Understanding the Role of Electrical Stimulation on Free Calcium Concentration, Activation of Calpain and Final Product Tenderness of Beef

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Authorization to Submit

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Abstract

Consumers report tenderness to be the most important palatability trait when consuming meat. Calpains are calcium activated proteases that are considered the primary proteolytic system involved in postmortem protein degradation, leading to improved tenderness. Calpain-1 is activated early postmortem while calpain-2 is activated after several days or even weeks postmortem. Activating calpain-2 earlier postmortem has the potential to improve tenderness after fewer days of aging. The objectives of this thesis are to 1) determine the effect of electrical stimulation on free calcium concentration, calpain activity and final product tenderness of the *longissimus lumborum* (LL) and *semimembranosus* (SM) and 2) introduce the unidentified 3rd band of calpain analyzed in this project. Twenty-three beef steers were harvested and stimulated (S) or not stimulated (NS) at exsanguination and at 1-hour postmortem, resulting in four stimulation treatments: NS-NS, NS-S, S-NS, or S-S. Overall, stimulation treatment was not shown to influence native calpain-1 or -2 activity, autolyzed calpain-2 activity, WBSF, or consumer sensory analysis in the LL. It did, however, impact autolyzed calpain-1 activity. In the SM, stimulation treatment did not influence free calcium concentration, calpain activity, WBSF or consumer sensory scores. Interestingly, during the analysis, an unidentified 3rd band of calpain was observed in many samples. Stimulation treatment was not significant in determining whether the 3rd band of calpain was observed. Analysis was conducted by the University of Idaho Mass Spectrometry Core Lab to classify the unidentified 3rd band using Liquid Chromatography-Mass Spectrometry (LC-MS), with little success. Further analysis needs to be conducted to characterize the unidentified 3rd band and determine its relation to calpain-1, if any. In the present study, stimulation treatment did not significantly improve free calcium

concentration, overall calpain activity, or final product tenderness. Other interventions need to be studied to activate calpain activity earlier postmortem and improve final product tenderness.

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Dedication

This thesis is dedicated specifically to my parents, Joe and Kim Buseman, for their constant support and encouragement in all I do. Thank you for instilling in me the importance of hard work and the value of education. I also want to recognize the rest of my family and friends who supported me in my move to Idaho, encouraged me through my program and have been praying for me faithfully. Thank you for being there for me and pushing me to be the best I can be.

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List of Abbreviations

CAT	Calcium-Activated Tenderness
EDTA	Ethylenediaminetetraacetic acid
ELV	Extra-Low Voltage
HV	High Voltage
LC-MS	Liquid Chromatography-Mass Spectrometry
LD	*Longissimus dorsi*
LL	*Longissimus lumborum*
LT	*Longissimus thoracis*
LV	Low Voltage
NS	Not Stimulated
PLGS	Protein Lynx Global Server
PM	*Psoas major*
PSE	Pale, Soft and Exudative
SM	*Semimembranosus*
S	Stimulated
USDA	United States Department of Agriculture
WBSF	Warner-Bratzler Shear Force

CHAPTER 1

Review of Literature

Introduction

Calpains are calcium activated proteases that play an important role in protein degradation in an organism both during life and postmortem (Goll et al., 1998). During life, calpains work alongside other protease systems to degrade misfolded, dysfunctional and undesired proteins. This allows essential proteins to accrue, thereby maintaining and increasing muscle mass of the animal. If proteases are hyperactive, desirable and functional proteins may be destroyed and muscle wasting can ensue (Goll et al., 1998). Postmortem, the calpain system is considered the primary proteolytic system involved in protein degradation (Koohmaraie et al., 1995). This degradation during aging results in a more-tender product demonstrated by improved Warner-Bratzler Shear Force values and consumer palatability reports (Colle et al., 2016). Consumers consistently report tenderness as the most important palatability trait when consuming meat (Koohmaraie et al., 1995; Mintert et al., 2000; Koohmaraie and Gesink, 2006). Therefore, understanding how calpains function and discovering a strategy to ensure earlier activation will allow for a more consistent and tender product. This review of the literature outlines the importance of tenderness, importance of the calpain system both pre- and post-mortem, the factors that influence its functionality and the effect of electrical stimulation on carcass quality traits.

Tenderness

Consumers report that tenderness is the most important quality trait they consider when consuming meat (Koohmaraie et al., 1995; Mintert et al., 2000; Koohmaraie and Gesink, 2006). So much so, many consumers are willing to pay premiums for product that is

"guaranteed tender" (Mintert et al., 2000). It is thought that there are three primary factors that regulate meat tenderness: background toughness, the toughening phase and the tenderization phase (Koohmaraie and Gesink, 2006). Background toughness is depicted by additional factors that could influence tenderness but are not related to sarcomere shortening, such as: levels and solubility of connective tissue within the muscle, organization of the perimysium, the amount of intramuscular fat (marbling) within the muscle and myofiber size (Strandine et al., 1949; Koohmaraie et al., 1995). The toughening phase is dependent on the level of sarcomere shortening that occurs during rigor (Koohmaraie et al., 1996). Finally, the tenderization phase is highly variable between carcasses due to the level of protein breakdown that can occur (Koohmaraie and Gesink, 2006). It is believed that proteases play a major role in this phase by determining the overall level of protein breakdown and rate of tenderization.

Connective tissue is a component of background toughness that is a contributing factor to final product tenderness (Cover et al., 1962; Cross et al., 1973). There are two primary forms of connective tissue found within the muscle, collagen and elastin. Collagen is the most common form and is heat-labile; whereas, elastin cannot be degraded by heat (Cover et al., 1962). Collagen levels play a large role in determining whether a muscle is known to be 'tender' or 'tough.' For example, the *biceps femoris* contains more connective tissue than the *longissimus dorsi* (LD) and was significantly more tough when cooked to 61^{o} and 80^{o}C (Cover et al., 1962; Cross et al., 1973). Additionally, levels of collagen have been shown to be a key driver of the influence that the overall level of connective tissue plays on influencing consumer perceptions of tenderness (Cross et al., 1973).

Degree of contraction was first shown to have a significant impact on tenderness and overall eating experience in a project conducted on Prime ox meat (Locker, 1960). Lewis et al. (1977) found that sarcomere length and tenderness within the *longissimus lumborum* (LL) and *psoas major* (PM) was positively related; with an increase in sarcomere length relating to a higher consumer sensory score for tenderness. However, though muscle contraction may be associated with tenderness, viewing it at one single point does not accurately reflect the contraction state of the entire muscle and therefore, does not fully depict the tenderness level of the entire carcass (Howard and Judge, 1968; Lewis et al., 1977). Cold shortening can play a major role in postmortem muscle contraction, leading to a tougher final product (Aberle et al., 2012). Although reducing muscle temperature rapidly postmortem is important to limit protein denaturation and microbial growth, too fast of a decline can lead to cold shortening (Aberle et al., 2012). These authors note that cold shortening occurs when muscle is rapidly cooled to $<15^{o}$ C before the onset of rigor. Locker and Hagyard (1963) found that at 0^{o} C, samples shortened to 47.7% of their original length. This can lead to tenderness problems in the final product (Locker and Hagyard, 1963; Aberle et al., 2012).

Skeletal muscle tissue consists of myofibers, connective tissue, blood vessels and extracellular tissue. Myofibers are specialized cells that account for 75-92% of muscle quantity (Aberle et al., 2012). Lewis et al. (1977) found that fiber diameter in the LL and PM was negatively related to consumer sensory analysis of tenderness. Meaning, as fiber diameter increased, tenderness values decreased, indicating a tougher product. This supported previous work by Herring et al. (1965), which found that fiber diameter was correlated with WBSF. Additionally, this study analyzed the relationship between fiber

diameter, sarcomere length and tenderness and found that fiber diameter and sarcomere length are responsible for approximately 12% of variation in tenderness (Herring et al., 1965).

The amount of marbling, or intramuscular fat, is one factor that influences carcass quality grade, or estimated palatability (Aberle et al., 2012; USDA, 2020). Marbling has been shown to increase final product tenderness by lowering the bulk density of each bite and providing lubrication to aid in digestion (Savell and Cross, 1988; Gruber et al., 2006; Emerson et al., 2013; Corbin et al., 2015). In fact, Emerson et al. (2013) found that marbling score explained 40% of variation in product tenderness based on analysis by a trained sensory panel. Interestingly, Wheeler et al. (1994) reported that the impact of marbling on tenderness may be smaller than expected, with marbling only representing 5% of variability in final product tenderness. Additionally, Razminowicz et al. (2008), found that there was no correlation between marbling score and Warner-Bratzler Shear Force (WBSF) in steaks aged 15 days. Although quality grade is a helpful predictor of palatability, it may not be able to increase consumer certainty that a product will be tender (Mintert et al., 2000; Aberle et al., 2012). Other factors such as myofiber size, connective tissue levels, or postmortem proteolytic activity can cause additional variation in final product tenderness (Herring et al., 1965; Koohmaraie et al., 1995; Yang et al., 2018). Because of this, other factors need to be considered when evaluating final product tenderness.

Koohmaraie et al. (1995) cited unpublished data from the Germplasm Evaluation Project, that predicted that the combination of intramuscular fat and connective tissue account for only 20% of variation in meat tenderness. At the time, it had been determined that a protease system, specifically calpains, was responsible for postmortem tenderization,

with the volume and activity level of these proteases being the major source of disparity in beef tenderness. Juárez et al. (2016) found that postmortem aging was the most influential player in tenderness. Because of this, it is important to evaluate the impact of other factors outside of marbling on product tenderness. Understanding the influence of these factors could help lead to a more consistent, tender product.

Certified Tender

The United States Department of Agriculture (USDA) offers an opportunity for processors to certify their products as "USDA Tender" or "USDA Very Tender". To fall into one of these programs, product must meet specific standards for WBSF or slice shear force (SSF) (USDA, 2012). To be deemed "Tender" or "Very Tender," WBSF value must be under 4.4 kg and 3.9 kg and the SSF value must be below 20.0 kg and 15.3 kg, respectively (Yates et al., N.D.). If accepted into one of these categories, there is a marketing opportunity to include a USDA-certified statement on the product label (USDA, 2012).

Calpain System

The calpain system requires calcium to initiate protein breakdown (Aberle et al., 2012). Although multiple forms exist, calpain-1 and calpain-2 are most active in skeletal muscle (Aberle et al., 2012). Calpain-1 requires 3-50 μM of calcium to be activated, whereas calpain-2 requires 400-800 μM of calcium for half maximal activity (Goll et al., 1995; Goll et al., 2003). Both calpain-1 and calpain-2 are formed of two subunits of molecular weights of 28 and 80 kDa (Croall and DeMartino, 1991; Goll et al., 2003). The 28 kDa subunit is identical between the two proteases and contains a C terminal holding amino acid sequences that allows for calcium binding (Lonergan et al., 2010). The 80 kDa subunit displays similarities between the two calpains, both containing four domains. Domain-1, has

no sequence homology specific to a polypeptide. Domain-2 serves as a catalyst and contains both cysteine and histidine residues (Lonergan et al., 2010). Domain-3 is catalytic and binds the catalytic binding site to calcium binding domains. Additionally, it is thought that domain-3 plays a role in regulating calpain activity through electrostatic interaction (Hosfield et al., 1999; Strobl et al., 2000; Goll et al., 2003). Finally, domain-4 is a calmodulin site and allows for calcium binding (Strobl et al., 2000; Goll et al., 2003). The domains work together to allow calcium to bind to these proteases to cause myofibrillar protein destruction.

As calpains bind with calcium and are activated, they themselves are autolyzed (Cong et al., 1989; Edmunds et al., 1991; Goll et al., 2003). When this happens, the amount of calcium needed to induce half-maximal activity is substantially lowered (50-150 μM calcium for calpain-2), while still allowing the enzyme to be active (Edmunds et al., 1991; Goll et al., 2003). Autolysis occurs quite rapidly, but still involves many steps: 1) the NH_2–terminal of the 80 kDa subunit of calpain-1 removes amino acids to create a 78 kDa subunit, which continues to breakdown to produce a 76 kDa autolyzed fragment, 2) the 80 kDa subunit of calpain-2 removes amino acids from the NH_2 –terminal to produce a 78 kDa autolyzed fragment, 3) the 28 kDa subunit first removes amino acids from the NH_2-terminal, producing a 22- to 23 kDa fragment, 4) autolysis continues until an 18 kDa autolytic fragment remains (McClelland et al., 1989; Goll et al., 2003). In calpain-2, the 28 kDa subunit is autolyzed more rapidly than the 80 kDa subunit; whereas, in calpain-1, the 80 kDa subunit is autolyzed quicker than the 28 kDa subunit (Brown and Crawford, 1993; Crawford et al., 1993; Goll et al., 2003). Calpains continue to function and work to degrade protein at lower rates until autolysis is complete.

Calpains are responsible for the fragmentation of myofibrillar proteins, including desmin, C-protein, tropomyosin, troponin T, troponin I, titin, nebulin, vimentin, gelsolin, vinculin and α-actinin, disrupting the sarcomere integrity (Goll et al., 1991; Huang and Forsberg, 1998). However, calpains do not degrade the contractile proteins, α-actin, f-actin, g-actin or myosin heavy chains (Goll et al., 1991; Goll et al., 1998; Huang and Forsberg, 1998). Once activated by calcium, the ability of calpains to degrade protein may be blocked by calpastatin (Goll et al., 1998). It is thought that calpains are located around the z-disc, allowing for calcium binding and destruction of myofibrillar proteins to increase cell degradation (Huang and Forsberg, 1998). In this way, calpains have access to free calcium and can work along the sarcomere to focus on myofibrillar remodeling and breakdown. Through myofibrillar breakdown, calpains can interact in the system to allow for protein turnover in living muscle tissue and improvements in final product tenderness.

Calpain Function Pre-Harvest

Muscle growth and accretion is important in living animals. Growth is dependent on three factors: 1) rate of muscle protein synthesis, 2) rate of muscle protein degradation and 3) number and size of skeletal muscle cells (Goll et al., 1998). The effect of these factors are impacted in many ways, such as genetic selection (Goll et al., 1998; Aberle et al., 2012), activity level of the protease system (Goll et al., 1998; Kemp et al., 2010) and growth promotant use (Parr, 1992; Goll et al., 1998; Lonergan et al., 2010). Although calpains are necessary for protein turnover, proper activity levels need to be maintained for the health of the organism and productive efficiency of livestock.

Protein formation and maturation is essential for organism health. An organism that undergoes muscle atrophy is at a higher risk for disease and death (Thomas and Mitch,

2013). A healthy organism relies on proteins which are embedded in the plasma membrane to send regulatory signals throughout the body (Kitzler et al., 2012). Unhealthy proteins need to be removed from the body, but degradation must be regulated to ensure healthy proteins are not also being destroyed. The calpain system partners with other protease systems, like the caspase system, to regulate the turnover of weak and injured proteins and encourage the accrual of healthy muscle tissue. Higher levels of calpastatin inhibit calpain activity. It is thought that caspases degrade calpastatin, decreasing the ability of calpastatin to disrupt calpain activity (Kemp et al., 2010). Although this interaction was demonstrated, it is still believed that if levels of calpastatin are too high, caspases may not be able to cause significant degradation. For example, in a callipyge lamb there are extreme levels of calpastatin and insufficient levels of caspases to noticeably decrease the stores to make an impact on calpain functionality (Kemp et al., 2010). This demonstrates how one-way protease systems work together to ensure healthy protein turnover.

Calpain Function Postmortem

Following harvest, muscle is converted to meat through rigor mortis (Aberle et al., 2012). As muscle is converted to meat, multiple changes occur, including depletion of usable energy, pH decline from nearly neutral to approximately 5.4-5.6, increase in ionic strength from halted functionality of ATP pumps and an increased ability to maintain reducing conditions (Lonergan et al., 2010). In addition to calcium availability, temperature, pH and oxidation may impact calpain function and will be further discussed in this section (Lonergan et al., 2010). During rigor, myosin heads bind to actin in the skeletal muscle creating the actomyosin complex. Cross bridges form between thick and thin filaments and leads to the irreversible contraction of the muscle fibers in a carcass, formed by a depleted

supply of ATP (Aberle et al., 2012). The role of the protease system is to cleave myofibrillar proteins and allow for degradation around the z-disc (Aberle et al., 2012). As meat ages, more time is allowed for protease activity, leading to higher amounts of protein breakdown, producing a more tender product demonstrated by lower WBSF values and higher consumer acceptability for tenderness (Colle et al., 2016).

Melody et al. (2004) found that rate of calpain autolysis mirrored pH decline. The muscle tissue with the most rapid drop in pH was found to have the earliest detectable autolysis of calpain. Pomponio and Ertbjerg (2012) saw rapid autolysis of calpains when muscle is held at higher temperatures. Higher temperatures increase the speed of enzymatic reactions, cause a more rapid pH decline and increases free calcium ion concentration, resulting in rapid autolysis of calpain (Pomponio and Ertbjerg, 2012). Additionally, a slight acceleration of pH decline may increase the rate of tenderization (Lonergan et al., 2010). When looking at pork, an accelerated pH decline can lead to a product that is pale, soft and exudative (PSE). Calpains are known to be most efficient at neutral pH levels, with the rate of activity changing based on the rate of pH decline (Melody et al., 2004). When PSE pork undergoes a rapid rate of pH decline, it is likely that proteins, including calpains, are denatured, leading to a lower rate of tenderization (Lonergan et al., 2010).

Both calpain-1 and calpain-2 contain histidine and cysteine residues at their active sites. When muscle is oxidized, histidine is converted to a carbonyl derivative and intra- and inter-protein disulfide cross-links form (Lonergan et al., 2010). If the proteins are oxidized and the calpain binding site is converted, the calpain is then modified and may become inactivated (Lonergan et al., 2010). Inactivation halts the proteolytic activity of calpains, thus lowering the potential for the product to be tender, decreasing consumer satisfaction.

Postmortem aging allows time for the calpain system to be active. Due to its relatively low calcium requirement (3-50 µM), calpain-1 is active early postmortem and is thought to be responsible for 95% of proteolytic activity in the first 7-14 days postmortem (Yang et al., 2018). Calpain-2 has been shown to activate after longer aging periods (Colle and Doumit, 2017). Calpain-2 requires higher levels of calcium to activate (400-800 µM); however, extended aging (>28 days) results in calpain-2 activation and increases the likelihood of a more tender product (Goll et al., 1995; Goll et al., 2003; Colle and Doumit, 2017). Figure 1.1 depicts some of the influencers of calpain activity both pre- and postmortem.

Influence of Calpastatin

Calpastatin is a known inhibitor of calpains and blocks the functionality of both calpain-1 and calpain-2 (Zór et al., 2012). When calpains are activated by calcium, calpastatin is able to block calpains from binding to proteins. Typically, calpastatin is an unstructured protein; however, when calpain is activated it transforms to block the calpain binding site (Kemp et al., 2010). Following the binding of calcium to calpains, calpastatin forms 4 inhibitory domains to limit calpain activity. Calpastatin wraps around the enzyme to block the active site, limiting function (Kemp et al., 2010). By doing this, calpains are unable to bind to the protein to cause degradation. As calpastatin is broken down by calpains, the subsequent protein fragments continue to inhibit calpain activity (Kemp et al., 2010).

It is estimated that calpastatin level causes up to a 40% variation in product tenderness (Kemp et al., 2010). Because of this, product palatability is tied to calpastatin activity and the impact it has on calpain functionality. Seeing the interaction between

calpains and calpastatin demonstrates that calpastatin levels depict the functionality of the calpain system (i.e. as calpastatin activity increases, muscle protein degradation decreases and skeletal muscle accrues) (Goll et al., 1998). Understanding the major role calpastatin has in influencing overall tenderness provides an opportunity to use it as a measure to predict final product tenderness (Zór et al., 2012).

Additional Factors Influencing Calpain Functionality

Effectiveness of the calpain system can be impacted by many things, including exogenous growth promotant administration and animal breed (Lonergan et al., 2010). Utilizing growth promotants, such as β-adrenergic agonists and hormonal growth promoters, during production has become common practice for many producers, with approximately 33% of producers utilizing implant technology to treat their livestock to increase growth efficiency (Stewart, 2013). β-adrenergic agonist additives impact the calpain system by increasing muscle calpastatin activity, therefore decreasing calpain function (Goll et al., 1998). It is thought that an increase in calpastatin activity caused by β-adrenergic agonists would suppress calpain-1 and -2 activity, resulting in limited protein breakdown and an increase in hypertrophy (Parr, 1992).

Livestock species can play a big role in the effectiveness of the calpain system. For example, *Bos indicus* cattle have higher levels of calpastatin activity than *Bos taurus* cattle (Goll et al., 1998; Aberle et al., 2012). Because of this, calpain activity of *Bos indicus* cattle will be relatively limited, increasing the likelihood of producing a tougher final product. Interestingly, genetic influence is thought to only causes about a 30% variation in beef tenderness, while 70% is influenced by environmental and non-additive gene effects (Koch

et al., 1982; Razminowicz et al., 2008). While calpain activity has genetic linkages, it can also be influenced by environmental factors and postmortem interventions.

Due to the need of calcium for calpains to be activated, outside calcium can be supplemented within the meat to increase the rate of tenderization (Wheeler et al., 1992; Koohmaraie et al., 1995). This process, known as Calcium-Activated Tenderization (CAT), is best used if injected uniformly through the meat and can be performed pre- or post-rigor (Koohmaraie et al., 1995). Wheeler et al. (1992) found that utilizing a postmortem calcium chloride injection resulted in earlier activation of calpain-2 and improved tenderness of the LL. Once activated, calpain-2 has been found to continually improve tenderness up to 42 days of aging (Colle and Doumit, 2017). Steaks injected with the calcium chloride solution and aged only 4 days were evaluated by a taste panel and found to be as tender as non-injected steaks aged 14 days (Colle et al., 2018). Calcium chloride injections are able to be used in specific cuts that are known to be tough, including meat from cull cows, *Bos indicus* influence, or bull meat (Koohmaraie et al., 1995). Finding that this treatment will consistently improve beef tenderness with little to no negative impact on other quality characteristics such as color and flavor, is a great opportunity for the meat industry to improve product consistency (Koohmaraie et al., 1995). Although calcium chloride injection has been found to improve product tenderness (Wheeler et al., 1992; Koohmaraie et al., 1995; Colle et al., 2018), injection leads to an increased risk of product contamination and introduction of a physical hazard (Food Processors Institute, 1999). Because of this, it is necessary to research other interventions that could be used to improve calpain activity and ultimately tenderness.

Additional studies have been completed evaluating the impact of freezing on product tenderness with varying results. Although product frozen and thawed displayed lower WBSF scores in some studies (Wheeler et al., 1992; Shanks et al., 2002), in another there was no difference in tenderness even though calpain-2 activity increased (Colle et al., 2018). It is thought that freezing and thawing meat may impact the muscle ultrastructure, influencing WBSF and calpain activity due to improved calcium uptake and enhanced calpain activation (Wheeler et al., 1992). Additionally, it has been shown that steaks that were thawed and then injected with calcium chloride had lower WBSF values than those not injected (Wheeler et al., 1992). This leads to the hypothesis that utilizing a combination of freezing and thawing techniques along with calcium chloride injections may be a method to improve tenderness. Figure 1.1 depicts additional influencers of calpain activity.

Electrical Stimulation

The use of electrical stimulation in meat production was first used by Benjamin Franklin in 1749, when he discovered that applying electrical stimulation to a turkey carcass early postmortem improved tenderness (Adeyemi and Sazili, 2014). Today, electrical stimulation is commonly used in beef processing facilities and serves multiple functions (Simmons et al., 2008):

1. Improves bleed following exsanguination (Adeyemi and Sazili, 2014).
2. Improves tenderness through muscle contraction (Will et al., 1980; Sorinmade et al., 1982; Luo et al., 2008).
3. Increases the rate of rigor (Chrystall and Hagyard, 1976).
4. Prevents cold shortening (Swatland, 1981; Luo et al., 2008; Kim et al., 2013).
5. Accelerates pH decline (Dutson et al., 1980).

Besides improving bleed, all these functions play a role in improving final product tenderness. Electrical stimulation causes a hard contraction of muscle fibers, causing physical disruption. Analysis has shown that electrically stimulated samples had stretched and broken contractile bands, leading to poorer structural integrity (Savell et al., 1978). Others, found that stimulation caused irregular contractile proteins in the LL, showing signs of stretching, tearing and fragmentation (Sorinmade et al., 1982). In another study, electrical stimulation caused cellular swelling, myofiber stretching and disrupted sarcomere integrity in the LL (Will et al., 1980). Additionally, when evaluating samples under electron microscopy, electrical stimulation and the use of delay-chilling caused the change of the sarcomere ultrastructure (Luo et al., 2008).

The use of electrical stimulation is thought to increase the rate of glycolysis, utilize the remaining ATP available in the system, accelerate rigor mortis and therefore prevent cold shortening (Chrystall and Hagyard, 1976; Lee et al., 2000). Additionally, accelerating glycolysis leads to lower pH values early postmortem in electrically stimulated carcasses. However, by 24 hours postmortem, pH values of stimulated and non-stimulated samples are similar (Chrystall and Hagyard, 1976; Uytterhaegen et al., 1992; Eilers et al., 1996). Interestingly, calpains are known to be most active at a neutral pH (Melody et al., 2004). Rapidly accelerating glycolysis has potential to denature calpains early postmortem, limiting their functionality. Finally, Uytterhaegen et al. (1992) found a correlation between electrical stimulation and calpain-1 activity. This leads us to believe that electrical stimulation can improve product tenderness through ultrastructure changes, rate of rigor onset, accelerated pH decline and improved protease activity (Lee et al., 2000).

There are three types of electrical stimulation utilized in beef processing: extra-low voltage (<100 V), low voltage (LV) (100-110 V) and high voltage (HV) (>110 V, often 500-1,000+ V) (Adeyemi and Sazili, 2014). Traditionally, LV and HV are the most common forms used and researched (Adeyemi and Sazili, 2014). LV stimulation is primarily used when there is a delay between bleeding and stimulation (10-20 min) and is usually applied prior to hide removal (Adeyemi and Sazili, 2014). HV is more commonly used if there is a large gap in time between bleed and stimulation (up to 60 minutes) (Adeyemi and Sazili, 2014). Traditionally, LV and HV are the most common forms researched; however, there is much variability between methods, including: voltage, impulse, duration, frequency, timing and location of electrode (Adeyemi and Sazili, 2014). In addition to the type of stimulation used, timing of stimulation, animal breed, animal age and other factors can influence the effectiveness of the stimulation (Adeyemi and Sazili, 2014).

Hwang and Thompson (2001) evaluated the effects of LV and HV stimulation at different time periods (HV/LV, 3- and 40-min postmortem; HV, 40- and 60- min postmortem; LV, 40 min postmortem) on meat tenderness and calpain activity. HV stimulation led to significantly lower calpastatin levels and a tendency for lower calpain-1 levels immediately following exsanguination than the LV treatment. This led to the belief that activation of the calpain system was likely linked to the type of stimulation system utilized. Levels of calpain-2 remained consistent, implying that calcium released during stimulation may not have reached the level required for initial activation. All stimulation methods, regardless of voltage or timing, showed an increase in meat tenderness and juiciness levels compared to the unstimulated control. Interestingly, this study evaluated tenderness (WBSF and consumer sensory panel) up to 14 days of age, but only evaluated

calpain activity prior to stimulation, post-stimulation and at 24 hours postmortem. Conversely, a study by Li et al. (2012) found that electrical stimulation (80 V, 35 sec, 30 min postmortem) after dressing improves WBSF values and activates calpain-1 earlier postmortem when compared to a non-stimulated control. Additionally, this study found that electrical stimulation accelerated meat tenderization specifically at 24 and 48 hours postmortem and tended to improve tenderness up to 7 days postmortem. Juárez et al. (2016) found that the use of high voltage stimulation caused approximately 12% variation in product tenderness. Additionally, the interaction of feeding system (calf-fed vs. yearling-fed) and stimulation accounted for ~5% of the variability in tenderness (Juárez et al., 2016).

Hwang and Thompson (2001) found that HV stimulation led to significantly lower calpastatin levels than the control sides. There were significant differences in calpain-1 levels between the stimulation treatments, where HV stimulation at exsanguination lowered calpain-1 values significantly compared to HV at 40-minutes postmortem, HV at 60-minutes postmortem, LV at exsanguination and LV at 40-minutes postmortem. This study demonstrates the role of electrical stimulation in tenderness improvements and the opportunity to advance the technology to initiate calpain activity earlier postmortem.

A study by Eikelenboom et al. (1985) argued that there was no difference in overall tenderness between LV and HV stimulation administered at exsanguination. However, consumers preferred both LV and HV treated LL samples over the non-stimulated control. Additionally, this study found that sarcomere length of the control carcasses was significantly shorter than both the LV and HV; potentially leading to the poorer tenderness scores. This study did not evaluate the influence of stimulation at multiple time points, rather focusing specifically on exsanguination.

Razminowicz et al. (2008) analyzed the effect of electrical stimulation and postmortem aging on various quality attributes of the (LD) of grass-fed steers. In this study, 16 carcasses had one side stimulated at 30 min postmortem (230 V, 60 Hz, 30 sec). This study found that the electrically stimulated LD had improved (lower) WBSF values than the non-stimulated side on day 2 and 15 postmortem. A big enough improvement was seen that day 2 stimulated steaks had a WBSF almost as low as the control steaks on day 15. By day 29, no difference in mechanical tenderness was seen between the stimulation treatments. Interestingly, electrical stimulation proved to be more effective as fat thickness decreased. This was especially noteworthy for grass-fed steers, as they are known to not finish with as much fat cover as those fed a high energy, grain diet (Razminowicz et al., 2008).

Although little research is available looking specifically at the effects of ELV stimulation, Powell et al. (1984) analyzed the effects of various ELV stimulation on beef carcasses (45 V, 90 sec, within 8 min of exsanguination; 60 sec, 3 sec on and 1 sec off, within 4 min of exsanguination; and 40 sec, 3 sec on and 1 sec off, within 4 min of exsanguination). Samples from carcasses stimulated 8 min postmortem were significantly tougher when evaluated by a consumer sensory panel than those stimulated 4 min postmortem. Additionally, there were improvements in WBSF values, irrespective of treatment type. Although an improvement in WBSF was seen when ELV stimulation was used, the WBSF value for the SM and (LD) was still 8 kg (Powell et al., 1984). No analysis was performed on the influence of ELV on free calcium concentration or calpain activity (Powell et al., 1984). Seeing improvements in calpain activity and final product tenderness regardless of stimulation treatment, provided an opportunity to further evaluate the effect of ELV stimulation on beef to determine its value to the industry.

Strip Loin and Top Round Properties

The LL ranks twelfth out of 40 in terms of Warner-Bratzler shear force values (4.07 kg) (Calkins and Sullivan, 2007). When evaluated by a sensory panel, the LL ranked third out of 14 for overall tenderness and juiciness (Calkins and Sullivan, 2007). The LL is shown to have improvements in tenderness up to 14 days of aging, with little improvements due to extended aging periods (Razminowicz et al., 2008; Colle et al., 2015, 2016) The limited improvement in LL tenderness due to extended aging is thought to be because the LL is inherently more tender than the *semimembranosus* (SM) (Colle and Doumit, 2017).

Muscles in the beef round are not known to offer the level of tenderness that consumers prefer, causing a consistency problem in the beef industry (Guelker et al., 2013; Martinez et al., 2017). Looking at a comparison of muscles, the SM ranks twenty-first out of 40 in terms of Warner-Bratzler shear force values (4.51 kg) (Calkins and Sullivan, 2007). Even at this ranking, the muscle still falls within the intermediate tenderness (3.9 kg < x < 4.6 kg) category (Calkins and Sullivan, 2007). In the same study, fourteen muscles were evaluated by a sensory panel where the SM ranked thirteenth for both tenderness and juiciness.

Previous studies evaluated how aging impacts top round tenderness and found improvements up to 42 days of aging (Colle et al., 2016). This improvement is likely caused by the activation of calpain-2 (Colle and Doumit, 2017). Although tenderness can improve up to 42 days postmortem, the average overall aging time for the top round is only 23.2 days, while 46.6% of top rounds in the retail store are sold after only 14 days of aging (Martinez et al., 2017). Since the top round likely does not reach its maximum tenderness before it is sold, consumers may be left with an unsatisfactory eating experience. Therefore,

a strategy to improve top round tenderness must be developed to ensure consistent customer satisfaction.

Aging Methods

Beef palatability traits, such as tenderness and flavor development, can be enhanced by aging. Common forms of this practice are dry-aging and wet aging (Sitz et al., 2006). Dry-aged product is stored in a refrigerated, open-air environment that allows moisture loss and microbial growth. This requires high levels of environmental control and product space (Parrish et al., 1991; Sitz et al., 2006). Conversely, wet aging encloses the product in a vacuum sealed bag, limiting the potential for product contamination (Sitz et al., 2006). This allows for limited microbial growth and moisture loss.

Although both aging methods provide a tender, palatable product; dry-aged beef has been found to require 5-10 times greater trim loss due to dehydrated and discolored surfaces compared to wet aged product (Parrish et al., 1991). In a study by Sitz et al. (2006) wet aged, Prime strip loin steaks were rated higher for tenderness than dry-aged steaks by a consumer sensory panel. Conversely, no significant differences were seen between the products when evaluated by Warner-Bratzler Shear Force. Wet aging tends to be the preferred method of aging utilized in the beef industry due to the additional trim loss incurred by dry-aged product and space required for storage (Dikeman et al., 2013); though both methods are a viable option to allow for calpain activity to improve final product palatability.

Calpain Analysis

Dayton et al. (1975) analyzed the usefulness of utilizing column chromatographic purification to analyze calpains. Five different columns were utilized in the following order;

1) 6% agarose; 2) DEAE-cellulose; 3) Sephadex G-200; 4) DEAE-cellulose; 5) Sephadex G-150. This analysis proved to show higher levels of calpain activity and was a more efficient process than previously reported analyses conducted by Huston and Krebs (1968) that used stepwise elution of DEAE-cellulose columns and batch extraction of protein solutions.

Today, casein zymography is a widely used method to analyze calpain activity. Raser et al. (1994) discussed the use of casein zymography as a method to study calpain-1 and calpain-2. Utilizing casein zymography allows for the separation and detection of levels of calpain-1 and calpain-2, while preventing calpastatin from inhibiting enzymatic activity. The gel contains casein, a known protein that is digested by calpains. Muscle samples are combined with a calcium chelator, ethylenediaminetetraacetic acid (EDTA), to prevent calpastatin from inhibiting calpain activity. Electrophoresis differentiates the enzymes based on their mobility. Calpain-2 has a higher mobility than calpain-1 and thus separates itself within the gel. Once the samples have been run through the gel, they are placed in an incubation buffer containing Dithiothreitol and calcium to activate the enzymes and allow them to breakdown the casein contained within the gel. Following incubation, the gels are stained with Coomassie blue and subsequently destained. This makes the calpain activity visible through a bright, white band, allowing for easy distinction between enzymes (Figure 1.2).

Not only does casein zymography differentiate between calpain-1 and calpain-2 while blocking the influence of calpastatin, it also separates native and autolyzed forms of calpain and is able to separate calpains from other known proteases. Due to the slightly basic pH used during the process (8+ for the running buffer and 7+ during incubation), lysosomal proteases, such as cathepsins are not seen because of their preference for an acidic

environment. When the same procedure was conducted using an incubation buffer of 5.5, cathepsin B was able to denature the casein; however, it was broken down into large fragments rather than small peptides, reducing the visibility of the bands (Raser et al., 1994).

Calcium Analysis

Calcium, a positively charged ion, is a necessary component for muscle contraction (Koohmaraie et al., 1995; Goll et al., 1998; Aberle et al., 2012). When Na^+ enters the cell, it sends an action potential down the sarcolemma. It travels down the T-tubule and causes calcium to be released from the sarcoplasmic reticulum. Calcium then binds to troponin-C and allows the myosin heads to bind to thin filaments, causing the muscle to contract (Aberle et al., 2012). When high enough levels of calcium are available (3-50 µM for calpain-1, 400-800 µM for calpain-2), calpains are activated and cause protein breakdown (Goll et al., 1995; Goll et al., 2003). In theory, the use of an electrical current should pull enough free calcium out of the sarcoplasmic reticulum to activate calpain-2.

Due to its positive charge, free calcium concentration can be analyzed using a calcium selective electrode and measured in mV. Utilizing calcium standards (50 µM, 100 µM, 500 µM, 1000 µM, 2000 µM and 8000 µM) allows for a concentration curve to be built and free calcium concentration to be analyzed.

Warner-Bratzler Shear Force

The need for an objective measure for meat tenderness was met in the 1920s by K.F. Warner, L.J. Bratzler and associates (Warner, 1952; Wheeler et al., 1997). In order to obtain an accurate measurement that both matched quality grade and consumer taste, cores must be taken from cooked product (Warner, 1952). Other factors can impact the results of WBSF analysis, such as orientation of muscle fibers, initial steak temperature (if steaks were

cooked from frozen or thawed state), speed of the shear, as well as cooking parameters (Wheeler et al., 1997). Steaks should be allowed to cool to a consistent temperature (room temperature, 23^{o}C), prior to removing cores (AMSA, 2016). A minimum of 6 cores, 1.27 cm in diameter, should be removed parallel to the muscle fiber. Cores should then be shorn perpendicular to the muscle fiber (Wheeler et al., 1997; AMSA, 2016). When performed correctly, WBSF is a widely used and respected measurement of meat tenderness within the meat science community (Wheeler et al., 1997).

Consumer Sensory Panel

One of the main goals of meat-focused research is to improve the eating experience of the end consumer. Consumers desire products that are affordable, convenient, healthy and most importantly, taste good (Chambers, 1994). In order to provide that, consumer sensory panels are utilized. In addition to providing feedback on product tenderness, consumer sensory panels can be used to analyze off-flavors and other quality characteristics, such as juiciness and mouth feel. The combination of lab analysis and consumer sensory analysis paints a clear picture as to what can be expected from the product. For example, when analyzing tenderness, WBSF and consumer sensory analysis are both important units of measurement. Miller et al. (1995) found that, as WBSF value decreased, consumer perception of tenderness improved. So much so, that consumers could detect > 0.5 kg difference in WBSF values (Miller et al., 1995; ASTM, 2011). Using both analyses provides objective measurements as well as final consumer feedback to help predict the overall acceptability of the product and therefore gauge willingness to purchase the product.

Reason for Study

Understanding the function and importance of the calpain system leads us to believe that developing a method to activate the system earlier postmortem will lead to improved product consistency and tenderness. Although CAT has been shown to provide high enough levels of free calcium to activate the calpain system and improve product tenderness, it has potential to introduce biological and physical hazards to the product. Because of this, developing another intervention to improve calpain activity and product tenderness is necessary. It is necessary to evaluate how electrical stimulation influences free calcium concentration, calpain activity and therefore, tenderness. Understanding this could potentially help develop a method to improve product consistency, pleasing consumers and driving the demand for high quality beef. Previous research demonstrates the influence of LV and HV stimulation on calpain activity and final product tenderness. Limited research about ELV stimulation of beef carcasses is available. Additionally, little information is available regarding the effect of electrical stimulation on free calcium availability in meat and calpain-2 activity. Identifying these voids in the literature led us to evaluate the use of ELV electrical stimulation at two different time points in the harvest process, exsanguination and one-hour postmortem and to identify the influence it has on free calcium availability, calpain activity, mechanical tenderness and consumer sensory analysis.

The specific objectives of this study were to 1) determine the influence of the timing of extra-low voltage electrical stimulation on free calcium concentration and calpain-2 activity on beef strip loin and top round steaks aged 0, 4 and 14 days and 2) measure overall product tenderness by evaluating WBSF and consumer sensory analysis of beef strip loin and top round steaks aged 4 and 14 days.

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Tables and Figures

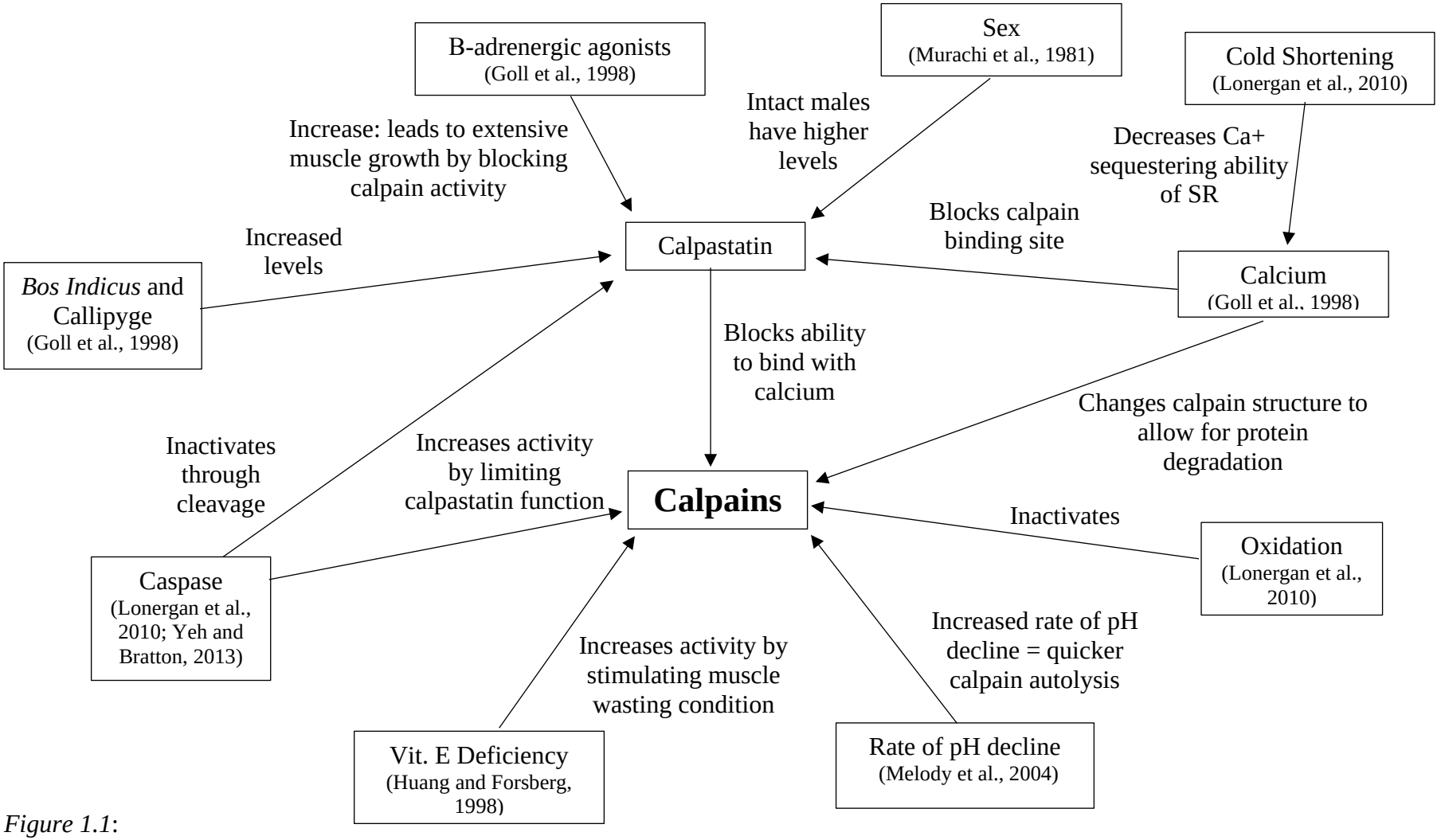

Figure 1.1:
This figure represents various factors that influence calpain activity both during life and postmortem.

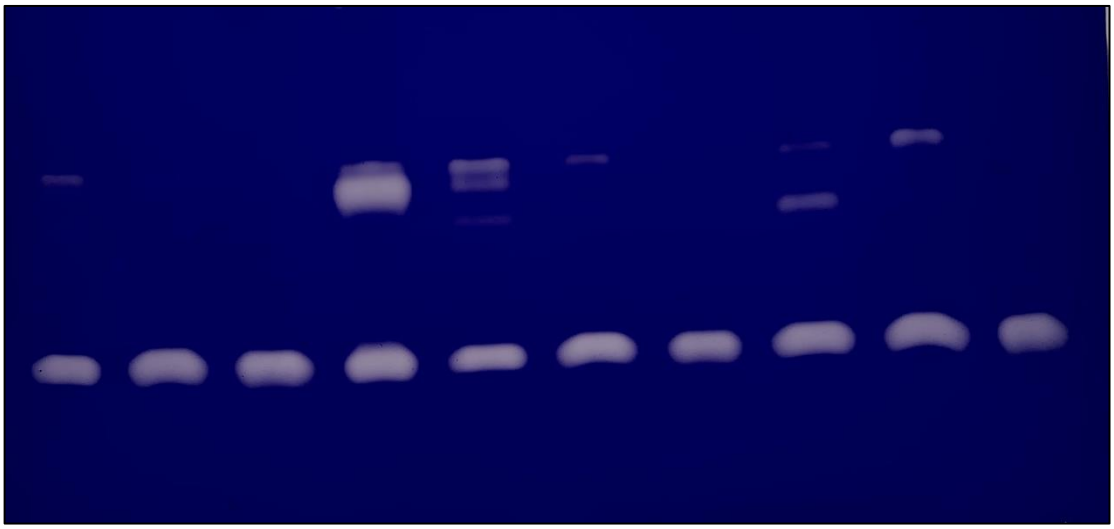

Figure 1.2:

Casein within the gel is dyed with Coomassie Blue, leaving bright, white bands where protein has been denatured by calpains.

CHAPTER 2

Free Calcium Concentration, Calpain-2 Activity and Final Product Tenderness of Electrically Stimulated Beef

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Abstract

The objective of this study was to evaluate timing of electrical stimulation on free calcium concentration, calpain-2 activity, Warner-Bratzler Shear Force (WBSF) and consumer sensory analysis. Twenty-three beef steers were harvested and stimulated (S), using extra-low voltage (ELV) or not stimulated (NS) at exsanguination and at 1-hour postmortem, resulting in four stimulation treatments: NS-NS, NS-S, S-NS, or S-S. Samples were cut from the *longissimus lumborum* (LL) and *semimembranosus* (SM) for free calcium and calpain-2 analysis on days 1, 4 and 14 postmortem. On day 4, steaks were cut from the LL and SM for WBSF and sensory analysis and assigned to an aging period of 4 or 14 days. Data were analyzed using the Mixed Model procedure of the Statistical Analysis System, with significance determined at $P < 0.05$. There was a trend for an aging period by stimulation treatment interaction for LL free calcium concentration ($P = 0.05$), and a significant difference between aging periods ($P < 0.01$). No difference was observed on free calcium concentration in the SM between stimulation treatments ($P = 0.44$); aging, however, significantly increased SM free calcium concentration ($P < 0.01$). Stimulation did not impact native calpain-2 activity in the LL ($P = 0.71$) or SM ($P = 0.89$). Furthermore, stimulation treatment did not improve WBSF for the LL ($P = 0.69$) or SM ($P = 0.61$). Additionally, stimulation treatment did not influence consumer sensory scores for tenderness in the LL ($P = 0.56$) or SM ($P = 0.36$). However, a longer aging period tended to increase calpain-2

activity in the SM ($P = 0.08$) and improve WBSF in the LL ($P = 0.09$) and significantly improve consumer tenderness scores in the SM ($P < 0.01$). In conclusion, the timing of electrical stimulation utilized in the current study did not influence free calcium concentration, calpain-2 activity or beef tenderness; however, aging did improve tenderness.

Introduction

Consumers have consistently reported that tenderness is the most important quality trait they consider when consuming beef (Koohmaraie and Gesink, 2006). There are three primary factors that regulate meat tenderness: background toughness, the toughening phase and the tenderization phase (Koohmaraie and Gesink, 2006; Veiseth-Kent et al., 2018). It is believed that proteases play a major role in the tenderization phase and determine the overall level of postmortem protein breakdown and rate of tenderization.

Calpains are calcium activated proteases that function to breakdown protein (Goll et al., 1998; Aberle et al., 2012). Postmortem, the calpain system is considered the primary proteolytic system involved in protein degradation (Koohmaraie et al., 1995). This degradation during aging results in a more tender product, which has been demonstrated by improved Warner-Bratzler Shear Force values and consumer palatability reports (Colle et al., 2016). Because of the role calpains play in improving product acceptability, it is necessary to research interventions that could be used to improve calpain activity and ultimately tenderness.

Calcium is a positively charged ion. In theory, the use of an electrical current should pull enough free calcium out of the sarcoplasmic reticulum to activate calpain-2. Little research has been conducted analyzing the influence of electrical stimulation on free

calcium concentration within muscle tissue. This, along with the lack of information available about the effect of ELV stimulation, provides an opportunity for further analysis.

Finding a way to influence protease activity during the tenderization phase to improve final product tenderness without affecting food safety is of utmost importance. The objectives of this study were to 1) determine the influence of the timing of extra-low voltage (<100 V), electrical stimulation on free calcium concentration and calpain-2 activity on beef LL and SM aged 0, 4 and 14 days and 2) measure overall product tenderness by evaluating WBSF and consumer sensory analysis of beef strip loin and top round steaks aged 4 and 14 days.

Materials and Methods

Human Subject Participation in Consumer Panel

The University of Idaho Institutional Review Board certified this project as exempt (Appendix A)

Animal Harvest and Stimulation Treatment

Twenty-three crossbred beef steers (Angus x Hereford x Simmental) were harvested at the University of Idaho Meat Laboratory under USDA inspection. Steers were harvested in six groups of three and one group of five over a 5-week period. Carcasses were systematically assigned a stimulation treatment to account for harvest day. Following exsanguination, approximately half of the carcasses (n = 11) were electrically stimulated (21 volts for 20 seconds) with a Jarvis, Model ES-4, Low Voltage Beef Stimulator (Middletown, CT, US) and the remaining carcasses (n = 12) were not electrically stimulated. At exsanguination, the stimulator clamp was positioned on the nose of the carcass. At one-hour postmortem, one side of each carcass was stimulated and the other side was not stimulated,

resulting in four stimulation treatments: stimulated-stimulated (S-S; n = 11 sides), stimulated-not stimulated (S-NS; n = 11 sides), not stimulated-stimulated (NS-S; n = 12 sides) and not stimulated-not stimulated (NS-NS; n = 12 sides). At one-hour postmortem, the clamp was positioned on the *rhomboideus* of the stimulated side. The stimulator was grounded to the rail which allowed the electrical current to travel through the entire carcass.

Carcass Measurements

Carcasses were ribbed between the 12^{th} and 13^{th} rib at 24 hours postmortem. Quality grade was determined after 20 min of bloom time on each side by trained University of Idaho personnel using USDA Quality Grade standards (USDA, 2020; Bertelsen, N.D.). Ribeye area and backfat were measured and kidney, pelvic and heart fat was estimated on each side to allow for USDA yield grade calculation (Bertelsen, N.D). USDA Yield Grade = 2.5 + (2.5 x 12^{th} Rib Backfat) + (0.0038 x Hot Carcass Weight) – (0.32 x Ribeye Area) + (0.2 x Kidney Pelvic and Heart Fat).

Calpain and Free Calcium Analysis Sampling Procedures

On day 1 postmortem, samples for calpain and calcium analyses were collected from the *semimembranosus* (SM) and *longissimus lumborum* (LL) muscles. Samples from the SM were removed from the open face of the top round. The outer crust of the subprimal was removed to ensure there was no outside influence from lactic acid spray or carcass dehydration. The LL samples were removed from a steak cut from the medial, anterior face of the LL following ribbing. The same sample was used to subsample on days 1, 4 and 14 to ensure consistency across sampling times. On days 1, 4 and 14, samples were finely diced, snap frozen in liquid nitrogen and then stored in 15 mL conical tubes at -76º C (Panasonic, MDF-C8V1-PA, Wood Dale, IL). Between the aging periods, the remaining sample was

vacuum packaged and stored at 0.5º C. A control sample to be analyzed on each calpain gel was obtained from the *sternocephalicus* muscle immediately following exsanguination on day 0 from the first NS carcass.

Fabrication

Carcasses were fabricated on day 2 postmortem. The top round (Institutional Meat Purchase Specifications (IMPS) 169A) and the strip loin (IMPS 180) were vacuum packaged and stored (0º C) until day 4 postmortem. On day 4 postmortem, four 2.54 cm thick steaks were cut from the proximal end of the LL and the anterior end of the SM. Steaks were assigned one of four treatments: Day 4 Warner-Bratzler Shear Force (D4 WBSF), Day 4 Taste Panel (D4 TP), Day 14 Warner-Bratzler Shear Force (D14 WBSF) and Day 14 Taste Panel (D14 TP). Steaks assigned to D4 WBSF or D4 TP were vacuum packaged (High-barrier, EVOH Vacuum Pouch) and frozen (-20º C) for future analysis. Steaks assigned to D14 WBSF or D14 TP were vacuum packaged and wet aged (0º C), until day 14 postmortem, when they were frozen (-20º C) and stored for future analysis.

pH

The pH was recorded using a portable pH meter for food testing (SX811-SS, Apera Instruments, LLC, Columbus, OH) with a spear, puncture type, pH electrode (LabSen753, Apera Instruments, LLC, Columbus, OH). The meter was calibrated on a 3-point scale, using standards of 4.0, 7.0 and 10.0. Measurements were taken at the open face of the *longissimus thoracic* (LT) of each side 24 hours postmortem. Additionally, final pH was measured on the anterior end of the LL and the open face of the SM when steaks were cut on day 4 postmortem.

Calcium Analysis

Two grams of frozen, finely diced sample were weighed and stored in a -76º C freezer. Samples were transferred to a -20º C freezer 68 hours before analysis. On the day of analysis, samples were placed in a 4º C refrigerator for 20 minutes before being centrifuged (Sorvall RT1 Centrifuge, Thermo Scientific, Waltham, MA) at 20,000 x g at 5° C for 40 min. Following centrifugation, 250 µl aliquots of the supernatant were mixed with 5 µl of Calcium Ion Strength Adjuster (Hanna Instruments, Woonsocket, RI). The solution was then incubated in a water bath for 5 minutes at room temperature.

The calcium selective electrode (PerfectION combination Ca^{2+}, Mettler Toledo, Woburn, MA) was attached to a portable ion meter and used to measure the ionic strength of the solution. The electrode was soaked in a 1 x 10^{-2} calcium solution for approximately one hour prior to calibration and reading samples. A calibration curve was formed prior to each run with calcium standards containing 10 µM, 50 µM, 100 µM and 500 µM calcium, using the Calcium ISE standard 1000 mg/1 as Ca^{2+} (Mettler Toledo, Woburn, MA). Samples were read and recorded along the calibration curve to determine total free calcium concentration (Hopkins and Thompson, 2001; Colle and Doumit, 2017; Appendix B).

Calpain Extraction

On the day calpains were extracted, 1.0 g of muscle sample was combined with 3.0 mL of extraction buffer (100 mM Tris, 10 mM EDTA, 10 mM DTT, pH 8.3) and homogenized (POLYTRON® PT 10-35 GT; PT-DA 12/2EC-B154, Radnor, PA, USA) on ice for three, 15 second bursts, with a 15-second cooling period between each burst. One mL of the homogenate was then pipetted into 1.7 mL centrifuge tubes (SafeSeal Microcentrifuge Tubes, Sorenson BioScience, Inc., Salt Lake City, UT) and centrifuged (Sorvall RT1

Centrifuge, Thermo Scientific, Waltham, MA) at 8,800 x g for 30 minutes at 4º C. The remaining supernatant was placed in 1.7 mL microcentrifuge tubes and stored in a -76º C freezer for later analysis (Colle and Doumit, 2017; Appendix C).

Casein Zymography

One mm, polyacrylamide gels were formed with a 12.5% separating gel containing 0.2% casein and overlaid with a 4% stacking gel. Casein gels were run at 100 volts (Mini-PROTEAN® Tetra Vertical Eletrophoresis Cell) for 15 minutes with running buffer in an ice bath (25 mM trisHCL, 1 mM DTT, 192 mM Glycine, 1 mM EDTA) prior to loading samples. Frozen, homogenized samples were thawed at room temperature while gels were poured. Once thawed, 40 µL of supernatant and 10µL of sample buffer (150 mM trisHCL, 20% glycerol, 10 mM DTT, 0.02% of 0.8% Bromophenol blue) were combined and mixed using a vortex mixer (VWR Vortexer 2, Scientific Industries, Inc., Bohemia, N.Y.). Twenty µL of sample and buffer combination were loaded into the casein minigels. One lane containing the D0 control sample was included on each gel. Gels were run at 100 volts for a minimum of 3.5 hours in an ice water bath. Gels were then incubated at room temperature in incubation buffer (50 mM Tris HCL, 10 mM DTT, 4 mM $CaCl_2$) for 17 hours with slow shaking. Incubation buffer was replaced twice (30 min and 1 hour). Incubation buffer was removed, the gels were rinsed and stained for 1 hour using Coomassie Blue R250 (BioRad). Following staining, gels were destained for 3 hours with Coomassie Blue R250 Destaining Solution (BioRad). Gels were analyzed on a BioRad ChemiDoc MP_{TM} System. Images were inverted and lanes and bands detected on high sensitivity with manual adjustment to ensure entire sample was read. The volume of each band was recorded as a percentage of the

control band ran on each gel (Pomponio and Ertbjerg, 2012; Colle and Doumit, 2017; Appendix D).

Cooking

Steaks were thawed at 0º C for 24 h prior to cooking. Steaks were cooked on a clamshell-style countertop grill (Cuisinart Griddler Deluxe Model GR-150). Steaks were removed once an internal temperature of 66º C was reached and could rest before a peak final temperature was recorded (32311-K EconoTemp$_{TM}$ Thermocouple, Atkins, Middlefield, CT). The average final peak temperature was 70.0º C ± 0.1. Raw steaks were weighed prior to grilling and cooked steaks were weighed once they reached room temperature to determine percent cook loss. $\text{Percent Cook Loss} = \frac{\text{raw weight} - \text{cook weight}}{\text{raw weight}} \text{ x } 100.$

Warner-Bratzler Shear Force

Following cooking, steaks were cooled to room temperature. Six, 1.27 cm cores were removed using an oscillating drill press (Shop Fox, W1667 8-1/2") with a 1.27 cm coring bit attachment) from both the superficial and deep portions of the SM and six cores were removed from the LL, parallel to the muscle fibers avoiding connective tissue and fat. Cores were sheared (200 mm/min, Mecmesin, Warner-Bratzler Meat Shear, G-R Manufacturing, Co.) perpendicular to the muscle fibers and peak shear force was recorded (Appendix E).

Consumer Sensory Panel

Two consumer taste panels, one for each muscle group, were implemented on the University of Idaho campus at the Margaret Ritchie School of Family and Consumer Sciences Mary Hall Niccolls Building Test Kitchen in accordance with the AMSA guidelines (AMSA, 2015; Appendices F, G and H).

Steaks were cooked as previously described. Five, 1.27 cm x 1.27 cm cubes were cut from each steak, avoiding the edge of the steak and excessive connective tissue. Samples were assigned to panelists using an incomplete block design. In addition to the sample, consumers were supplied with water and unsalted soda crackers as palate cleansers between samples.

To limit sample fatigue, panelists (n = 92/panel) were presented with 5 samples representing various stimulation treatment groups and aging periods and were asked to consume the samples in a predetermined order. Steaks were systematically assigned to panelists to ensure variation between treatments being sampled. Each sample was evaluated on a 10-point scale for overall acceptability, tenderness, juiciness and flavor (1 = dislike extremely, 10 = like extremely).

Statistical Analysis

Data were analyzed using a generalized linear mixed model assuming either a normal distribution (calpain activity, WBSF, cook loss and consumer sensory analysis) or a lognormal distribution (pH and free calcium concentration; Stroup 2014). Within each model, carcass and side within carcass were random effects and age, stimulation treatment and their interaction were fixed effects. The relationship between calpain-2 activity and WBSF was assessed using Pearson Correlation analysis. Significance was determined at ($P < 0.05$) For significant fixed effects, means were separated using pair-wise comparisons. All statistical analyses were carried out using SAS V9.4.

Results

Carcass Data

The average yield grade of the carcasses was 2.94 ± 0.22 and the average marbling score was 460 ± 21.17 (Ch^{-}). Stimulation treatment was not significant for final yield grade ($P = 0.70$) or marbling score ($P = 0.29$).

pH

Stimulation treatment did not influence 24-hour pH ($P = 0.38$), with an average final value of 5.5 ± 1. Additionally, no differences were seen between stimulation treatments on final pH in the LL (pH = 5.47; $P = 0.13$) or SM (pH = 5.47; $P = 0.45$).

Calcium Analysis

There was a trend for an interaction between aging period and stimulation treatment for LL free calcium concentration ($P = 0.05$; Table 2.1). While all treatments had similar initial and final values, the NS-NS treatment showed a rapid increase relative to the other treatments at 4 days of aging. There was no difference between stimulation treatments ($P = 0.36$; Table 2.2) on free calcium concentration in the LL, but a significant difference between aging periods was observed ($P < 0.01$; Table 2.3). There was no interaction between aging and stimulation treatment for SM free calcium concentration ($P = 0.54$). Furthermore, free calcium concentration in the SM was not observed to be influenced by stimulation treatment ($P = 0.44$; Table 2.2). However, aging did significantly increase SM free calcium concentration ($P < 0.01$), with day 4 and day 14 free calcium concentration being higher than day 1 (Table 2.3).

Calpain Analysis

There was no aging period by stimulation treatment interaction observed for native calpain-2 activity in the LL ($P = 0.57$) or the SM ($P = 0.70$). Furthermore, stimulation treatment did not significantly influence native calpain-2 activity in the LL ($P = 0.71$) or the SM ($P = 0.89$; Table 2.2). In the current study, aging period did not reach significance in increasing native calpain-2 activity in the LL ($P = 0.11$); however, a larger sample size may show differences. Aging did show higher native calpain-2 activity in the SM on day 14 ($P = 0.08$; Table 2.3).

Stimulation treatment did not show an interaction with aging on autolyzed calpain-2 activity in the LL ($P = 0.46$) or the SM ($P = 0.44$). Stimulation treatment did not significantly influence autolyzed calpain-2 activity in the LL ($P = 0.67$) or the SM ($P = 0.76$; Table 2.2). Aging period tended to increase activity in the LL ($P = 0.06$) and significantly increased autolyzed calpain-2 activity in the SM ($P = 0.03$; Table 2.3).

Warner-Bratzler Shear Force

No aging period by stimulation treatment interaction was observed for WBSF in the LL ($P = 0.09$) or SM ($P = 0.40$). Additionally, stimulation treatment was not significant for WBSF in the LL ($P = 0.69$) or SM ($P = 0.61$; Table 2.2). There was a significant difference between aging period in the LL ($P < 0.01$), with steaks aged 14 days being more tender than those aged only 4 days (Table 2.4). Aging period was not observed to be significant in influencing WBSF values of the SM ($P = 0.61$; Table 2.4). Calpain-2 activity was not correlated with tenderness in terms of WBSF in the LL ($P = 0.66$) or SM ($P = 0.34$).

Cook Loss

No interaction was observed between aging period and stimulation treatment on cook loss in the LL ($P = 0.77$). An interaction between aging period and stimulation treatment was observed for cook loss in the SM ($P < 0.01$; Table 2.5). Day 4 samples showed the highest cook loss in the S-NS treatment. There was, however, not one stimulation treatment that differed significantly with regard to Day 14 samples. Interestingly, there were no differences between NS-NS and S-S in either aging period (Table 2.5). In the LL, cook loss was not observed to be influenced by stimulation treatment ($P = 0.76$; Table 2.2) or aging period ($P = 0.57$; Table 2.4).

Consumer Sensory Panel

Demographics of the two consumer sensory panels are summarized in Table 2.6. In the LL, no interaction was observed between aging and stimulation treatment in terms of consumer sensory analysis for overall acceptability ($P = 0.65$), tenderness ($P = 0.60$), juiciness ($P = 0.78$), or flavor ($P = 0.56$). Additionally, no interaction was observed in the SM between aging and stimulation treatment in terms of consumer sensory analysis for overall acceptability ($P = 0.89$), tenderness ($P = 0.52$), juiciness ($P = 0.12$), or flavor ($P = 0.18$). No influence by stimulation treatment on consumer sensory analysis was observed within the LL or SM for overall acceptability ($P = 0.54$; $P = 0.53$), tenderness ($P = 0.56$; $P = 0.36$), or flavor ($P = 0.85$; $P = 0.82$), respectively (Table 2.7). Although there was no detectable stimulation treatment influence on consumer sensory analysis for juiciness in the LL ($P = 0.9$), there was a tendency for stimulation to decrease consumer acceptability for juiciness in the SM ($P = 0.08$). In the LL, aging did not show a significant influence on overall acceptability ($P = 0.35$), tenderness ($P = 0.71$), juiciness ($P = 0.86$), or flavor ($P =$

0.90) (Table 2.8). Consumers preferred the tenderness of SM samples aged 14 days over those aged 4 days ($P < 0.01$) but did not distinguish differences in overall acceptability ($P = 0.43$), juiciness ($P = 0.73$), or flavor ($P = 0.29$).

Discussion

Electrical stimulation is commonly used in packing plants to increase tenderness prior to entering the cooler by causing extreme muscle contractions and subsequent muscle fiber tearing (Luo et al., 2008). There are three types of electrical stimulation utilized in beef processing: extra-low voltage (ELV; <100 V), low voltage (LV; 100-110 V) and high voltage (HV; >110 V, often 500-1,000+ V; Adeyemi and Sazilli, 2014). Although most studies analyzing the effect of electrical stimulation have seen an improvement in product tenderness regardless of stimulation type (Eilers et al., 1996, Hwang and Thompson, 2001; Powell et al., 1984), traditionally, LV and HV are the most common forms researched, with a lack of research available analyzing ELV stimulation. Great variability exists between methods of stimulation, including voltage, impulse, duration, frequency, timing and location of electrode (Adeyemi and Sazilli, 2014).

Electrical stimulation is associated with accelerating glycolysis, leading to lower pH values early postmortem (Chrystall and Hagyard, 1976). However, similar to the observations in the current study, by 24 hours postmortem, pH values of stimulated and non-stimulated samples have been shown to be similar between treatments (Chrystall and Hagyard, 1976; Uytterhaegen et al., 1992; Eilers et al., 1996).

Contrary to the hypothesis of this study, on day 4 postmortem, the LL NS-NS treatment group tended to have higher free calcium levels than that of the S-NS and S-S treatment groups. Samples from the S-NS and S-S stimulation treatment groups tended to

show higher levels of free calcium up to 14 days of aging, while NS-NS and NS-S samples did not show signs of improvement after 4 days. This differs from the observations of Hwang and Thompson (2001), who speculated that massive catabolism caused by muscle contraction during stimulation can lead to higher levels of free calcium.

In the current study, stimulation treatment of the SM samples did not significantly alter free calcium concentration, but aging period did. Free calcium concentration did not increase in the SM after 4 days of aging. This observation differs from previous research conducted by Colle et al. (2018), who showed no differences in free calcium concentration between aging periods (1, 4 and 14 days).

Calpains are considered the leading protease in postmortem tenderization (Koohmaraie et al., 1995). Free calcium must be available in order to activate calpains (Goll et al., 1995; Goll et al., 2003). Due to a relatively low free calcium concentration requirement for activation (3-50 µM) calpain-1 is active early postmortem and is thought to be responsible for 95% of proteolytic activity in the first 7-14 days postmortem (Yang et al., 2018). Calpain-2 requires higher levels of free calcium (400-800 µM) for activation (Goll et al., 1995; Goll et al., 2003); therefore, extended aging (>28 days) results in calpain-2 activation and increases the likelihood of producing a more tender product (Colle and Doumit, 2017). In this study, calcium levels were highest on day 4 and 14 in the SM (116.75 ± 1.06 and 127.74 ± 1.06 µM, respectively). Although free calcium concentration never reached the required levels for activation, as described by Goll et al. (2003), calpain-2 was still observed in all samples across stimulation treatments and aging periods. No increase in native or autolyzed calpain-2 activity was observed in the LL or SM when electrical stimulation was used. This observation is supported by the work of Li et al. (2012), who

found that calpain-2 activity did not change with low-voltage stimulation (80 V, 35 sec) 30 minutes postmortem. In the present study, there were significantly higher levels of autolyzed calpain-2 in SM samples aged 14 days rather than those aged 1 or 4 days. Additionally, the LL tended to have higher levels of autolyzed calpan-2 in samples aged 14 days than those only aged 1 or 4 days. The observations of this study are supported by Colle and Doumit (2017), who showed higher levels of autolyzed calpain-2 as the product is aged. This was also supported by Hwang and Thompson (2001), who evaluated the effects of various voltage levels at different time periods (HV/LV, 3- and 40-min postmortem; HV, 40- and 60-min postmortem; LV, 40-min postmortem). Levels of calpain-2 remained consistent irrespective of stimulation type or time. Interestingly, the study by Hwang and Thompson (2001) only evaluated calpain activity prior to stimulation, post-stimulation and at 24 hours postmortem.

No significant interaction was observed between stimulation treatment and aging period on WBSF values of the LL or SM. This differs from the observations of Razminowicz et al. (2008), who found that the *longissimus dorsi* (LD) from electrically stimulated sides showed lower WBSF values at days 2 and 15 postmortem than unstimulated sides. However, Razmiowicz et al. (2008) only looked at one high voltage (230 V, 60 Hz, 30 sec) stimulation at 30 min postmortem. Additionally, Li et al. (2012) found that electrical stimulation (80 V, 35 sec, 30 min postmortem) accelerated meat tenderization specifically at 24 and 48 hours postmortem and tended to improve tenderness up to 7 days postmortem.

Hwang and Thompson (2001) noted that electrical stimulation increased meat tenderness, but early application of stimulation (3 min postmortem) had higher WBSF values than that stimulated at 40- or 60-min postmortem. While little research is available

specifically evaluating the effects of ELV stimulation, Powell et al. (1984) did report improvements in WBSF values when analyzing three different ELV stimulation treatments (45 V, 40 HZ, 90 sec continuous; 60 sec, 2 sec on and 1 sec off; or 40 sec, 3 sec on and 1 sec off), irrespective of treatment type. Although an improvement in WBSF was observed when ELV stimulation was used, the WBSF value for the SM and LD was 8 kg, depicting a very tough product (Powell et al., 1984). Additionally, Eikelenboom et al. (1985) observed no difference in overall tenderness between type of stimulation administered at exsanguination, with consumers preferring both LV and HV treated samples over the non-stimulated control. Based on previous research and observations of this study, utilizing LV or HV stimulation treatments following a post-exsanguination delay may be the most effective method to improve tenderness. More research needs to be conducted to identify the ideal voltage and timing of stimulation to maximize final product tenderness.

Tenderness values for SM WBSF were not significantly different between stimulation treatments; however, consumers were able to detect a difference in tenderness between aging periods in the SM. This observation is inconsistent with the findings of Miller et al. (1995), who found that, as WBSF value decreased, consumer perception of tenderness improved. When eating steaks cooked at home, consumers can detect a > 0.5 kg difference in WBSF (Miller et al., 1995; ASTM, 2011), which aligns with the difference seen in the SM steaks (1.14 kg) in the present study.

Aging treatment in this study improved LL WBSF, with steaks aged 14 days being more tender (lower WBSF) than steaks aged 4 days, similar to Hwang and Thompson (2001). This is likely because LL tenderness has been found to improve during aging for up to 14 days (Eilers et al., 1996; Bratcher et al., 2005; Colle et al., 2016). Another study

showed lower WBSF values in LL steaks aged 12 days than those aged 6, but no difference between 12 and 18 days of aging (Eilers et al., 1996). Interestingly, consumers in this study were not able to detect differences in tenderness in the LL. This was surprising, considering there was an improvement of 0.51 kg of WBSF in steaks aged 14 days compared to those aged only 4 days. Though there was no observable difference detected, it is important to note that consumers consider 4.3 kg WBSF acceptable for beef tenderness (Miller, et al. 1995). All LL steaks in the current study, regardless of stimulation treatment or aging period, fell below the threshold to qualify to be marketed as *Certified Very Tender* (WBSF < 3.9 kg; ASTM, 2011). Improvements in livestock genetics, animal handling and processing is likely leading to improved product tenderness prior to stimulation.

Electrical stimulation can disrupt muscle fibers, leading to poorer structural integrity (Savell et al., 1978). In addition to impacting tenderness, muscle fiber disruption as well as accelerated pH decline may allow for higher levels of purge cook loss. In this study, there was a tendency observed for stimulation treatment to influence consumer perception of juiciness with S-S steaks tending to be less juicy than NS-NS steaks. Interestingly, day 4 postmortem, S-NS SM steaks had the highest level of cook loss. By day 14 postmortem, however, no differences were seen between NS-NS, S-NS and S-S treatments. Other researchers have observed varied responses when evaluating the influence of electrical stimulation on juiciness; some found no influence (Lee et al., 2000; Hwang and Thompson, 2001), while others observed a decrease in juiciness when stimulation was used (Savell et al., 1978). Inconsistencies such as this implicate factors outside of stimulation treatment, such as freezing and thawing technique and cooking method, to impact cook loss and therefore final product juiciness.

Conclusion

Although electrical stimulation plays an important role in overall carcass quality, in this study, ELV electrical stimulation was not observed to be an effective method of improving free calcium concentration, calpain-2 activity, or final product tenderness in the LL or SM. These findings could be due to the small sample size evaluated in this project. Stimulation treatment did, however, support the fact that aging leads to a higher concentration of free calcium, increased calpain activity and therefore improved consumer perceptions of product tenderness, specifically within the SM. Knowing the role that tenderness plays in consumer satisfaction pushes researchers to find ways to improve product tenderness to offer a more consistent eating experience to consumers

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Tables and Figures

Table 2.1: LL Estimated Free Calcium Concentration (μM) for the Tendency for the Stimulation Treatment by Aging Period Interaction

	Stimulation Treatment					
Aging Period	NS[1]-NS	NS-S[2]	S-NS	S-S	SEM	*P*-Value
1	23.10	22.87	25.03	29.37	1.19	0.05
4	56.83	42.95	25.53	30.88		
14	58.56	59.74	57.40	57.40		

All statistical inferences are based on log transformed data
[1]Not Stimulated
[2]Stimulated (21 V for 20 seconds)

Table 2.2: Estimated Mean Values for Calpain Activity, Final pH, Free Calcium Concentration (μM), WBSF (kg) and Cook Loss (g) by Stimulation Treatment

	Stimulation Treatment					
	NS[1]-NS	NS-S[2]	S-NS	S-S	SEM	*P*-Value
Longissimus lumborum						
Native Calpain-2[3]	53.94	51.39	62.55	62.64	7.31	0.71
Autolyzed Calpain-2	0.06	0.06	0.00	0.00	0.38	0.67
Free Calcium Concentration[4]	42.58	38.94	33.10	37.29	1.11	0.36
Warner-Bratzler Shear Force	3.12	3.11	2.88	3.03	0.20	0.69
Cook Loss	63.47	63.86	67.91	66.30	3.01	0.76
Semimembranosus						
Native Calpain-2	63.82	65.95	66.02	63.50	6.80	0.89
Autolyzed Calpain-2	1.01	0.17	4.89	5.60	3.66	0.76
Free Calcium Concentration[4]	102.51	113.30	121.51	113.30	1.08	0.44
Warner-Bratzler Shear Force	3.83	5.08	3.84	3.95	0.82	0.61

[1]Not Stimulated

[2]Stimulated (21 V for 20 seconds)

[3]Values are percentages of Calpain-2 compared to the control, day zero, *sternocephalicus* samples.

[4] Statistical inferences are based on log transformed data

Table 2.3: Estimated Mean Calpain Activity and SM Free Calcium Concentration (µM) by Aging Period

	Aging Period				
	1	4	14	SEM	*P*-Value
Longissimus lumborum					
Native Calpain-2[1]	62.69	55.43	54.77	5.11	0.11
Autolyzed Calpain-2	0.00	0.00	0.08	0.03	0.06
Free Calcium Concentration[2]	24.99[c]	37.20[b]	58.20[a]	1.09	<0.01
Semimembranosus					
Native Calpain-2	63.62	62.68	68.17	4.72	0.08
Autolyzed Calpain-2	0[b]	1.64[b]	7.72[a]	2.78	0.03
Free Calcium Concentration[2]	95.58[b]	116.75[a]	127.74[a]	1.06	<0.01

[a-c]Means within a row within a muscle group without a common superscript differ ($P < 0.05$)

[1]Values are percentages of Calpain-2 from day zero, *sternocephalicus* samples.

[2]Statistical inferences are based on log transformed data

Table 2.4: Estimated Mean WBSF (kg) and LL Cook Loss (g) by Aging Period

	Aging Period			
	4	14	SEM	*P*-Value
Longissimus lumborum				
Warner-Bratzler Shear Force	3.29[a]	2.78[b]	0.12	<0.01
Cook Loss	64.81	65.96	1.99	0.57
Semimembranosus				
Warner-Bratzler Shear Force	4.75	3.61	0.56	0.61

[a-b]Means within a row without a common superscript differ ($P < 0.05$)

Table 2.5: Estimated Mean SM Cook Loss (g) for the Aging Period by Stimulation Treatment Interaction

	Treatment					
Aging Period	NS-NS[1]	NS-S	S-NS	S-S	SEM	*P*-Value
4	170.58^{efg}	174.54^{dg}	205.56^{abc}	187.95^{cde}	7.28	<0.01
14	191.89^{ad}	166.78^{g}	178.05^{dg}	187.53^{bdf}		

[a-e]Means without a common superscript differ ($P < 0.05$)

[1]Not Stimulated

[2]Stimulated (21 V for 20 sec)

Table 2.6: Consumer Panel Demographics (n = 92/panel)

	Longissimus lumborum		*Semimembranosus*	
	n	%	n	%
Age				
18-19	20	21.7	26	28.3
20-29	52	56.5	44	47.8
30-39	8	8.7	10	10.9
40-49	1	1.1	3	3.3
50+	11	12.0	8	8.7
Not Indicated			1	1.1
Gender				
Male	40	46.5	34	37.0
Female	52	56.5	58	63.0
Beef Meals/wk[1]				
0-1	10	10.9	10	10.9
2-4	44	47.8	50	54.3
5-7	34	37.0	24	26.1
8+	4	4.3	8	8.7
Most Consumed[2]				
Ground	63	68.5	61	66.3
Roast	2	2.2	3	3.3
Steak	24	26.1	21	22.8
Other	1	1.1	4	4.3
Not Indicated	2	2.2	3	3.3

[1]Please indicate the number of meals a week in which you consume beef: 0-1, 2-4, 5-7, 8+
[2]Please indicate the form in which you most commonly consume beef: Ground, Roast, Steak, Other

Table 2.7: Estimated Mean Consumer Sensory Panel Scores by Stimulation Treatment

	Stimulation Treatment					
	NS[1]-NS	NS-S[2]	S-NS	S-S	SEM	*P*-Value
Longissimus lumborum						
Acceptability[3]	6.9	7.1	7.1	6.9	0.2	0.54
Tenderness	6.6	6.6	7.0	6.6	0.3	0.56
Juiciness	6.4	6.5	6.6	6.3	0.3	0.90
Flavor	6.5	6.6	6.7	6.8	0.2	0.85
Semimembranosus						
Acceptability	6.1	6.1	5.9	5.8	0.2	0.53
Tenderness	5.7	5.5	5.5	5.2	0.2	0.36
Juiciness	6.2	5.7	5.6	5.2	0.3	0.08
Flavor	5.9	5.9	5.7	5.8	0.2	0.82

[1] Not Stimulated

[2] Stimulated (21 V for 20 seconds)

[3] Scale: 1 = dislike extremely (not at all tender, extremely dry and dislike flavor extremely); 10 = like extremely (extremely tender, extremely juicy and like flavor extremely)

Table 2.8: Estimated Mean Consumer Sensory Panel Scores by Aging Period

	Aging Period			
	4	14	SEM	*P*-Value
Longissimus lumborum				
Acceptability[1]	6.9	7.0	0.2	0.35
Tenderness	6.7	6.7	0.2	0.71
Juiciness	6.5	6.5	0.2	0.86
Flavor	6.6	6.6	0.2	0.90
Semimembranosus				
Acceptability	5.9	6.0	0.2	0.43
Tenderness	5.2[b]	5.7[a]	0.2	<0.01
Juiciness	5.7	5.6	0.2	0.73
Flavor	5.7	5.9	0.2	0.29

[a-b]Means within a row without a common superscript differ ($P < 0.05$)
[1]Scale: 1 = dislike extremely (not at all tender, extremely dry and dislike flavor extremely); 10 = like extremely (extremely tender, extremely juicy and like flavor extremely)

CHAPTER 3

Effect of Electrical Stimulation on Calpain-1 Activity in Beef

Abstract

The objective of this study was to evaluate the timing of electrical stimulation on calpain-1 activity. Twenty-three beef steers were harvested and stimulated (S), using extra-low voltage (ELV) or not stimulated (NS) at exsanguination and at 1-hour postmortem, resulting in four stimulation treatments: NS-NS, NS-S, S-NS, or S-S. Samples were cut from the *longissimus lumborum* (LL) and *semimembranosus* (SM) for calpain-1 analysis on days 1, 4 and 14 postmortem. Data were analyzed using the Mixed Model procedure of the Statistical Analysis System, with significance determined at $P < 0.05$. Stimulation treatment did not show a significant impact on native calpain-1 concentration in the LL ($P = 0.19$) or SM ($P = 0.94$). It did, however, influence autolyzed calpain-1 concentration in the LL ($P = 0.03$) but not the SM ($P = 0.60$). Interestingly, during the analysis an unidentified 3rd band of calpain was seen in many samples. Stimulation treatment was not significant in determining whether the 3rd band of calpain was seen in the LL ($P = 0.94$) or SM ($P = 0.83$). In the LL aging period was significant in influencing native calpain-1 ($P < 0.01$), autolyzed calpain-1 ($P < 0.01$) and the unidentified 3rd band of calpain ($P < 0.01$), with day 1 having significantly higher concentrations than days 4 or 14. Additionally, in the SM, aging period was significant in influencing native calpain-1 ($P < 0.01$), autolyzed calpain-1 ($P < 0.01$) and the unidentified 3rd band of calpain-1 ($P < 0.01$), with day 1 having significantly higher concentrations than day 4 and 14. Analysis was conducted by the University of Idaho Mass Spectrometry Core Lab to classify the unidentified 3rd band using Liquid Chromatography-Mass Spectrometry (LC-MS), with little success. Further analysis needs to be conducted to

characterize the unidentified 3rd band and determine its relationship to calpain-1. Electrical stimulation did not prove to effect calpain-1 activity, but aging period did.

Introduction

Calpains are calcium activated proteases that function to breakdown protein (Goll et al., 1998; Aberle et al., 2012). Postmortem, the calpain system is considered the primary proteolytic system involved in protein degradation (Koohmaraie et al., 1995). Although multiple forms of calpain exist, calpain-1 and calpain-2 are most active in skeletal muscle (Aberle et al., 2012). Calpain-1 requires 3-50 μM of calcium to be activated, whereas calpain-2 requires 400-800 μM of calcium for half maximal activity (Goll et al., 1995; Goll et al., 2003). Due to its low calcium requirement, calpain-1 is active early postmortem and is thought to be responsible for 95% of proteolytic activity in the first 7-14 days postmortem (Yang et al., 2018). As calpains bind with calcium and are activated, they themselves are autolyzed (Cong et al., 1989; Edmunds et al., 1991; Goll et al., 2003). Autolysis occurs quite rapidly but involves many steps of fragmentation. Calpains continue to degrade protein until total autolysis is complete and function is lost (Cong et al., 1989; Edmunds et al., 1991; Goll et al., 2003).

Because of the role calpains play in improving product acceptability, it is necessary to research interventions that could be used to improve calpain activity and ultimately tenderness. This study analyzed the influence of ELV stimulation on calpain-1 concentration.

Materials and Methods

Human Subject Participation in Consumer Panel

The University of Idaho Institutional Review Board certified this project as exempt (Appendix A).

Animal Harvest and Stimulation Treatment

Animal harvest and stimulation treatment was conducted following the procedure described in Chapter 2.

Calpain Sampling Procedures

Calpain sampling was conducted following the procedure described in Chapter 2.

Calpain Extraction

Calpains were extracted following the procedure described in Chapter 2 (Appendix C).

Casein Zymography

Initial calpain analysis was completed following the procedure described in Chapter 2 (Appendix D).

Third Band Analysis

To isolate the unidentified 3rd band, gels were formed following the procedure for casein zymography as described in Chapter 2 with minor modifications. Three gels were formed following said protocol and three were formed without the inclusion of casein within the gel. Samples were run in duplicate with one gel containing casein and one without. Gels without casein were not incubated in the incubation buffer. Once gels completed the destain process, pairs were matched to aid in band identification. Gels with casein served as the guide to isolate the bands in gels not containing casein (Figure. 3.1). Gels were viewed on a

Scienceware® Mini Light Box (Bel-Art, Radnor, PA) and bands were cut in groups of matching samples (Figure 3.2). Samples from what visually appeared to be native calpain-1, autolyzed calpain-1, native calpain-2, autolyzed calpain-2 and the unidentified 3rd band, were placed in 1.7 ml microcentrifuge tubes (SafeSeal Microcentrifuge Tubes, Sorenson BioScience, Inc., Salt Lake City, UT) and brought to the University of Idaho Mass Spectrometry Core Lab. Samples then underwent tryptic digestion and Liquid Chromatography-Mass Spectrometry (LC-MS) (Appendix I), performed by the Mass Spectrometry Core Lab staff.

Statistical Analysis

Data were analyzed using a generalized linear mixed model assuming a normal distribution. Carcass and side within carcass were random effects and age, stimulation treatment and their interaction were fixed effects. Significance was determined at ($P < 0.05$) All statistical analyses were carried out using SAS V9.4.

Results and Discussion

Native Calpain-1

In this study, there was not a significant interaction between aging period and stimulation treatment in the LL ($P = 0.25$) or the SM ($P = 0.57$). Additionally, stimulation treatment did not significantly impact native calpain-1 activity in the LL ($P = 0.19$) or the SM ($P = 0.94$; Table 3.1). However, aging period did significantly influence native calpain-1 activity in the LL ($P < 0.01$) and the SM ($P < 0.01$); both showing the highest levels on day 1 and no further differences between day 4 and 14 (Table 3.2). This aligns with previous research that depicted a relatively low free calcium concentration requirement (3-50 μM) for calpain-1 (Goll et al., 1995; Goll et al., 2003). Calpain-1 is active early postmortem and is

thought to be responsible for 95% of proteolytic activity in the first 7-14 days postmortem (Yang et al., 2018).

Autolyzed Calpain-1

No significant interaction was seen between aging period and stimulation treatment in the LL ($P = 0.07$) or the SM ($P = 0.72$). Interestingly, stimulation treatment did show significant differences in autolyzed calpain-1 levels in the LL ($P = 0.03$), but not in the SM ($P = 0.60$; Table 3.1). Aging period significantly impacted autolyzed calpain activity in the LL ($P < 0.01$) and the SM ($P < 0.01$); both having the highest levels of autolyzed calpain present on day 1, with no differences seen between day 4 and 14 (Table 3.2). This aligns with previous research showing that calpain-1 is active early on postmortem due to the low calcium requirement for activation. Calpain is known to self-autolyze, which aligns with the high levels of autolyzed calpain-1 on day 1 and low levels on days 4 and 14 demonstrated in this study (Cong et al., 1989; Edmunds et al., 1991; Goll et al., 2003).

Unidentified 3rd Band

When analyzing calpains, calpain-1 and -2 are often displayed in their native form (prior to autolysis) and as an autolyzed fragment; leading to four measurable forms (native calpain-1, autolyzed calpain-1, native calpain-2 and autolyzed calpain-2). During this study, a 3rd band was identified under what was thought to be autolyzed calpain-1 (Figure 3.3). There was no interaction between stimulation treatment and aging period on the third band in the LL ($P = 0.97$) or SM ($P = 0.97$). No differences were seen between stimulation treatments in the LL ($P = 0.94$) or the SM ($P = 0.83$). There was a difference seen between aging periods in the LL ($P < 0.01$) and SM ($P < 0.01$), with the highest levels of the third band being seen on day 1 and no differences seen between days 4 and 14.

Further analysis was conducted to try to identify the unidentified 3rd band. The first round of analysis used only samples from gels that contained casein. Individual bands were removed from the gel to be analyzed. When further analyzed by the Mass Spectrometry Lab, there were strong hits for casein and other meat proteins, but not calpain. This was hypothesized to be due to the autolysis that calpain undergoes as it works to denature available protein. Prior to samples being removed, the gels were incubated in a calcium solution for 17 hours, allowing time for calpain activation and denaturation.

The results from the first round of analysis led to the decision to remove casein from the gels to try to isolate the calpain without interfering casein. The incubation step was excluded to try to limit calpain autolysis. Additionally, multiple replicates of the same sample were run on each gel. Samples were then removed as a group of bands rather than individually to try to increase the available concentration of calpain to be more easily detected. A duplicate gel was run following the original procedure to serve as a reference for band location for removal from the gel not containing casein.

In the second round of analysis, there were no strong matches to calpain in either Protein Lynx Global Server (PLGS) or Mascot, which reports protein and peptide matches from database searches. Expression analysis was then performed to try to identify missing peptides. The SwissProt database search with the expression data didn't yield good results on calpain. However, three samples, native calpain-1, native calpain-2 and autolyzed calpain-2 had hits using the calpain database. The protein hits had good scores, but the individual peptide scores were not as strong as desired.

To further test the matches, the best protein match from the expression analysis was used to create a targeted "include list" for expected proteins, where ms/ms spectra are

acquired preferentially from ions on the include list to try to confirm if the spectra are from the expected proteins. The included list was prepared using the expected peptide m/z values from the protein with the accession number AAA30422.1 (Deobald, 2020). This analysis did not provide further support for calpain identification.

The second round of analysis did not provide positive results in identifying the 3rd band of calpain-1. Although there was no casein in the second set of samples analyzed, other proteins may have been more prominent than calpain. The incubation step during casein analysis is beneficial as it not only allows calpain activation but holds the gels in a pH of 7.5 which promotes calpain activity but decreases presence of lysosomal proteases due to their preferences of an acidic environment (Raser et al., 1994). The presence of other proteases may dilute the concentration of calpain in these samples. Further analysis needs to be conducted to determine the identity of the third band of calpain that was observed in this study.

Conclusion

Electrical stimulation did not show an influence on native or autolyzed calpain-1, nor did it appear to influence the level of the unidentified 3rd band of calpain in the LL and SM. However, aging period did significantly influence native and autolyzed calpain-1 as well as the unidentified 3rd band of calpain in both the LL and SM with day 1 showing the highest levels. Although various analyses were used, the unidentified 3rd band of calpain was not able to be further classified in this study. Further analysis needs to be done to identify and categorize the unidentified 3rd band of calpain.

Acknowledgements

We gratefully acknowledge Lee Deobald, Director of the University of Idaho Mass Spectrometry Core Lab, for his guidance and assistance in the further analysis of the unidentified 3rd band of calpain.

Future Research

Calpain-1 and calpain-2 are the most active forms of calpain found in skeletal muscle. When utilizing casein zymography, typically both forms, as well as their autolyzed forms, may be observed. Through this study, an unidentified 3rd band of what appeared to be calpain-1 was observed in multiple samples. Though initial LC-MS analysis was conducted, strong results for characterization of the band were not found. Further analysis needs to be done to determine if the unidentified 3rd band is, in fact, a form of calpain or if it is another active protease. If this band is determined to be a form of calpain, analysis needs to be conducted for further classification, such as determining calcium required for activation as well as the influence on final product tenderness.

Additionally, ELV stimulation was not determined to be a significant influencer of free calcium concentration, calpain-2 activity and final product tenderness. Although much research has been done looking at LV and HV stimulation, much variability is seen between the specific methods used (timing of stimulation, voltage, length of stimulation, etc.). This provides an opportunity to develop methods that best enhance carcass quality characteristics while also capturing the most value for the processor.

Finally, researching other interventions to improve calpain activity postmortem to improve product tenderness is of great importance. Although CAT has proved to be a

valuable method, it increases risk of product contamination. Finding a safe, practical method to improve tenderness will increase eating satisfaction for consumers.

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Tables and Figures

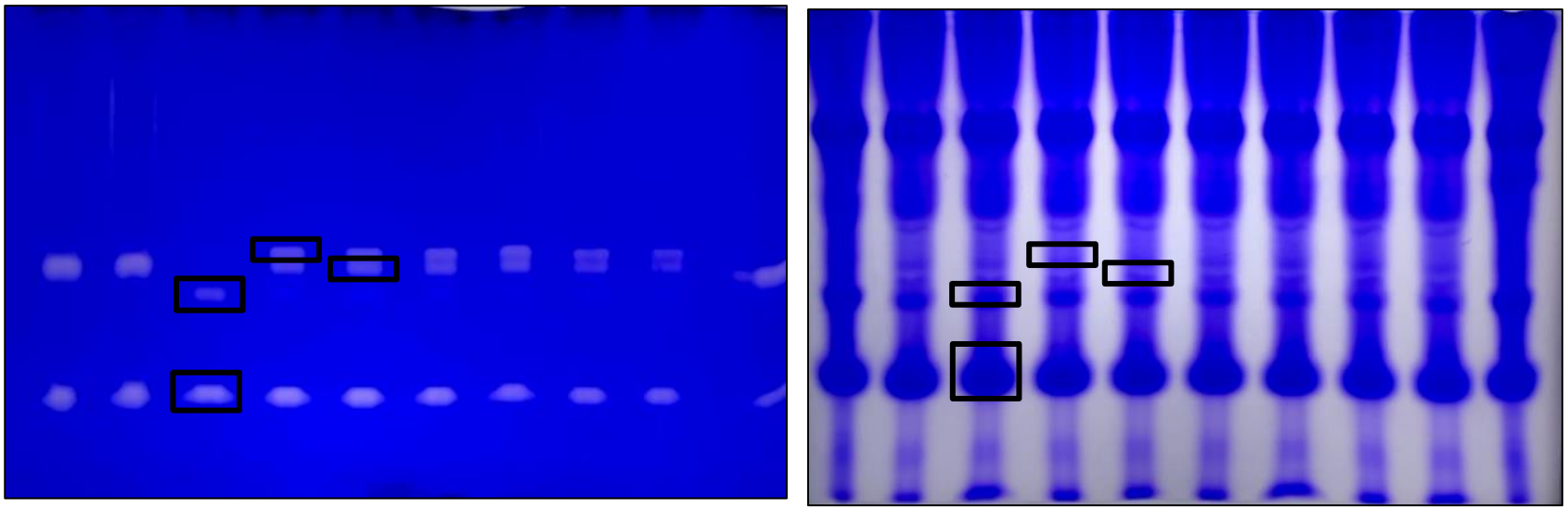

Figure 3.1:

Casein zymogram showing the unidentified 3rd band of calpain. The gel on the left contained casein. The bands shown are where calpain was active and denatured available protein. This served as the reference for the bands to be removed from the gel without casein. The gel on the right did not include casein. Various bands are indicated with black boxes on both gels.

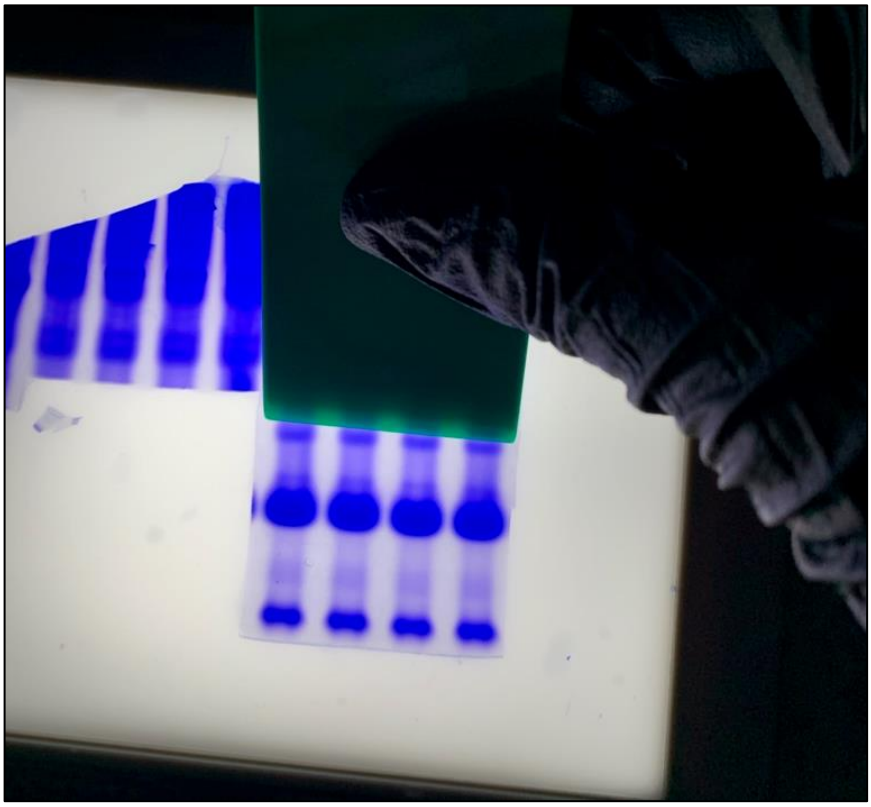

Figure 3.2:

Samples were run in replicate. Samples were removed from the gels in groups in an effort to increase calpain concentration for further analysis. The image above shows how a group of unidentified 3rd bands of calpain were removed from the gel for further analysis.

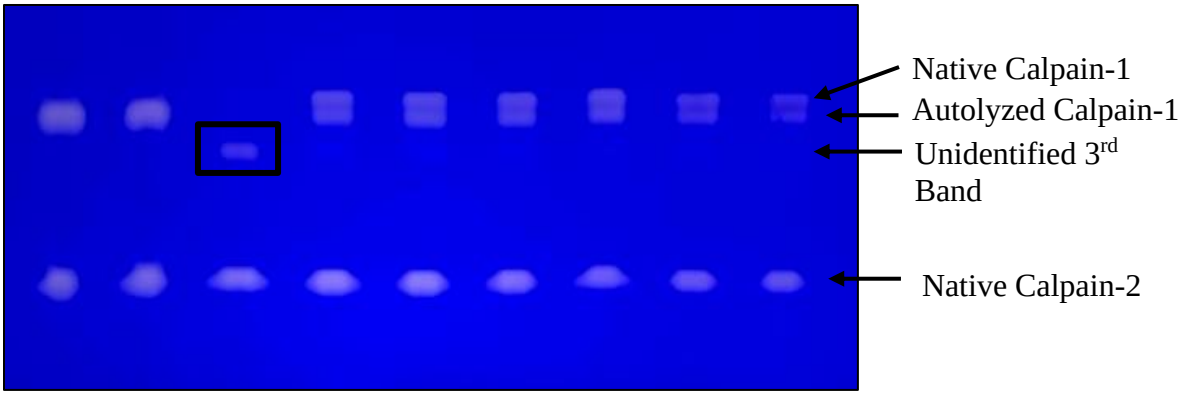

Figure 3.3:

The casein zymogram shows native calpain-1, autolyzed calpain-1, the unidentified 3rd band and native calpain-2. The unidentified third band is indicated with a label and black box.

Table 3.1: Estimated Mean Calpain Activity by Stimulation Treatment

	Stimulation Treatment					
	NS[1]-NS	NS-S	S[2]-NS	S-S	SEM	*P*-Value
Longissimus lumborum						
Calpain-1[3]	7.10	4.30	2.40	0.80	2.17	0.19
Autolyzed Calpain-1	6.25[a]	1.39[b]	1.33[b]	0.42[b]	1.49	0.03
Unidentified 3rd Band	0.83	1.17	1.24	1.12	0.58	0.94
Semimembranosus						
Calpain-1	4.96	7.87	6.70	6.77	4.21	0.94
Autolyzed Calpain-1	1.85	8.50	5.55	10.31	5.22	0.60
Unidentified 3rd Band	4.54	4.52	5.78	3.46	2.15	0.83

[a-b]Means within a row within a muscle group without a common superscript differ ($P < 0.05$)

[1]Not Stimulated

[2]Stimulated (21 V for 20 seconds)

[3]Values are percentages of Calpain-1 from day zero, *sternocephalicus* samples.

Table 3.2: Estimated Mean Calpain Activity by Aging Period

	Day of Aging				
	1	4	14	SEM	*P*-Value
Longissimus lumborum					
Native Calpain-1[1]	10.21[a]	0.67[b]	0.00[b]	1.82	<0.01
Autolyzed Calpain-1	5.54[a]	1.51[b]	0.00[b]	1.08	<0.01
Unidentified 3rd Band	2.49[a]	0.78[b]	0.00[b]	0.43	<0.01
Semimembranosus					
Native Calpain-1	14.91[a]	0.00[b]	3.38[b]	2.15	<0.01
Autolyzed Calpain-1	18.88[a]	0.76[b]	0.01[b]	4.32	<0.01
Unidentified 3rd Band	9.26[a]	3.76[b]	0.70[b]	2.83	<0.01

Means within a row within a muscle group without a common superscript differ ($P < 0.05$)

[1]Values are percentages of Calpain-1 from day zero, *sternocephalicus* samples.

Appendix A: Exempt certification for IRB project number 19-181

University of Idaho
Office of Research Assurances
Institutional Review Board
875 Perimeter Drive, MS 3010
Moscow, ID 83844-3010
Phone: 208-885-6162
Fax: 208-885-6014
Email: irb@uidaho.edu

To: Michael James Colle

From: University of Idaho Institutional Review Board

Approval Date: September 05, 2019

Title: Understanding the Role of Electrical Stimulation on the Activation of Calpain-2

Project: 19-181
Certified: Certified as exempt under category 6 at 45 CFR 46.104(d)(6).

Appendix B: Free Calcium Concentration Analysis

Adapted from Hopkins and Thompson (2001) with minor modifications.

276 Samples

Procedure:

1. Place samples (2g) stored at -80°C in a -20°C freezer at least 36 hours prior to calcium measurement.
2. Following equilibration to -20°C samples place samples in a refrigerator (4°C) for 20 min before finely dicing, placing on ice and then centrifuging (Sorvall RT1 Centrifuge, Thermo Scientific, Waltham, MA) at 20,000 g at 5°C for 40 min.
3. Mix 250 µl aliquots of the supernatant with 5 µl of Calcium Ion Strength Adjuster (Mettler Toledo, Woburn, MA).
4. Incubate in a water bath at room temperature for 5 min.
5. Use a calcium selective electrode (PerfectION combination Ca^{2+}, Mettler Toledo, Woburn, MA) attached to a portable ion meter (Seven2Go pro, Mettler Toledo, Woburn, MA) to measure the calcium concentration.
6. Allowed the electrode to soak in a 1×10^{-2} calcium solution at least 1 h prior to calibration and reading samples.

Create a calibration curve prior to each run. Calcium standards containing 8000 µM, 2000 µM, 1000 µM, 500 µM, 100 µM and 50 µM calcium need to be made from Calcium ISE standard 1000 mg/l as Ca^{2+} (Mettler Toledo, Woburn, MA).

Appendix C: Calpain Extraction

Extraction buffer pH 8.3 (50ml)

Tris 0.6056g (100mM)

EDTA 0.1461g 10mM)

DTT 0.0772g (10mM)

Sample preparation

1. Homogenize 1g muscle in 3mL extraction buffer in 15 ml centrifuge tubes three times on ice for 15s with 15s cooling between bursts.
2. Pipet the homogenate into 1.7 ml microcentrifuge tubes. 1.0 ml/tube, 2 tubes per sample and centrifuge at 8,800xg for 30 min @ 4ºC
3. Pour supernatant into 1.7 ml microcentrifuge tubes and freeze in -80 freezer.

Appendix D: Casein Zymography

276 Samples

Solutions *Those containing DTT Make fresh Daily*

1.5M Tris base pH 8.8

18.15g/100ml H2O – bring up to 100mL w/ water

pH to 8.8 with HCl. Filter and store at 4ºC

0.5M Tris base pH 6.8

6g/100ml H2O – bring up to 100mL w/ water

pH to 6.8 with HCl. Filter and store at 4ºC

Stock acrylamide 30%

25 ml of 37.5:1

7.3g acrylamide

0.1948g bisacrylamide

Or (we will be using 75:1)

25 ml of 75:1

7.4013g acrylamide

0.0988g bisacrylamide

10% ammonium persulfate

1g/10ml H2O – bring up to 10mL w/ water

Store in dark bottle @ 4ºC

Water saturated butanol (60ml)

50ml n-Butanol and 10ml H2O

Sample buffer pH 6.8 (15 ml)

trisHCL 0.27255g (150mM)

glycerol 3ml (20%)

DTT 0.02315g (10mM)

Bromophenol blue (0.02%) 0.375ml of 0.8% bromophenol blue

Running buffer pH 8.3 (2 liter)

trisHCL 6.055g (25mM)

DTT 0.3086g (1mM)

Glycine 28.8g (192mM)

EDTA 0.5844 g (1mM)

Incubation buffer pH 7.5 (375ml)

Tris HCL 2.2707g (50 mM)

DTT 0.5784g (10mM)

CaCl2 0.1665g (4mM)

Procedure

Enough for 6 1 mm Gels**

Separating gel 12.5%

1.5M Tris ph 8.8	9.375 ml
30% Acrylamide	15.66 ml (75:1 or 37.5:1; 75:1 worked better in the trial round)
H2O	11.91 ml
Casein	0.75g
APS	187.5µl
Temed	18.75µl

Stacking gel 4%

0.5M Tris pH 6.8	2.82 ml
30% Acrylamide	1.5ml (75:1 or 37.5:1; 75:1 worked better in the trial round)
H2O	6.825ml

APS	75µl
Temed	11.25µl

1. The height of the separating gel is 5.5cm
2. Mix separating gel and degas 15 min. Add APS and TEMED and mix immediately before pouring gel (step 3)
3. Pour gel (5.5cm) overlay with water saturated butanol and allow to polymerize 1 hour
4. Make stacking gel and degas 15 minutes. Add APS and TEMED and mix immediately before stacker is to be poured (See step 5)
5. Pour off water saturated butanol and rinse well with distilled water. Remove any residual water with a kimwipe. Place comb in between plates. Pour the wells, allow to polymerize 30 min.
6. Remove comb and rinse wells with water, remove residual water with kimwipe.

Sample preparation

1. Thaw samples at room temp while pouring gels
2. Add 10µl of sample buffer to 40µl of supernatant

Running the gels

1. The casein minigels (1.0 mm) were run at 100V for 15min in an ice bath with running buffer before loading samples (the first gels in June 10µl was used in 0.75mm gels. We now need to use 20µl in 1.0mm gels)
2. Run gels at 100V for 5 hours in an ice water bath
3. Incubate in incubation buffer at room temp with slow shaking for 1 h (2 changes of buffer) followed by 16 h incubation in same buffer at room temp.
4. Rinse and stain gels for 1 hour in Coomassie Blue R250
5. Rinse and destain gels for 3 hours (longer if necessary) in Coomassie Blue R250 destaining solution
6. Remove, analyze using gel doc in biotech.
7. Dry using gel air drying frame and cellophane for 3h w/ no heat, 1h w/ heat.

Appendix E: Warner-Bratzler Shear Force

Prior to:

1. Thaw steaks for at least 24 hours prior to cooking 4° C (39° F).
2. Remove steaks from packaging.
3. Record raw weight.

Cooking:

1. Insert Cuisinart Grill (Cuisinart Griddler Deluxe Model GR-150) plates into the grill, flat side up.
2. Heat both the upper and lower plates to 400° and allow to preheat.
3. Place temperature probes into the geometric center of the steak.
    a. Place steak on flat surface.
    b. Insert the probe through into the center of the edge of the steak until the tip has reached the center of the steak. Be careful not to squeeze the edges or press on the top of the steak. This can cause the steak to become disproportionate and impact cooking/temp measurement.
4. Place steaks on grills and close the lid (should be able to do 2/grill, depending on steak size).
5. Remove steaks from the grill once they have reached the internal temperature of 65° C (149° F).
6. Keep temp probes in and record peak temperature (as steaks rest, the temperature will continue to rise. It should end at approximately 71° C (160° F))

Coring:

1. Allow steaks to cool to room temperature approximately 25° C (77° F).
2. Record cooked weight of each steak.
3. Remove at least 6 cores from each steak/location of focus running parallel to the muscle fibers.
4. Inspect cores to ensure no large veins of fat or connective tissue that could skew the reading.

Shearing:

1. Shear cores perpendicular to the muscle fibers.
2. Make sure to reset the reader between every sample to ensure accuracy.
3. Wipe off blade between samples to ensure no lingering tissue.
4. Shear at least 6 cores. If one reading is an apparent outlier (possibly due to connective tissue/fat) shear one more sample.
5. Record all shear values.

Appendix F: Consumer Sensory Panel Consent Form

CONSUMER EVALUATION OF BEEF QUALITY

1. The University of Idaho Institutional Review Board has reviewed and found this study to be exempt.
2. The objective of this study is to evaluate the effects of electrical stimulation of beef on beef quality. The samples will be prepared under the Research Guidelines for Cookery, Sensory Evaluation and Instrument Tenderness Measurements of Fresh Meat, as outlined by the American Meat Science Association.
3. You will be asked to evaluate 5-6 samples (approximately 1" x ½" x ½") per session for acceptability (1 = dislike extremely to 10 = like extremely), tenderness (1 = extremely tough to 10 = extremely tender), juiciness (1 = dry to 10 = juicy) and flavor (1 = bland to 10 = intense) using a 10 point scale. It is not necessary that samples be ingested. The study should take approximately 15 to 20 minutes.
4. Although there are no or minimal risks associated with the project, it is possible that some samples will have one or more qualities that may not be appealing to you (e.g. flavor, tenderness, or juiciness that is less than you would prefer).
5. With your help, society can benefit from our attempt to improve the understanding of beef tenderness.
6. We anticipate that samples will be well received by panelists. However, if we find during the course of the taste panel that samples are unappealing, we will stop the evaluation process.
7. To maintain anonymity of the data collected during this evaluation, all the information you provide will be placed in a locked file with Dr. Colle.
8. If you have questions about the taste panel, you can ask the investigator during the evaluation, when the evaluation is complete or at a time you feel is appropriate.
9. Contact information for the University of Idaho faculty member leading this research:

   Dr. Michael Colle
   University of Idaho
   Department of Animal and Veterinary Science
   Moscow, ID 83844
   208-885-6007
10. During the course of this taste panel, you may terminate participation at any time. If you choose to do so, please notify the investigator that you no longer wish to participate.
11. If you choose to terminate participation in this evaluation, there will be no penalties associated with your withdrawal.

I have reviewed this consent form and understand and agree to its contents.

Participant Name: _____ Date: _____

Signature: _____

Appendix G: Consumer Sensory Panel Demographic Questionnaire

CONSUMER EVALUATION OF BEEF QUALITY

Panelist #: _____ Date: _____

Age: _____ Gender: _____

Please indicate the number of meals a week in which you consume beef:

0-1 2-4 5-7 8+

Please indicate the form in which you most commonly consume beef:

Ground Roast Steak Other

Appendix H: Consumer Sensory Panel Questionnaire

BEEF CONSUMER SENSORY PANEL QUESTIONNAIRE

Sample ID #:_____

1. **OVERALL ACCEPTABILITY OF SAMPLE:** This is based on your overall acceptability of the sample

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

(Dislike extremely) (Like extremely)

2. **TENDERNESS:** This is based on your overall opinion of the sample's tenderness

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

(Dislike extremely) (Like extremely)

3. **JUICINESS:** This is based on your overall opinion of the sample's juiciness

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

(Dislike extremely) (Like extremely)

4. **FLAVOR:** This is based on your overall opinion of the sample's flavor

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

(Dislike extremely) (Like extremely)

5. **OFF-FLAVOR:** This is based on your ability to detect an off-flavor of the sample

NO YES

6. CONSUMER SATISFACTION: Would you be willing to purchase this product?

NO YES

7. IF APPLICABLE, please circle the trait you liked **least** about this product.

Flavor Tenderness Juiciness Texture/Mouth Feel

8. IF APPLICABLE, please circle the trait you liked **most** about this product.

Flavor Tenderness Juiciness Texture/Mouth Feel

9. Overall Comments on Product:

Thank you for taking the time to participate in this sensory panel

Appendix I: Proteomic Analysis of Calpain Polyacrylamide Electrophoresis Gel Bands

Polyacrylamide electrophoresis gel bands that were stained with Coomassie brilliant blue R-250 were received and prepared for Liquid Chromatography-Mass Spectrometry (LC-MS) by doing in gel trypsin digestion. This is a multistep process contained in the attached protocol. To briefly summarize, the gel bands were cut into small cubes about 1 mm on a side using a scalpel and returned to the microcentrifuge that they arrived in. The pieces were destained with the usual Coomassie destain, reduced with dithiothreitol (to reduce disulfide bonds), alkylated with iodoacetamide (to alkylate cysteine residues and prevent reformation of disulfide bonds) and digested overnight with trypsin. The resulting peptides were extracted from the gel pieces with aqueous acetonitrile, dried in a centrifugal concentrator (Speedvac) and redissolved in LC-MS sample solution (5% ACN, 95% water, & 0.1% formic acid).

LC-MS-MS analysis of the peptides was done using a Waters Nanoaquity UPLC interfaced with a Waters Q-Tof Premier quadrupole-time of flight mass spectrometer. One or two microliters of sample were injected and peptides were accumulated onto a 100 µm x 20 mm C18 trap column (Waters P/N 186007496) for 3 min at 8 uL/min with 100% solvent A. After trapping, peptides were desorbed from the trap column and separated on a 100 µm x 100mm BEH C18 analytical column (Waters P/N 186007485) using a gradient starting at 97% solvent A (water with 0.1% formic acid) and 3% solvent B (acetonitrile with 0.1% formic acid) at a flow rate of 0.4 uL/min. The solvent B composition was increased using the steps summarized in the table below to elute the peptides.

Gradient Table

Time	%A	%B
0	97	3
1	97	3
11	87	13
21	75	25
31	61	39
41	45	55
51	25	75
56	10	90
61	10	90
71	97	3
90	97	3

The mass spectrometer was programmed to acquire ms/ms spectra of the eluting peptdies using either data dependent analysis (DDA) methods or Expression methods. The DDA methods detect multiply charged analytes (which are expected from trypsin digestion) in the eluent stream in an ms survey acquisition where the mass spec switches to ms/ms mode and acquires ms/ms spectra from those analytes having sufficiently high peak intensities. In the Expression method, data is acquired by alternately acquiring ms data with a low collision energy and elevated collision energy over the full mass spectrum range (50 to 1990 Da). In expression mode, the quadrupole operates in Rf only mode allowing all ions to pass (i.e. non-selective). The resulting data from both methods was analyzed using Protein Lynx Global Server (PLGS), ver. 2.4 (Waters Corporation) which reports protein and peptide matches from database searches. PLGS also outputs a peak list file (.pkl format) from the processed raw data that was used for database searching using the Mascot software which is available online using a NCBI nr database. The PLGS data was searched using a SwissProt database as well a custom database of 263 protein sequences downloaded from NCBI using the search terms “calpain” and “Bos Taurus”.

Protocol:

Reagents (All reagents prepared fresh).

1. Destain: 50% methanol/5% acetic acid in water.

2. 100 mM ammonium bicarb: 100 mM ammonium bicarbonate in distilled water: 0.158 g/20 ml.

3. 50 mM ammonium bicarb: 50 mM ammonium bicarbonate in distilled water.

4. Acetonitrile.

5. 10 mM DTT: 1.5 mg/mL in 100 mM ammonium bicarb.

6. 50 mM iodoacetamide: 10 mg/mL in 100 mM ammonium bicarb.

7. Trypsin solution (on ice): 20 ng/µL Promega sequencing grade modified trypsin in 50 mM ammonium bicarbonate.

8. Extraction solution: 5% formic acid in 50% acetonitrile.

9. Microfuge tubes. We use plain 1.5 ml tubes and low binding 0.65 ml. Rinse all tubes with water, ethanol, water, ethanol. It appears that thorough washing removes acetonitrile soluble material which forms a layer on aqueous solutions and interferes with evaporation.

Procedure:

When removing solution from gel pieces, microfuging does not seem to make any significant difference in the amount of liquid removed.

With faintly stained gel pieces, watch carefully to ensure that the gel piece stays in the tube and does not stick to a pipet tip.

When removing wash and alkylation solutions, using the same pipet tip does not seem to give cross contamination, although as a precaution, the tip is changed between different groups of samples. Use a fresh tip for each sample when removing peptides.

Day 1

1. Cut bands from gel as closely as possible. Divide into smaller pieces.

2. Destain the bands in 500 µL Destain overnight at room temperature.

Day 2

3. Remove Destain and replace with 200 µL Destain for 2 to 3 h.

4. Remove the Destain (discard) and dehydrate gel slices in 200 µL acetonitrile. Gel pieces should turn opaque-white within 5 minutes. If they don't, then remove acetonitrile and add another 200 µl of acetonitrile.

5. Remove acetonitrile (discard) and evaporate any residual acetonitrile in SpeedVac (2 to 3 min).

6. Reduce the gel pieces in 30-50 µL 10 mM DTT for 30 min. at RT.

7. Remove DTT solution.

8. Alkylate in 30-50 µL 50 mM iodoacetamide for 30 min. at RT.

9. Remove iodoacetamide solution.

10. Wash with 100 µL 100 mM ammonium bicarb for 10 min.

11. Remove wash.

12. Dehydrate gel slices in 200 µL acetonitrile. Gel pieces should turn opaque-white within 5 min. If they don't, then remove acetonitrile and add another 200 µl of acetonitrile.

13. Remove acetonitrile and rehydrate by swelling in 100 µL 100 mM ammonium bicarb for 10 min.

14. Remove ammonium bicarb.

15. Dehydrate gel slices in 200 µL acetonitrile.

16. Remove acetonitrile and add another 200 µL aliquot of acetonitrile.

17. Remove acetonitrile.

18. Dry gel pieces in SpeedVac (2 to 3 min).

19. Prepare trypsin. 20 µg of Promega trypsin in 1000 µL ice cold 50 mM ammonium bicarb (trypsin concentration = 20 ng/µL). Keep ice cold.

20. Add 30 to 50 µL of the trypsin solution to cover the gel pieces and incubate for 5 to 10 min on ice. Watch that gel pieces appear re-swollen. (The idea is to allow the trypsin to move into the gel but not begin digestion.)

21. Remove any excess trypsin solution and add 5-20 µl 50 mM ammonium bicarb. React overnight at 37 °C.

Day 3

22. Extract with 30µl 100 mM ammonium bicarb. Vortex. Incubate 10 min., microfuge and take off supernatant to a clean 0.5 mL microfuge tube.

23. Extract the peptides by adding 30 µL extraction solution. Incubate for 10 min and collect the extract to the same microfuge tube.

24. Repeat the extraction with a second aliquot of the extraction solution, combining the extracts in the microfuge tube.

25. Evaporate the sample to 20 µL (do not go to complete dryness). Commonly tubes are evaporated to 20 µl after 45 min, but at times may take much longer. To MS.

Analysis and methods prepared by Lee Deobald, Director of the University of Idaho Mass Spectrometry Core Lab.

Reference:

Shevchenko, M. Wilm, O. Vorm and M. Mann. 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Analytical Chemistry 68:850-858