotic translation initiation factor 5A-1. Nine protein spots over-abundant ($P < 0.05$) in LL from IMP-treated heifers (Table 4.8) were identified as serum albumin, serotransferrin, creatine kinase M-type, phosphoglycolate phosphatase (2 different spots), peroxiredoxin-6, triosephosphate isomerase (2 different spots), and protein deglycase DJ-1.

Myofibrillar Fraction

Image analyses of the 2D DIGE gels identified 7 differentially abundant protein spots (Table 4.5 and Fig. 4.3) among CON, OPT, and IMP comparisons. Four protein spots over-abundant ($P < 0.05$) in CON-treated LL (Table 4.6) were identified as myosin binding protein H, actin (alpha cardiac muscle 1), ATP synthase subunit beta (mitochondrial), and myosin light chain 1/3. Four protein spots over-abundant ($P < 0.05$) in LL from heifers subjected to the OPT treatment (Table 4.7) were identified as heat shock 70 kDa protein 1A (2 different spots), myosin binding protein H, and actin (alpha cardiac muscle 1). Five spots over-abundant ($P < 0.05$) in LL from IMP-treated heifers (Table 4.8) were identified as heat shock 70 kDa protein 1A (2 different spots), myosin binding protein H, and actin (alpha cardiac muscle 1), and troponin-T (fast skeletal muscle).

Discussion

Few proteins demonstrated a shift from theoretical p*I*, which may be due to possible post-translational modifications (Jia et al., 2009). In addition, multiple spots were representative of the same protein [aldehyde dehydrogenase (2 spots), phosphoglycolate phosphatase (2 spots), triosephosphate isomerase (2 spots), creatine kinase M-type (2 spots), and protein deglycase DJ-

1 (2 spots)], which is often seen in 2D DIGE gels, thought to result from protein fragmentation of post-translational modifications (Hamelin et al., 2007).

The differentially abundant proteins identified in both sarcoplasmic and myofibrillar fractions of the bovine LL included metabolic enzymes, antioxidant proteins, chaperone proteins, and muscle recovery proteins. Overall, the LL from OPT and IMP-treated heifers exhibited amplified expression of glycolytic and metabolic enzymes. Noticeably, heifers receiving exogenous growth promotants demonstrated overabundance of antioxidant, anti-apoptotic, regenerative proteins, such as carbonic anhydrase III, protein deglycase DJ-1, peroxiredoxin-2, peroxiredoxin-6, aldehyde dehydrogenase, heat shock 70 kDa protein 1A, myosin binding protein H, and eukaryotic translation initiation factor 5A-1.

While we saw no upregulation of chaperone and muscle recovery-related proteins in the myofibrillar fraction from CON-treated LL muscle. We did observe abundance increases in structural proteins needed for maintenance of muscle contraction including myosin binding protein H and Actin (alpha cardiac muscle 1), similar to those identified in OPT and IMP treatments, indicating the importance of maintaining skeletal muscle integrity proteins during the finishing period. The increased abundance of glycolytic proteins (phosphoglucomutase) and metabolic enzymes (phosphoglycolate phosphatase, adenosine kinase) observed in the LL muscle from heifers in the CON-treated group demonstrates a shift in metabolism to a more glycolytic muscle, also similar to that observed in OPT and IMP-treated muscle. For the purpose of this discussion on effects of growth promotant programs, we will focus on identified proteins overexpressed in OPT and IMP treatments in response to RAC with or without anabolic implant treatment.

Table 4.4. Identification by LC-MS/MS of 2D DIGE protein spots from sarcoplasmic fractions differentially abundant in *Longissimus lumborum* muscle of crossbred heifers subjected to three exogenous growth promoting programs.

Spot[A]	Protein	Uniprot Name	Matched peptides	pI (theo/exp)	MW (kDa) (theo/exp)	Location
123	Serum albumin	ALBU_BOVIN	16	5.60/5.82	66/70	cytoplasm, nucleus
141	Serotransferrin	G3X6N3	7	7.13/6.75	79/80	plasma membrane
185	Phosphoglucomutase-1	PGM1_BOVIN	6	6.36/6.36	62/62	cytoplasm
257	Aldehyde dehydrogenase	ALDH2_BOVIN	6	6.05/7.55	54/57	cytosol and organelles
266	Adenosylhomocysteinase	SAHH_BOVIN	3	5.88/5.88	48/48	cytoplasm
282	Alpha-enolase	ENOA_BOVIN	6	6.37/6.37	47/48	cytoplasm, cell membrane
284	Adenosine kinase	F1N468	7	5.85/5.85	39/39	cytoplasm
302	Actin, alpha cardiac muscle 1	ACTC_BOVIN	5	5.23/5.23	42/42	muscle
307	Actin, cytoplasmic 1 or 2	ACTB_BOVIN	4	5.29/5.31	42/42	cytoplasm, cytoskeleton
314	Beta-enolase	ENOB_BOVIN	23	7.74/6.73	47/47	cytosol
327	Creatine kinase M-type	KCRM_BOVIN	19	6.68/6.63	43/43	cytoplasm
328	Creatine kinase M-type	KCRM_BOVIN	5	6.68/6.63	43/43	cytoplasm
362	Phosphoglycolate phosphatase	PGP_BOVIN	6	5.18/5.18	34/35	cytosol
380	Malate dehydrogenase, cyto.	MDHC_BOVIN	8	6.15/6.16	36/37	mitochondria, cytoplasm
393	Carbonic anhydrase 3	CAH3_BOVIN	5	7.84/7.71	29/30	cytosol
395	Peroxiredoxin-6	PRDX6_BOVIN	8	6.02/6.00	25/25	cytoplasm, lysosome
403	Heat shock protein beta-1	HSPB1_BOVIN	5	5.98/5.98	22/22	nucleus, cytoplasm
412	Triosephosphate isomerase	TPIS_BOVIN	11	6.45/6.45	27/27	nucleus, extracellular space
422	Triosephosphate isomerase	TPIS_BOVIN	28	6.45/6.45	27/27	nucleus, extracellular space
427	Protein deglycase DJ-1	PARK7_BOVIN	13	6.84/6.84	20/20	ubiquitous
435	Protein deglycase DJ-1	PARK7_BOVIN	15	6.84/6.84	20/20	ubiquitous
440	Peroxiredoxin-2	PRDX2_BOVIN	5	5.37/5.37	22/22	cytoplasm
476	Phosphoglycolate phosphatase	PGP_BOVIN	6	5.18/5.18	34/35	cytosol
478	Apolipoprotein A-1	APOA1_BOVIN	12	5.36/5.71	28/30	secreted
486	Eukaryotic translation initiation factor 5A-1	IF5A1_BOVIN	3	5.08/5.08	17/17	cytoplasm

[A] Spot number refers to the numbered spots in gel image (Fig. 4.2). For each spot, different parameters related to protein identification are provided: Uniprot name, number of matched peptides, theoretical and experimental isoelectric point (pI), theoretical and experimental protein mass (MW), and location.

Table 4.5. Identification by LC-MS/MS of 2D DIGE protein spots from myofibrillar fraction differentially abundant in *Longissimus lumborum* muscle of crossbred heifers subjected to three exogenous growth promoting programs.

Spot[A]	Protein	Uniprot Name	Matched peptides	pI (theo/ exp)	MW (kDa) (theo/exp)	Location
184	Heat shock 70 kDa protein	HS71A_BOVIN	7	5.68/5.70	70/70	Ubiquitous
185	Heat shock 70 kDa protein	HS71A_BOVIN	8	5.68/5.70	70/70	Ubiquitous
190	Myosin binding protein H	Q0VBZ1_BOVIN	5	5.71/6.30	53/54	A-band of myofibril
192	Myosin binding protein H	Q0VBZ1_BOVIN	3	5.716.30	53/54	A-band of myofibril
259	Actin, alpha cardiac muscle 1	ACTC_BOVIN	4	5.23/5.20	42/42	cytoplasm, cytoskeleton
268	ATP synthase subunit beta, mito.	ATPB_BOVIN	8	5.00/5.15	52/56	mitochondria
322	Actin, alpha cardiac muscle 1	ACTC_BOVIN	11	5.23/5.20	42/42	cytoplasm, cytoskeleton
364	Troponin-T, fast skeletal muscle	TNNT3_BOVIN	3	5.99/6.00	32/32	muscle
449	Myosin light chain 1/3	MYL1_BOVIN	3	4.96/5.00	21/19	myofibril, myosin complex

[A] Spot number refers to the numbered spots in gel image (Fig. 4.3). For each spot, different parameters related to protein identification are provided; Uniprot name; number of matched peptides; theoretical and experimental isoelectric point (pI); theoretical and experimental protein mass (MW), and location.

Table 4.6. Functional roles of sarcoplasmic and myofibrillar proteins upregulated in CON-treated *Longissimus lumborum* from beef heifers collected 2 h post mortem.

Spot[A]	Protein	Comparison	Percent change[B]	*P*-Value	Function
Sarcoplasmic fraction					
185	Phosphoglucomutase	CON/OPT	39	0.01	glycolysis
284	Adenosine kinase	CON/OPT	33	<0.001	removal of adenosine, regulation of blood
		CON/IMP	21	0.04	flow to skeletal muscle
302	Actin, alpha cardiac muscle 1	CON/OPT	18	0.03	ATPase activity, ATP binding, actin-myosin
		CON/IMP	21	0.02	filament sliding
307	Actin, cytoplasmic 1 or 2	CON/OPT	16	0.01	ATP binding
		CON/IMP	20	0.005	
362	Phosphoglycolate phosphatase	CON/IMP	23	<0.001	carbohydrate metabolism
403	Heat shock protein beta-1	CON/OPT	48	<0.001	modulators of muscle contraction, cell
		CON/IMP	42	<0.001	migration, and cell survival
476	Phosphoglycolate phosphatase	CON/IMP	34	<0.001	carbohydrate metabolism
478	Apolipoprotein A-1	CON/OPT	28	0.002	elastase activity
		CON/IMP	21	<0.001	
Myofibrillar fraction					
192	Myosin binding protein H	CON/OPT	45	<0.001	localization of the A-band, formation of
		CON/IMP	19	0.07	myofibrils
259	Actin, alpha cardiac muscle 1	CON/OPT	42	<0.001	ATPase activity, ATP binding, actin-myosin
		CON/IMP	14	0.02	filament sliding
268	ATP synthase subunit beta,	CON/OPT	28	<0.001	ATP binding, proton transporting ATPase
	mitochondrial	CON/IMP	27	<0.001	activity
449	Myosin light chain 1/3	CON/OPT	34	0.003	muscle contraction

[A] Spot number refers to the numbered spots in gel images for sarcoplasmic and myofibrillar fractions (Fig 4.2 and 4.3), respectively).

[B] Positive values indicate an increase in the CON-treated LL over that of the comparative treatment.

Table 4.7. Functional roles of sarcoplasmic and myofibrillar proteins upregulated in OPT-treated *Longissimus lumborum* from beef heifers collected 2 h post mortem.

Spot[a]	Protein	Comparison	Percent change[b]	*P*-Value	Function
Sarcoplasmic fraction					
123	Serum albumin	OPT/CON	23	<0.001	negative regulation of apoptosis, transport
141	Serotransferrin	OPT/CON	34	0.02	transport
257	Aldehyde dehydrogenase	OPT/CON	40	<0.001	aldehyde oxidation, degradation of amino acids and
		OPT/IMP	25	<0.001	lipids, glycolysis, respond to oxidative stress
266	Adenosylhomocysteinase	OPT/CON	28	0.009	regulator of methylation
		OPT/IMP	23	0.03	
282	Alpha-enolase	OPT/CON	33	0.01	glycolysis
		OPT/IMP	26	0.001	
314	Beta-enolase	OPT/CON	19	0.02	muscle development and regeneration
		OPT/IMP	18	0.02	
327	Creatine kinase M-type	OPT/CON	15	0.007	ATP regeneration
328	Creatine kinase M-type	OPT/CON	25	<0.001	ATP regeneration
380	Malate dehydrogenase, cyto.	OPT/IMP	12	0.02	TCA cycle, oxidoreductase
393	Carbonic anhydrase	OPT/CON	30	<0.001	response to oxidative stress
		OPT/IMP	20	0.001	
395	Peroxiredoxin-6	OPT/CON	29	<0.001	redox regulation, lipid degradation
		OPT/IMP	14	0.01	
412	Triosephosphate isomerase	OPT/CON	30	0.01	glycolysis, gluconeogenesis
422	Triosephosphate isomerase	OPT/CON	30	<0.001	glycolysis, gluconeogenesis
427	Protein deglycase DJ-1	OPT/CON	25	0.01	antioxidant/oxidative stress sensor, quenching of
		OPT/IMP	18	0.02	ROS, upregulation of molecular chaperones
435	Protein deglycase DJ-1	OPT/CON	36	<0.001	antioxidant/oxidative stress sensor, quenching of
		OPT/IMP	17	0.02	ROS, upregulation of molecular chaperones
440	Peroxiredoxin-2	OPT/CON	24	0.03	redox regulation, regulation of apoptosis
		OPT/IMP	26	<0.01	
486	Eukaryotic translation	OPT/CON	24	0.04	muscle regeneration, skeletal muscle stem cell
	initiation factor 5A-1	OPT/IMP	15	0.006	differentiation

Table 4.7. Functional roles of sarcoplasmic and myofibrillar proteins upregulated in OPT-treated *Longissimus lumborum* from beef heifers collected 2 h postmortem. (Continued).

Spot[A]	Protein	Comparison	Percent change[B]	*P*-Value	Function
Myofibrillar fraction					
184	Heat shock 70 kDa protein 1A	OPT/CON	18	0.001	stress response, apoptosis inhibition, protection of muscle damage
185	Heat shock 70 kDa protein 1A	OPT/CON	33	<0.001	stress response, apoptosis inhibition, protection of muscle damage
190	Myosin binding protein H	OPT/CON	27	0.01	localization of the A-band, formation of myofibrils
268	Actin, alpha cardiac muscle 1	OPT/CON	28	<0.001	ATPase activity, ATP binding, actin-myosin filament sliding
		OPT/IMP	19	<0.001	

[A] Spot number refers to the numbered spots in gel images for sarcoplasmic and myofibrillar fractions (Fig 4.2 and 4.3), respectively.
[B] Positive values indicate an increase in the OPT-treated LL over that of the comparative treatment.

Table 4.8. Functional roles of sarcoplasmic and myofibrillar proteins upregulated in IMP-treated *Longissimus lumborum* from beef heifers collected 2 h post mortem.

Spot[A]	Protein	Comparison	Percent change[B]	P-Value	Function
Sarcoplasmic fraction					
123	Serum albumin	IMP/CON	19	0.006	negative regulation of apoptosis, transport
141	Serotransferrin	IMP/CON	27	0.004	transport
328	Creatine kinase M-type	IMP/CON	18	0.004	ATP regeneration
362	Phosphoglycolate phosphatase	IMP/OPT	23	<0.001	carbohydrate metabolism
395	Peroxiredoxin-6	IMP/CON	14	0.02	redox regulation, lipid degradation
412	Triosephosphate isomerase	IMP/CON	28	0.03	glycolysis, gluconeogenesis
422	Triosephosphate isomerase	IMP/CON	22	<0.001	glycolysis, gluconeogenesis
435	Protein deglycase DJ-1	IMP/CON	16	0.02	antioxidant/oxidative stress sensor, quenching of ROS, upregulation of molecular chaperones
476	Phosphoglycolate phosphatase	IMP/OPT	30	<0.001	carbohydrate metabolism
Myofibrillar fraction					
184	Heat shock 70 kDa protein 1A	IMP/CON	16	0.012	stress response, apoptosis inhibition, protection of muscle damage
185	Heat shock 70 kDa protein 1A	IMP/CON	27	<0.001	stress response, apoptosis inhibition, protection of muscle damage
190	Myosin binding protein H	IMP/CON	57	<0.001	localization of the A-band, formation of myofibrils
		IMP/OPT	24	0.01	
259	Actin, alpha cardiac muscle 1	IMP/OPT	24	<0.001	ATPase activity, ATP binding
364	Troponin-T, fast skeletal muscle	IMP/CON	53	<0.001	regulation of muscle contraction, calcium-dependent ATPase activity
		IMP/OPT	45	0.006	

[A] Spot number refers to the numbered spots in gel images for sarcoplasmic and myofibrillar fractions (Fig 4.2 and 4.3), respectively.

[B] Positive values indicate an increase in the IMP-treated LL over that of the comparative treatment.

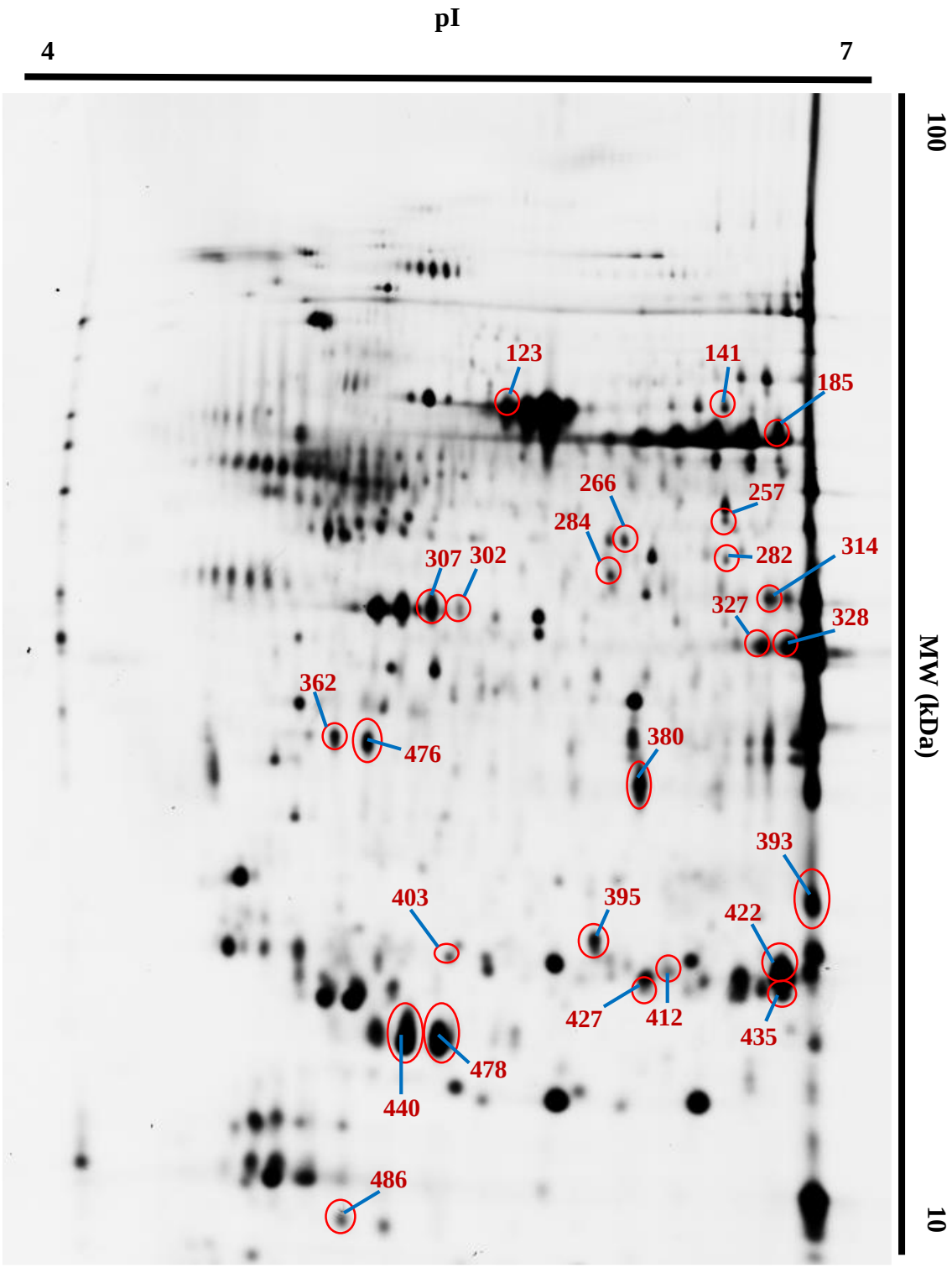

Figure 4.2. A representative 2D DIGE gel from bovine *Longissimus lumborum* (LL) sarcoplasmic fraction showing identified proteins. A total of 45 µg of CyDye-labeled protein (15 µg of each CyDye 2, 3, and 5) was loaded onto a 11-cm pH 4-7 IPG strip for the first dimension, and the second dimension was run on a 12.5% SDS-PAGE gel. Proteins labeled with CyDye5 are shown.

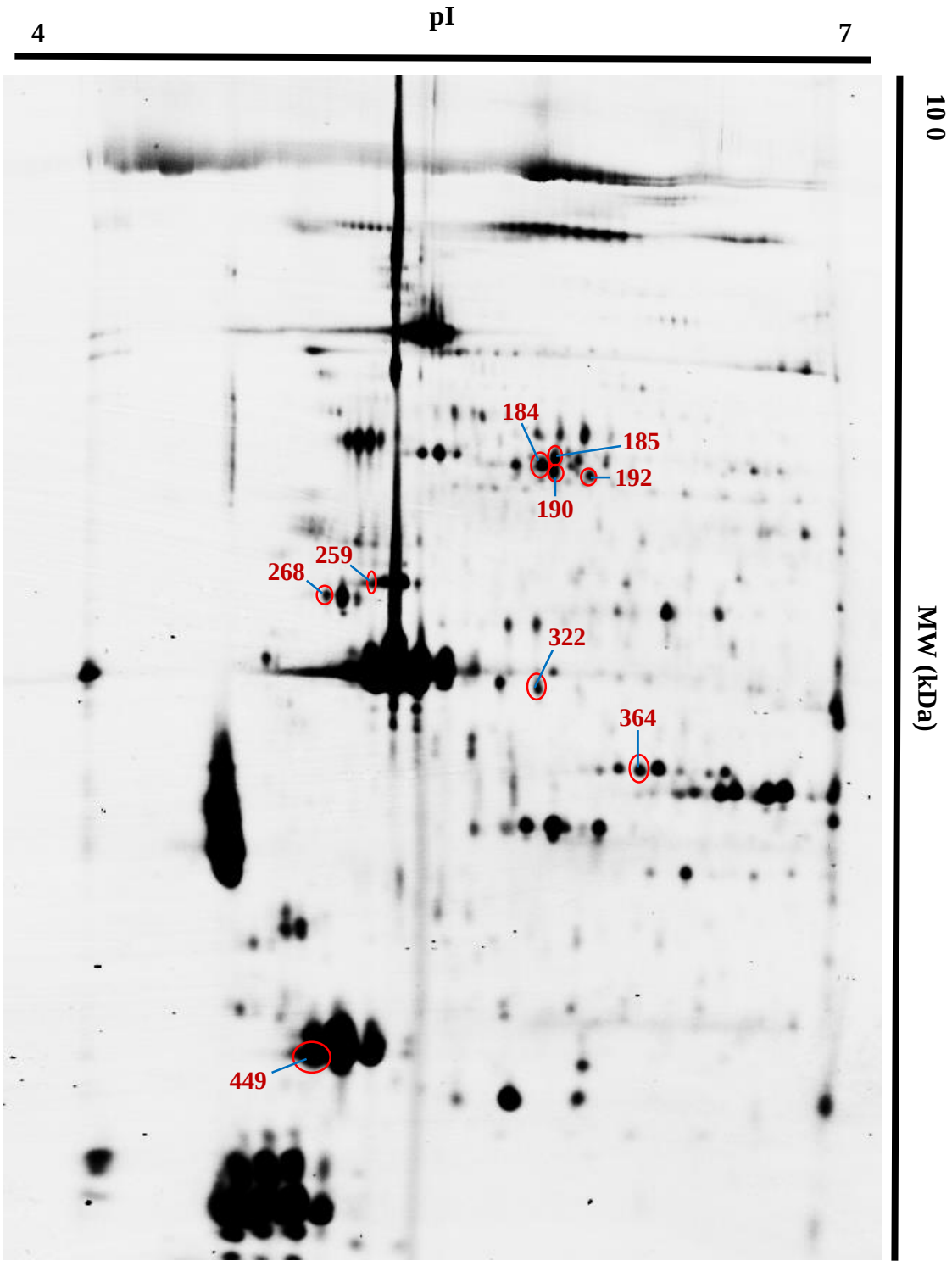

Figure 4.3. A representative 2D DIGE gel from bovine *Longissimus lumborum* (LL) myofibrillar fraction showing identified proteins. A total of 45 µg of CyDye-labeled protein (15 µg of each CyDye 2, 3, and 5) was loaded onto a 11-cm pH 4-7 IPG strip for the first dimension, and the second dimension was run on a 12.5% SDS-PAGE gel. Proteins labeled with CyDye3 are shown.

Protein Functionality and Potential Relationship with Exogenous Growth Promotants

Metabolic enzymes

Regarding muscle metabolic behavior, we can note from differential protein abundance in both treatments subjected to growth promoting programs (IMP and OPT), that comparisons show a progressive regulation of the principal 'reloading' mechanism of muscle fibers, as suggested by the upward trend of creatine kinase M-type (Tables 4.7 and 4.8 for OPT and IMP treatments, respectively). The mitochondrial enzyme is responsible for synthesis of the high-energy compound, phosphocreatine, needed for correct functioning of the creatine-phosphate shuttle to diffuse phosphocreatine from mitochondria to myofibrils. In addition to mitochondrial creatine kinase, the overabundance of the glycolytic enzyme, triosephosphate isomerase (OPT and IMP, Tables 4.7 and 4.8, respectively), alpha-enolase (OPT, Table 4.7), and beta-enolase (OPT, Table 4.7) signify a pronounced glycolytic metabolism in the LL of heifers treated with exogenous growth promotants.

Oxidative resistance

The overexpression of metabolic enzymes indicates increased protein turnover in LL from heifers subjected to β-agonists and/or anabolic implants. Such a rapid increase in the demand for energy in order to accommodate amplified muscle growth results in an increased glycolytic metabolism. Glycolysis is the metabolic pathway in mitochondria that converts glucose/glycogen into pyruvate and hydrogen. The pyruvate is then transformed into acetyl-CoA and enters the Krebs cycle. When ADP is transformed to ATP as a result of the Krebs cycle and Electron Transport Chain, otherwise known as oxidative phosphorylation, reactive oxygen species (ROS) build up as a byproduct. Although the aerobic metabolism of skeletal muscle is continuously generating ROS, the inability to manage ROS capacity leads to elevated oxidative

stress and cell damage. Due to this, cells have developed an antioxidant defense system comprised of antioxidant vitamins, glutathione, sulfhydryls, and antioxidant enzymes (Maclin and Bendich, 1987). Seven differentially abundant proteins (Tables 4.7 and 4.8) were expressed in LL from IMP and OPT treated-heifers demonstrating antioxidant properties in skeletal muscle tissue.

Carbonic anhydrase III (CAIII) is abundant in red skeletal muscle (8% of soluble protein) and has been shown to respond to oxiradicals during oxidative stress through oxidizable sulfhydryls that serve as a first responder in skeletal muscle (Zimmerman et al., 2004). In Zimmerman's study, CAIII exhibited a reparative function, with irreversible oxidation of CAIII found to indicate non-recoverable muscle damage. The cell redox state has also been suggested to regulate apoptosis and programmed cell death. When exposed to hydrogen peroxide, Raisenen and others (1999) demonstrated cells lacking CAIII showed rapid increase in ROS, whereas no increase was seen in CAIII-introduced cells, indicating a protective action on apoptotic-induced cells. Upregulated in the LL from OPT-treated hiefers, CAIII abundance was increased by 30 percent ($P < 0.001$) when compared with CON muscle and increased by 20 percent when compared with LL from IMP-treated muscle.

Another protein with anti-apoptotic properties, protein deglycase DJ-1 has recently been shown to protect cells from oxidative damage by regulating cellular responses to oxidative stress in skeletal muscle from humans and mice (Taira et al., 2004; Andres-Mateos et al., 2007; Dodson and Guo, 2007). In a study that treated cells with various concentrations of hydrogen peroxide, Taira and collaborators observed the acitivity of DJ-1 oxidizing itself in response to hydrogen peroxide introduction. Through the activity of oxidizing itself, DJ-1 eliminated hydrogen peroxide and protected cells from induced apoptosis. Mutations of DJ-1 were found to

cancel this activity and increase cell's vulnerability to apoptotic events (Taira et al., 2004). An X-ray crystal structure of DJ-1 reveals the functionality of a dimer and the possession of protease activity. Overabundant in both IMP and OPT treatment comparisons, increased levels of DJ-1 in this study suggest enhanced effort to make muscle less susceptible to oxidative damage.

The role of DJ-1 in oxidative stress has been associated with the similar function of peroxiredoxin-like peroxidase (Andres-Mateos et al., 2007). Peroxiredoxins are a family of peroxidases that reduce hydrogen peroxide to water and alcohol (Rhee et al., 2001). Specifically, peroxiredoxin 2 (PRDX2) and peroxiredoxin 6 (PRDX6) are powerful antioxidants in skeletal muscle (Wood et al., 2003; Kwasiborski et al., 2008). PRDX2 expression increased during mouse skeletal muscle cell differentiation and regeneration in response to extracellular hydrogen peroxide (Won et al., 2012). Knockdown, or elimination of cellular PRDX2 increases expression of other antioxidant enzymes, such as thioredoxin, but also allows buildup of intracellular ROS during muscle differentiation (Won et al., 2012). In a study investigating the adaptive response in skeletal muscle of knockout mice lacking endothelial nitric oxide synthase (eNOS), a common superoxide scavenger in skeletal muscle, Da Silva-Azevedo and others (2009) observed up-regulated PRDX6 in mice lacking eNOS. Their data supports the adaptive response of PRDX6 to counteract the absence of eNOS in scavenging of ROS. In the current study, both PRDX2 and PRDX6 were upregulated in LL from OPT and IMP treatment comparisons. Expression of PRDX2 increased 24% ($P < 0.03$) in OPT-LL when compared with CON and 26 percent ($P < 0.01$) when compared with IMP. PRDX6 with increased when comparing OPT to CON and OPT to IMP (29 and 14 percent, respectively; $P < 0.01$). The LL from IMP also demonstrated increased expression of PRDX6 when compared with CON (14%, $P < 0.020$. Taken together, these data suggest heightened response to oxidative stress through ROS scavengers.

Overabundant in LL from heifers subjected to the OPT treatment, aldehyde dehydrogenase (ALDH) expression increased 40% when compared with CON and 25% when compared with LL from IMP-treated heifers ($P < 0.001$, Table 4.7). Lipid peroxidation, or the oxidative breakdown of lipid membranes, produces toxic aldehydes that inflict oxidative insult on the surrounding environment. Aldehyde dehydrogenases reduce damaging effects of protein oxidation in reactions with aldehydes by catalyzing their oxidation to non-reactive acids (Singh et al., 2012). Past studies have shown the protective capabilities of ALDH in mice (Isse et al., 2005; Li et al., 2004, 2006). Using a transgenic mouse model, ALDH metabolized 94% of acetaldehyde concentrations in blood, brain, and liver after ethanol administration. Li and others (2006) used ALDH to investigate acetaldehyde-induced tissue and cell injury. In their study, ALDH overexpression significantly reduced acetaldehyde-induced oxidative stress and apoptosis in umbilical vein epithelial cells and human cardiac myocytes. These date suggest ALDH is a major enzyme in acetaldehyde metabolism and cellular detoxification. Several studies have also shown a link of ALDH and the prevention of cardiac disease (Chen et al., 2010; Ma et al., 2010; Zhang and Ren, 2011), but little has been done to elucidate the function of mitochondrial ALDH in beef skeletal muscle. Past research into the anti-oxidative properties of ALDH in mice and humans calls for further animal research on the muscle lipid profile and possible protective attributes of mitochondrial ALDH to counteract cellular oxidation in skeletal muscle.

Similar to ALDH with antioxidant function active in lipid metabolism, bovine serum albumin (BSA) was found to be overabundant ($P < 0.01$) in LL from heifers receiving OPT (+23% change, Table 4.7) and IMP (+19% change, Table 4.8) treatments when compared to cattle from the CON treatment receiving no exogenous growth promotants. Albumin protects cells scavenging ROS by trapping them, and investigations in beef and swine have deemed

albumin a major circulating antioxidant (Bergen et al., 1989; Mills et al., 2003; Castillo et al., 2005; Fukuzawa et al., 2005). Similar to the results from our study, investigation (Costa-Lima et al., 2015) of 200 Berkshire pigs receiving RAC for 14 and 28 d in fishing diets identified overabundant serum albumin in the *longissimus thoracis* muscle collected 24 h post mortem. Costa-Lima and others (2015) reasoned that increased levels observed may be a mechanism to protect proteins from ROS products of lipolysis in RAC-fed animals.

The presence of BSA in the sarcoplasmic fraction samples is somewhat expected because water-soluble plasma proteins from the residual blood left in the muscle are homogenized in buffer during protein extraction. However, the increased expression of BSA observed in LL from both IMP and OPT treatments could be a physiological response to the increased development of skeletal muscle tissue in animals receiving RAC and anabolic implants (Krizanovic et al., 2008). In a study of naturally occurring blood antioxidants in Simmental young bulls, Krizanovic and others (2008) observed significantly increased BSA during the finishing/fattening period, suggesting increasing muscle mass and double bonds in cell membranes resulted in increased blood antioxidants.

Taken together, the discussion of proteins and data presented indicate LL from cattle treated with exogenous growth promoting programs appear to have more anti-oxidative capacities. Many of the proteins identified in the current study are multifunctional and it is important to question the cause of the oxidative state of muscle observed in our study. If the muscle cannot produce adequate ATP (to accommodate rapid muscle growth) through the Krebs cycle, energetic reserves and an associating anaerobic metabolism will be utilized. The byproduct of glycolysis, lactic acid, will build up in the muscle, resulting in pH drop. When ATP is reduced, inflammatory processes increase, primarily as a result of oxidative stress. In

combination with other growth factors (anabolic implants, β-agonists), inflammation derived ROS may also contribute to the oxidative state of skeletal muscle and participate in a cascade of events leading to muscle regeneration and repair (Barbieri and Sestilli, 2012).

In the current study, eukaryotic translation initiation factor 5A-1 (eIF5A), a muscle protein with recovery functions, was overabundant in LL from heifers subjected to the OPT treatment ($P < 0.04$, Table 4.7) when compared with LL from CON and IMP-treated cattle. Several eukaryotic translation initiation factors (eIFs) have been identified, but eIF5A is unique in that it has a polyamine-derived amino acid, hypusine/N^{ε}-(4-amino-2-hydroxy-butyl) lysine, in its primary structure (Lee et al., 2009). Initially, eIF5A is synthesized as an inactive precursor and post-translational modification of the amino acid, hypusine, is needed to convert it to its active form. The only source of free hypusine comes from the degradation of eIF5A. Decreased expression of eIF5A was associated with reduced muscle protein synthesis in the gastrocnemius of tumor-bearing rats (Ventrucci et al., 2007). When the rats were given a leucine-rich diet, protein synthesis was increased, the authors hypothesized, through the activation of eIFs (Ventrucci et al., 2007). Comparable to our study, Spurlock and others (2006) used the β-agonist, clenbuterol, to investigate the physiological pathways that potentially facilitate induced skeletal muscle growth 24 h and 10 d after administration. Two eIFs had significantly increased mRNA abundance 24 h after clenbuterol administration, increasing cellular translation and associated muscle growth. Collectively with the results of the current study, a pathway of increased skeletal muscle growth in association with the treatment of exogenous growth promoting programs may be partially attributable to increased eIF5A expression.

The current study also observed overabundant expression of myofibrillar fraction protein, myosin binding protein H (MBPH), in LL from heifers receiving both OPT and IMP treatments

when compared with CON-treated cattle. We saw a +57% change (P <0.001, Table 4.8) in LL from IMP when compared with CON, and a +24% change (P = 0.01, Table 4.8) when compared with LL from OPT-treated heifers. MBPH was also overabundant (+27% change) in LL from OPT when compared with LL from CON (P = 0.01, Table 4.7). In skeletal muscle, MBPH is distributed in the cross-bridge zone of the A-bands of myofibrils and is most commonly associated with fast twitch muscle fibers (Bennet et al., 1986; Vaughan et al., 1993). While MBPH has traditionally been shown to play a major role in formation of myofibrils and location of the A-band (Gilbert et al., 1999), increased expression of MBPH has recently been associated with possible regenerative skeletal muscle potential.

Past studies in mice have shown the ability of MPBH to cluster and act as a "myosin stabilizer" within the sarcomere, maintaining myofiber integrity after strenuous muscle activity or injury. In a study investigating the molecular mechanisms of skeletal injury using histopathological evaluation and real-time RT-PCR, myogenic stress-responsive factors were measured for both direct destruction of muscle tissue and induced contractile overload in mice (Warren et al., 2007). Their results demonstrated that minimal myofiber destruction did not stimulate genes coding for MBPH. However, they observed increased expression of MBPH and chaperones following induced contractile overload, suggesting that the treatment induced early expression of genes coding for proteins with the purpose of repairing modified muscle proteins, restoring myofiber integrity, and acting to recover muscle function. More recently, a study observed increased expression of MBPH in the skeletal muscle of ALS patients and has proposed the protein to be a potential marker of muscle alteration and, possibly, a mechanistic attempt to regenerate atrophied muscle resulting from the disease (Conti et al., 2014).

In the present study, we have identified overabundant expression of proteins possessing both oxidative and regenerative/recovery characteristics in LL from heifers receiving both IMP and OPT treatments. Rapid protein turnover due to growth promoting programs can cause an imbalance, with more protein synthesis than proteolysis, resulting in an anabolic state that encourages the lean tissue growth we are observing in cattle treated with RAC and/or anabolic implants. The newly synthesized mitochondrial proteins may be susceptible to oxidative damage, causing a call-to-action in expression for many of the proteins observed in this study. Further investigation into protein vulnerability to free radical damage during transportation from the cytosol to mitochondria as well as measures of protein assembly with regards to oxidative stress is needed.

Chaperone proteins

Stress induces synthesis of protective proteins called heat shock proteins (HSP) which protect proteins against denaturation and malfunction (Kultz, 2003). A family of multifunctional proteins, HSP play important roles in protein folding, protein transport to mitochondria, cell survival (anti-apoptosis), inhibition of ROS, and restoration of damaged proteins. We observed overabundance in two spots (184 and 185, Fig. 4.3) from the myofibrillar fraction of the LL identified as heat shock 70 kDa protein-1A (HSP70-1A) in OPT and IMP (Tables 4.7 and 4.8) treated muscle when compared with the CON treatment receiving no exogenous growth promotants. Guillemin and others (2011a) analyzed the cellular pathways of tenderness with the purpose of increasing understanding of the many established beef tenderness markers, including HSP70-1A, and how they interact in muscle cells. Functional analysis of HSP70-1A demonstrated involvement in several cellular mechanisms, but particularly that of apoptosis and regulation, nucleic acid metabolism, and protein metabolism. According to most studies, the

increase in HSP levels results in meat toughening (Cassar-Malek et al., 2011; Guillemin et al., 2011b).

These findings are concordant with the oxidative status we have observed in the present study constituted by overexpression of CAIII, DJ-1, PRDX2, PRDX6, ALDH, eIF5A, and MPBH in addition to decreased meat tenderness (Table 4.3) in the LL muscle from heifers subjected to exogenous growth promoting programs. These proteins could all be responsible for the balance between apoptotic and/or stress response pathways stimulated by global metabolic shifts in the skeletal muscle of RAC and/or anabolic implant-treated animals. Results from our study and others question if anti-apoptotic activity of many of these proteins causes muscle to be more resistant to death while maintaining normal levels of muscle growth, or, if drastic breakdown is followed by rapid protein accretion, stimulating the proteins identified in the present study to have more of a recovery/regenerative function. Several questions still remain surrounding the mechanisms responsible for the upregulation of HSP70-1A with regards to adrenergic β-agonists and anabolic implants.

Concluding Thoughts

MS and 2D DIGE proved a successful method to identify proteins from the sarcoplasmic and myofibrillar LL muscle influenced by anabolic implant treatment with or without RAC treatment in beef heifers during the finishing period. The results of the present study suggest that exogenous growth promoting programs influenced the abundance of glycolytic enzymes and proteins possessing oxidative resistant, protective, regenerative, recovery, and anti-apoptotic properties. Particularly, the overabundance of these proteins in the LL from heifers receiving the OPT treatment demonstrated increased muscle proteins corresponding to cellular defense mechanisms. What, if any, physiological stimuli is causing the stimulation of the observed

cellular pathways? While the overabundant protein functionality is discussed in the present study, further insight is needed to measure the timing of cellular defense mechanisms during the dosage period, withdrawl time, and conversion of muscle to meat. The LL samples in the present study were collected 2 h post mortem, but the timing of exogenous growth promoting programs' influence on muscle resistance to oxidation, apoptosis, or alternative stressors are yet to be elucidated.

When comparing OPT and IMP treatments to CON, the major shift in the LL to a more glycolytic metabolism may result in alterations to fatty acid profile, fiber type, collagen content, and lipid metabolism. Investigations into the vulnerability of proteins involved in this metabolic switch to circulating ROS could not only help explain oxidative state of the muscle during exogenous growth promoting programs, but also reveal insight into their effect on cell death pathways. Presently, we do not have a full grasp of oxidation and the role it plays in protein turnover, muscle remodeling, and overall growth, particularly in response to growth promotant treatment programs. The signs of stress we observed in this study may not be able to be measured in gross measurements of stress (lactate, heart rate, creatine phosphokinase, etc.) and smaller, more diminutive changes may have a large impact.

Proteomic data analysis in the field of animal science has proven to be useful for increasing our understanding of the conversion of muscle to meat and for the characterization of cellular and molecular mechanisms behind meat quality as well as for the discovery of biological traits and diseases in farm animals. Major changes in the skeletal muscle proteome are well established, and consideration must be given to certain challenges when utilizing the technology in livestock research. Currently, limitations exist in the number of proteins that have been sequenced, thus far, in livestock species. However, the majority of the proteins identified in this

study have been discussed prior to our observations in past investigations on human, livestock, and mice, indicating the need to develop strategies to identify novel muscle proteins and take a more in-depth look at protein modification. The level of sample purification will be increasingly important as future studies sharpen focus onto protein-protein interaction and protein compartmentalization/translocation in order to identify previously unrecognized proteins. Furthermore, our current understanding will be improved by the advanced integration of proteomics and transcriptomics in the study of complex biological systems, including the skeletal muscle investigated in the present study.

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CHAPTER 5. OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

The three studies discuss in this dissertation used different techniques to achieve the ultimate goal of improving animal efficiency and meat quality in beef. Results from genetic technologies utilized to investigate the influence of myostatin mutations in beef cattle increased our knowledge of how the double-muscling condition affects beef carcass characteristics in meat palatability traits. The positive influence on HCW, REA, and YG indicates the need for further investigations into the development of crossbred programs that optimize utilization of the double-muscling condition in beef production systems.

Results from the application of a modified processing technique early post mortem indicated differences in meat quality across superficial and deep portions of the beef *semimembranosus*. However, difficulty altering the rate of temperature decline in the deep portion of the muscle in our small cooling chamber indicates the need for adequate space and consistent temperature in the cooling chamber when partially hot-boning the beef round. Further studies are needed to elucidate the ideal environmental conditions needed to influence the rate of temperature decline in deep locations of the *semimembranosus* muscle when using a partial hot-boning technique.

The treatment of anabolic implants with or without ractopamine hydrochloride treatment during the finishing period affected protein expression in the *longissimus lumborum* of beef heifers. Our data suggest that the treatment of exogenous growth promotant programs enhanced glycolytic metabolism. In addition, skeletal muscle from heifers subjected to anabolic implants and/or β-agonists during the finishing period had increased expression of proteins with oxidative resistant, anti-apoptotic, cellular defense, and regenerative functions, suggesting a response to possible oxidative insult. Further study measuring the timing of cellular response mechanisms

during the dosage period, withdrawl time, and conversion of muscle-to-meat are needed to elucidate more diminutive responses to the treatment of growth promotants in beef cattle.