IMPROVING SHELF-LIFE AND TENDERNESS OF BEEF DURING EXTENDED AGING

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Authorization to Submit Thesis

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Abstract

Extended aging (>28 days) leads to improved tenderness of the *semimembranosus* (SM), while *longissimus lumborum* (LL) tenderness does not improve past 14 days of aging. Unfortunately, extended aging reduces color stability, increases browning, and increases lipid oxidation. The objectives of this dissertation are to 1) determine the effect of 0.05% ascorbic acid and 0.1% rosemary extract on color stability, microbial growth, lipid oxidation, and consumer sensory acceptability of extended aged beef LL and SM, 2) determine the effect of extended aging on calpain-1 and -2 activity in beef LL and SM, and 3) determine the effect of calcium chloride injection or post rigor freezing on sarcoplasmic calcium concentrations and calpain-2 activity in beef LL and SM. Ascorbic acid and rosemary extract treatment increased the lightness but decreased the redness and yellowness of LL steaks. Additionally, treated LL steaks were less brown on day 4 of retail display. The antioxidant treatment did not affect microbial growth, lipid oxidation, or sensory flavor scores. Calpain-1 had minimal activity by day 4 postmortem and was not active following 14 days of aging. Native calpain-2 activity decreased, while autolyzed calpain-2 activity increased with longer aging. Free calcium levels in the calcium, frozen, and control steaks averaged 1256, 127, and 121µM for the LL and 1520, 120, and 111µM for the SM, respectively. LL native calpain-2 activity was lower in calcium and frozen steaks than control steaks, while SM native calpain-2 activity was lowest in calcium steaks and intermediate in frozen steaks. LL calcium steaks were more tender than control steaks. Therefore, ascorbic acid and rosemary extract led to minor improvements in shelf-life of extended aged beef. Both calpain-1 and calpain-2 may be responsible for the postmortem improvement of beef tenderness, with calpain-1 being responsible for the tenderness

improvement early postmortem and calpain-2 responsible for the improvement during extended aging. Calcium chloride injection and freezing are effective strategies to activate calpain-2 earlier postmortem even though free calcium concentration never reached >300μM in frozen steaks. In conclusion, manipulating calpain-2 to improve tenderness earlier postmortem is a more feasible strategy to improve product quality than extended aging.

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Dedication

This dissertation is dedicated to my fiancée, Megan, my parents and my brothers. They have motivated and pushed me to be the best that I can be.

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List of Abbreviations

AA	Ascorbic Acid
AMSA	American Meat Science Association
CA	Calcium Injected
CAF	Calcium-Activated Factor
CANP	Calcium-Activated Neutral Protease
CASF	Calcium-Activated Sarcoplasmic Factor
CAT	Calcium Activated Tenderization
CFU	Colony Forming Unit
CIE	Commission International de I'Eclairage
CO	Control
DEAE	Diethylaminoethyl
FR	Frozen
KAF	Kinase Activating Factor
IMPS	Institutional Meat Purchase Specifications
LD	*Longissimus dorsi*
LL	*Longissimus lumborum*
LSM	Least Squares Mean
MDA	Malondialdehyde
MMb	Metmyoglobin
MRA	Metmyoglobin Reducing Activity
NAMP	North American Meat Processors
OC	Oxygen Consumption Rate
OMb	Oxymyoglobin
RE	Rosemary Extract

SM	*Semimembranosus*
ST	*Semitendinosis*
TBARS	Thiobarbituric Acid Reactive Substances
USDA	United States Department of Agriculture
WBSF	Warner-Bratzler Shear Force
WHC	Water Holding Capacity

CHAPTER 1

Review of Literature

Introduction

The 2010/2011 National Beef Tenderness Survey revealed that aging times for beef retail subprimals in cold storage ranged from 1 to 358 days post-fabrication (Guelker et al., 2013). Aging beef improves tenderness (Bratcher et al., 2005; Colle et al., 2015; 2016; Dixon et al., 2012; Eilers et al., 1996; Gruber et al., 2006; Tatum et al., 1999) but reduces shelf-life of the product (Colle et al., 2015; 2016). Specifically, extended aging reduces shelf-life but has positive effects on consumer perception of tenderness of the strip loin, top sirloin, top round, and bottom round (Colle et al., 2015; 2016). The strip loin and top round represent extremes for both tenderness and color stability in response to extended aging (> 28 days) (Colle et al., 2015; 2016). Likewise, Phelps et al. (2016) found that eye of round tenderness improved up to 70 days of aging. These researchers did not evaluate shelf-life of extended aged steaks. Further research is needed to 1) determine a method to improve shelf-life of extended aged product, 2) better understand the role of calpain in meat tenderization, and 3) manipulate calpains to make meat more tender earlier postmortem.

Shelf-life

Shelf-life is the amount of time that food is stable and desirable (Doyle, 2007). Color, microbial growth, and lipid oxidation are all factors that affect shelf-life (Tarladgis et al., 1960; Kraft, 1986; Jensen et al., 2003; Mancini and Hunt, 2005; McKenna et al., 2005; Campo et al., 2006; AMSA, 2012; English et al., 2016). Faustman and Cassens (1990) note that zero oxygen consumption, maximum reducing capacity, zero lipid oxidation, sterility,

dark storage, oxygen-permeable and moisture-impermeable packaging, and low temperature storage are critical in maximizing color stability of fresh meat. Improving color stability and decreasing lipid oxidation results in improved shelf-life (Gómez et al. 2016). Recently, the beef industry has been focused on improving shelf-life and color stability of beef (Rogers et al. 2014).

Color

Color plays an important role in a consumer's decision when purchasing meat products (Luño et al., 2000; Mancini and Hunt, 2005; AMSA, 2012; English et al., 2016). Accordingly, discoloration results in meat being perceived as old and less wholesome by consumers (Faustman and Cassens, 1990), and therefore must be discounted or discarded (Suman et al., 2014). These discounts result in and economic loss to the United States beef industry of over $1 billion annually (Smith et al. 2000).

Myoglobin is the protein in meat that is largely responsible for meat color. The ligand bound at the 6th coordination site on myoglobin along with the oxidation state of the iron determines meat color (Figure 1.1) (Mancini and Hunt, 2005). Binding of oxygen to the 6th coordination site of myoglobin results in the formation of oxymyoglobin and the meat being bright cherry red in color, while the absence of a ligand leads to the formation of deoxymyoglobin and the meat being purple in color (Faustman and Cassens, 1990). When carbon monoxide is bound the meat is bright red in color and called carboxymyoglobin (Mancini and Hunt, 2005). The oxidation of iron converts ferrous iron (+2) into ferric iron (+3) resulting in the formation of metmyoglobin which is brown in color (Faustman and Cassens, 1990). Consumers prefer beef to be red in color (Carpenter et al., 2001) and thus

the myoglobin in the oxymyoglobin state. Therefore, it is critical to prevent oxidation of myoglobin as well as increase the reducing activity in meat in order to improve the color stability and shelf-life of the product.

Metmyoglobin reducing activity and oxygen consumption rate

Metmyoglobin reducing activity (MRA) and oxygen consumption rate (OC) are two main factors in determining color stability in meat (Ledward, 1985; Faustman and Cassens, 1990; Mckenna et al., 2005; English et al., 2016). McKenna et al. (2005) classified the *longissimus dorsi* (LD) and *semimembranosus* (SM) as "high" and "intermediate" color stability muscles, respectively. High color stability muscles have greater MRA and lower OC (Mckenna et al., 2005). Madhavi and Carpenter (1993) observed that color stability decreased with a decrease in MRA. Likewise, Reddy and Carpenter (1991) noted that the muscles with the greatest MRA were the most color stable. Meanwhile, the ratio of MRA to OC may be the best determining factor of color stability (McKenna et al., 2005). English et al. (2016) found that MRA and OC decreased with increased aging time. Additionally, McKenna et al. (2005) noted that nitric oxide MRA decreased over retail display time in beef muscles. Extended aging and retail display time reduce MRA and therefore color stability.

Microbial growth

Microbial growth is one of the main causes of meat spoilage (Dave and Ghaly, 2011). Likewise, Lambert et al. (1991) noted that microbial growth is the most important factor affecting shelf-life of meat. Slime formation, off odors, and a change in color are indicators of microbial growth (Lambert et al., 1991; Dave and Ghaly, 2011). Aerobic and

anaerobic microbial growth increases during 28 days of vacuum packaged storage of wholesale beef cuts (Hodges et al., 1974; Wicklund et al., 2005). Additional, Seideman et al. (1976) noted the brighter surface color observed with longer aging periods of the knuckle may be due to lactic acid production by *lactobacilli*. It is important to note that meat spoils somewhere around 10^6 CFU/cm^2 (Jensen et al., 2003) or 10^7 colony forming units (CFU)/g (Kraft, 1986). Even after 63 days of aging and 4 days of retail display no steaks reach the spoilage point of 10^6 CFU/cm^2 (Colle et al., 2015; 2016). Storing meat at cooler temps, in the dark, or using antioxidants can help to reduce microbial growth (Dave and Ghaly, 2011). Additionally, vacuum packaging and 100% CO_2 modified atmosphere packaging decrease microbial growth and therefore improve shelf-life (Borch et al., 1996). Minimizing microbial growth results in improved shelf-life, safety, and overall product quality.

Lipid Oxidation

Lipid oxidation is another factor involved in meat spoilage (Dave and Ghaly, 2011). Autoxidation of lipids results in the development of free radicals leading to myoglobin oxidation and off flavors (Dave and Ghaly, 2011). Lipid oxidation and myoglobin oxidation are closely related (Faustman and Cassens, 1990). An increase in lipid oxidation and at the same time an increase in myoglobin oxidation likely plays a role in discoloration of steaks during retail display. Besides affecting color, lipid oxidation also affects product flavor (Dave and Ghaly, 2011). Lipid oxidation is commonly measured using the Thiobarbituric Acid Reactive Substances (TBARS) assay (Dave and Ghaly, 2011). Lipid oxidation increases with aging and retail display time (Colle et al., 2015; 2016). Similarly, McKenna et al. (2005) and Campo et al. (2006) observed an increased in lipid oxidation with longer retail display times. There is a wide range of reported TBARS values for the threshold of

when off flavors are detected (Tarladgis et al., 1960; McKenna et al., 2005; Campo et al., 2006). McKenna et al. (2005) noted that off flavors in beef can be detected when TBARS values reach 1.0 mg malondialdehyde (MDA)/kg meat. Another study found that 2.3 mg MDA/kg meat is the threshold for lipid oxidation detection (Campo et al., 2006). Even though lipid oxidation increases with aging and retail display time, Colle et al. (2015; 2016) noted that LL and SM steaks aged for up to 63 days plus 4 days of retail display did not reach the 1.0 mg MDA/kg meat threshold. Antioxidants are one strategy to decrease lipid oxidation (Rojas and Brewer, 2007). Reducing lipid oxidation is important to producing a high quality meat product.

Using antioxidants to improve shelf-life

Treating steaks with a natural antioxidant prior to retail display could improve the color stability and shelf-life of extended aged beef without negatively affecting the positive sensory attributes of extended aged beef. Antioxidants inhibit lipid oxidation by removing free radical catalysts (Dave and Ghaly, 2011). Ascorbic acid is a reducing and chelating agent that prevents myoglobin oxidation (Ahn et al., 2004; Brewer, 2008). Djenane et al. (2003) found that LD steaks sprayed with a 0.1% rosemary/0.05% ascorbic acid solution significantly delayed the decrease in Commission International de l'Eclairage (CIE) a* values (redness) compared untreated steaks. Furthermore, they observed a decrease in lipid oxidation, myoglobin oxidation, as well as less metmyoglobin formation in steaks treated with the antioxidant solution. Ahn et al. (2007) found that rosemary reduced lipid oxidation of cooked ground beef after 9 days of storage. SM steaks injected with a sodium ascorbate solution resulted in improved retail display lean color stability (Wheeler et al., 1996). Additionally, with longer retail display times the treated steaks were redder (higher a*

value) and had less surface discoloration (Wheeler et al., 1996). Interestingly, at low levels, ascorbic acid acts as a prooxidant (Buettner and Jurkiewicz, 1996; Wheeler et al., 1996). Ascorbic acid acts in this manner by reducing ferric iron to ferrous iron, the latter being a pro-oxidant (Buettner and Jurkiewicz, 1996). Natural antioxidants have the potential to improve shelf-life of extended aged beef.

History of the calpain system

Guroff (1964) was the first to note the finding of a neutral, calcium-activated proteolytic enzyme. The enzyme was located in the soluble portion of rat brain and found to breakdown casein. Furthermore, the author noted that mercaptoethanol activated this enzyme, while heavy metals inhibited the enzyme. They noted that in small amounts EDTA stimulates the enzyme by chelating these heavy metals. Additionally, Guroff (1964) found the optimum pH of the purified enzyme to be between 7.1 and 7.3.

Huston and Krebs, (1968) found a calcium-requiring proteolytic enzyme in rabbit skeletal muscle. This enzyme was referred to as kinase activating factor (KAF) due to its believed role in the activation of phosphorylase kinase by calcium (Huston and Krebs, 1968). Since KAF was found to be a proteolytic enzyme, the authors note it is unlikely that KAF plays a role in the activation of phosphorylase kinase. EGTA was found to inhibit KAF, but the addition of calcium reversed the inhibition (Huston an Krebs, 1968). Davey and Gilbert (1969) noted that EDTA addition prevented the dissolution of myofibrils at the Z-lines likely by binding calcium ions.

Busch et al. (1972) discovered calcium-activated sarcoplasmic factor (CASF) which is an endogenous enzyme than can degrade the Z-line in meat. The optimum pH for CASF

activity is 7.0 and the factor requires 0.1 mM calcium for activation, which is a level higher than that in living muscles (Busch et al., 1972). However, the calcium released from the sarcoplasmic reticulum postmortem may be enough to activate CASF (Busch et al., 1972). Busch et al. (1972) noted that EGTA completely prevents the breakdown of Z-lines, while EDTA causes the Z-lines to broaden but still remain.

Reddy et al. (1975) and Dayton et al. (1976a and b) purified the same enzyme as Busch et al. (1972) from rabbit and porcine skeletal muscle, respectively. These authors referred to it as calcium-activated factor (CAF) or calcium-activated protease. The procedure used by Dayton et al. (1976a) created a 17,800-fold increase in specific activity of CAF. They found CAF consists of an 80,000 and 30,000-dalton subunit and there is 3.4µg CAF per g of muscle. Reddy noted optimal conditions for CAF activity were a pH 6.9 and 10mM of calcium while, Dayton et al. (1976b) found that the optimal conditions for CAF activity were a pH of 7.5, 1mM calcium, and at least 2mM 2-mercaptoethanol. On the other hand Mg^{2+}, Mn^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+} and iodoacetate inhibit CAF (Dayton et al., 1976b). Reville et al. (1976) found that CAF is not located within an organelle but is in direct contact with the cytoplasm and therefore intracellular calcium must regulate CAF activity to prevent continuous breakdown of myofibrillar proteins. Meanwhile, Dayton et al. (1975) noted that CAF degraded troponin, tropomyosin, and C-protein but did not degrade actin, myosin, or α-actinin. Additionally, the optimum pH of the calcium-activated enzyme in cardiac muscle is 7.0 to 8.0 which suggests the enzyme is nonlysosomal (Waxman and Krebs, 1978).

At this same time an inhibitor to CAF was being discovered (Waxman and Krebs, 1978; Nishiura et al., 1978). Waxman and Krebs (1978) discovered a ~270,000 mw

inhibitor of calcium-activated protease in bovine cardiac tissue. Nishiura et al. (1978) found a ~300,000 mw specific inhibitor of calcium-dependent protease in rat liver tissue and in unpublished data observed a comparable protease inhibitor in rat brain. The inhibitor interacts directly with the CAF and does not inhibit by chelating calcium (Waxman and Krebs, 1978; Nishiura et al., 1978). Additionally, in bovine cardiac muscle the inhibitor is present in much larger quantities than CAF (Waxman and Krebs, 1978). Nishiura et al. (1978) note the inhibitor is heat and acid stable and is likely ubiquitous just like calcium-dependent protease.

Later, Ishiura et al. (1980) referred to CAF as calcium-activated neutral protease (CANP). These authors found that approximately 4 percent of CANP was bound to myofibrils, mostly at the Z-line (Ishiura et al., 1980). Likewise, Slinde and Kryvi (1986) later noted that CANP's main target was the Z-line. Mellgren (1980) then identified two forms of the calcium dependent protease in canine cardiac muscle. One form required approximately 40μM for half maximal activity, while the other required 800μM (Mellgren, 1980). Still, both forms had an optimal ph of 7.0 (Mellgren, 1980). Goll et al. (1989) noted that both forms breakdown the same myofibrillar proteins.

Murachi et al. (1981) proposed to call the calcium activated proteolytic enzyme calpain, with 'cal' referring to calcium and 'pain' coming from thiol proteases including papain, clostripain, and bromlain, while the calpain inhibitor would be called calpastatin. The two types of calpain would be called calpain-1 and calpain-2 based on the order they are eluted from a DEAE-cellulose column at pH 7.5 (Murachi et al., 1981). Murachi et al. (1981) notes that only the calcium activated form of calpain can be inhibited by calpastatin. Furthermore, the authors note that the relative abundance of calpain to calpastatin differs

from one tissue to the next and also within a tissue based on sex, age, and stage of differentiation (Murachi et al. 1981).

Cong et al. (1989) then referred to the calcium dependent proteinases as µ- and m-calpain based on their micromolar and millimolar calcium requirements, respectively. Cong et al. (1989) noted the level of free calcium in the cell, 300-1200 nm, is much lower than the concentration required for activation of µ- or m-calpain.

As reviewed by Goll et al. (2003), the calpain system consists of µ-calpain, m-calpain, and calpastatin. These proteins are located intracellularly and largely associated with myofibrils in skeletal muscle. The calpains are calcium dependent, cysteine proteases, while calpastatin is a specific inhibitor of µ- and m-calpain. Both calpains are heterodimers consisting of a unique 80 kDa subunit and a common 28 kDa subunit. The calcium requirements for half maximal activity of µ- and m-calpain are 3-50 µM and 400 to 800 µM, respectively. Their optimum pH is 7.2 to 8.2. Both µ- and m-calpain undergo autolysis when incubated in the presence of calcium. Autolysis reduces the calcium requirement for half maximal activity of µ- and m-calpain to 0.5-2.0 µM and 50 to 150 µM, respectively, but does not affect either enzymes activity. The 80 kDa subunit of µ- and m-calpain is reduced to 76 kDa and 78 kDa, respectively. The common 28 kDa subunit is reduced to 18 kDa. (Goll et al., 2003)

A single gene encodes calpastatin in bovine (Goll et al., 2003). Calpastatin isoforms vary in molecular weight and range from 17.5 kDa to 87 kDa (Goll et al., 2003). These authors note calpastatin does require calcium to bind to calpains, but the amount required depends on the calpain. Doumit and Koohmaraie (1999) used a calpastatin antibody to

identify a ~130 kDa undegraded protein in lamb *longissimus* along with degradation products of ~100, 80, 65, 54, 32, and 29 kDa. These authors found µ-calpain, m-calpain, cathepsin B, proteosome, trypsin, and chymotrypsin degrade calpastatin. However, only the calpastatin degradation products from µ-calpain and m-calpain digestion resembled those in postmortem lamb *longissimus.* Interestingly, calpastatin loses its activity rapidly during frozen storage while calpains remain stable throughout frozen storage (Koohmaraie, 1990).

Based on Campbell and Davies's (2012) recommendation, throughout the remaining portion of this dissertation µ-calpain will be referred to as calpain-1 and m-calpain will be referred to as calpain-2. This reduces confusion since both calpain-1 and -2 require micro molar levels (3-50 and 400-800, respectively) of calcium for activation (Campbell and Davies, 2012).

Measurement of calpains

Guroff (1964) was the first to find and purify a neutral, calcium activated proteinase that ended up being calpain. The author purified the calpains by dialysis and then fractioned using a DEAE-cellulose column. The fractions were precipitated from the column by ammonium sulfate. Enzyme activity was determined by following the increase in acid soluble tyrosine resulting from the hydrolysis of casein after 20 minutes of incubation. One unit of calpain activity is the amount of calpain that resulted in an increase of 1 µM in tyrosine content. Therefore, an increase in acid soluble tyrosine meant an increase in calpain activity.

Dayton et al. (1975) noted column chromatography was utilized to purify calpains. The calpain crude fraction was purified through five columns: 6% agarose, DEAE-

Cellulose, Sephadex G-200, DEAE-cellulose, and Sephadex G-100. Dayton et al. (1976a) used three methods to determine the calpain activity of different fractions collected during purification. Each method used the same controls (no calcium control, calcium control, calpain no calcium control) along with the calpain calcium assay. First the authors used phase microscopy to follow the removal of Z-lines. The second assay used casein as a substrate to measure calpain activity. This assay measured the absorbance of the control or calpain solutions 30 min after the addition of the fractions. The degradation of casein resulted in an increase in absorption at 278 nm. The third method quantified the release of soluble materials from myofibrils caused by calpains. This material is soluble in KCl and absorbs at 278 nm. The authors noted that the results from the three methods were similar to one another and therefore they used the casein assay results throughout the paper.

Similarly, Koohmaraie (1990) utilized both hydrophobic and ion-exchange chromatography to determine calpain activity. For hydrophobic chromatography, samples were loaded onto a Phenyl-Sepharose column and then washed with equilibrating buffer to remove unabsorbed proteins. The calpains were then eluted off with an ethylene glycol, Tris, EDTA, and MCE buffer. For ion-exchange chromatography, samples were loaded onto a DEAE-Sephacel column and washed with equilibrating buffer until unabsorbed proteins were eluted. A continuous gradient of NaCl was used to elute the bound proteins (calpain-1, calpain-2, and calpastatin). Calpain activities were determined by using casein as a substrate. One unit of calpain activity is the amount of calpain that increases the absorbance by 1.0 at 278 nm in 60 min at 25°C. This procedure measures the caseinolytic activity or the extent of peptide removal from casein by calpain. The author noted that the ion-exchange chromatography was much better than hydrophobic chromatography.

More recently casein zymography has become a popular method for determining calpain activity (Raser et al., 1995; Veiseth et al., 2001; Geesink et al., 2006; Pomponio et al., 2008; Pomponio and Ertbjerg, 2012; Phelps et al., 2016). Casein zymography is able to differentiate between calpain-1 and -2 as well as the native and autolyzed forms of these enzymes (Veiseth et al., 2001; Geesink et al., 2006). Casein zymography utilizes nondenaturing polyacrylamide gels that contain casein (Raser et al., 1995). The calpain samples are run utilizing a Tris-glycine buffer that contains EGTA to stabilize calpains. Following the run, gels are incubated in a calcium containing buffer which activates the calpain enzymes leading to breakdown if the casein that has been incorporated into the gel. Where the calpains have degraded the casein clearing zones appear on the gel after staining (Figure 1.2). Calpain-2 migrates further than calpain-1 on the gel because calpain-2 has a greater net negative charge (Raser et al., 1995).

Calpains role in meat tenderization

A proteolytic system involved in the postmortem proteolysis of meat must be endogenous to skeletal muscle, located within the cell, mimic postmortem effects on myofibrils, and have access to myofibrils (Koohmaraie, 1988; 1992). Calcium dependent proteases (calpains) have these characteristics. Calpains are likely involved in the postmortem improvement of meat tenderness (Goll et al., 1983; Koohmaraie, 1988; 1992; Koohmaraie et al., 1995; Pringle et al., 1997; Goll et al 1998). A review by Goll et al. (1983) summarizes evidence for calpain involvement in improving tenderness; 1) muscle ultrastructure (degradation of Z-line, desmin, and titin) and contractile proteins (no degradation of myosin or actin and degradation of troponin T) are affected similarly by calpains and postmortem storage; 2) tenderness improves to a greater extent in muscles with

higher levels of calpains; 3) both calpain treatment and postmortem storage change the actin-myosin interaction by increasing the magnesium modified and calcium modified myofibril ATPase activity; 4) degradation of titin is dependent upon calcium, which is needed to activate calpains; and 5) freeze-dried muscle rehydrated with a saline solution containing calpains is more tender than when rehydrated with a solution without calpains. Furthermore, a review by Koohmaraie (1992) provides additional evidence for the role of the calcium dependent proteolytic system (calpains) in postmortem proteolysis; 1) incubating muscle tissue with calcium chloride accelerates proteolysis; 2) incubating muscle tissue with calcium chelators (EDTA and EGTA) inhibits proteolysis; 3) injecting carcasses with calcium chloride expedites proteolysis so aging of carcasses is no longer needed to ensure tenderness; 4) injecting carcasses with zinc chloride (calpain inhibitor) inhibits proteolysis; and 5) feeding β-andrenergic agonists to lambs reduces the calpain systems activity and therefore its ability to break down proteins which leads to tougher meat.

Aging effect on calpains

Aging has an effect on both calpain-1 and -2 in beef (Koohmaraie et al., 1987; Boehm et al., 1988). Koohmaraie et al. (1987) found that calpain-2 had 80.2% of its original activity after 14 days of storage, while calpain-1 and calpastatin lose their activity rapidly. These authors note calpastatin had 20.7% of its original activity after 24 hours and almost no activity after 6 days postmortem. Similar to Koohmaraie et al. (1987), Boehm et al. (1998) found that on day 1 postmortem calpain-1 and -2 had 20 and 83 percent of the day 0 value, respectively, while on day 7 postmortem calpain-1 and -2 had less than 4 and 63 percent of the day 0 value, respectively. Interestingly, Phelps et al. (2016) found that in the beef *semitendinosus* (ST) muscle calpain-1 activity decreased with extended aging and was

detected up to 42 days of aging, while calpain-2 activity decreased from day 42 to 70 of aging. Additionally, Boehm et al. (1998) found the calpastatin activity on day 1 and 7 ranged from 17 to 67 percent and 15 to 38 percent of the day 0 value, respectively. Since myofibrillar fragmentation increases to a large extent during the first 24 hours postmortem and calpain-1 activity but not calpain-2 activity changes during that time, only calpain-1 may be responsible for the improvement in tenderness postmortem (Koohmaraie et al., 1987). Koohmaraie et al. (1987) also note both calpains undergo autolysis in vitro with ample calcium available for activation, unfortunately, early postmortem calcium concentrations are too low (~10 μM) to activate calpain-2. Even with longer postmortem storage, free calcium concentrations may not be ample to activate calpain-2 (Koohmaraie et al., 1987). Therefore, it is widely believed that calpain-1 is largely if not solely responsible for the postmortem tenderization of beef (Koohmaraie et al., 1987; Geesink et al., 2006; Koohmaraie and Geesink, 2006).

In 75 pigs, Pomponio et al. (2008) found that at 3 days postmortem calpain-2 autolysis was observed in 37% of LD and 80% of SM muscles. The native calpain-2 activity was not different between the two muscles, while the autolyzed calpain-2 was greater in the SM (Pomponio et al., 2008). These authors also found LD calpain-2 activity decreased from 100% on day 1 postmortem to 61% on day 6 postmortem. In a separate study, Pomponio and Etbjerg (2012) found that incubation of calpain-1 and calpain-2 for longer times and at higher temperatures led to increased autolysis. Autolysis of calpain-2 started later and happened at slower rate than autolysis of calpain-1, furthermore calpastatin activity decreased to a greater extent with longer incubation time and higher temperature

(Pomponio and Etbjerg, 2012). Calpain-2 seems to be activated earlier postmortem in pork than in beef.

Other management factors affecting calpains

Besides aging, muscle location, castration, and the callipyge mutation may affect calpain activity (Morgan et al., 1993; Koohmaraie et al., 1995; Phelps et al., 2016). Phelps et al. (2016) found that location within the ST did not affect calpain-1 or -2 activities. Additionally, Morgan et al. (1993) found that calpain-1 and -2 activity is not affected by castration but calpastatin activity is greater in meat from intact male cattle. Furthermore, Koohmaraie et al. (1995) found that immediately after slaughter callipyge lambs had higher calpastatin and calpain-2 activities than normal lambs, while calpain-1 activity was not different between callipyge and normal lambs. Additionally, callipyge lambs had greater calpastatin activity than normal lambs following 7 and 21 days of aging (Koohmaraie et al., 1995). This increase in calpastatin activity reduces postmortem proteolysis by calpain-1 and -2 during aging (Koohmaraie et al., 1995).

Calcium

Calpains require calcium for activation. Calpain-2 has been shown to require 400 to 800 μM calcium for half maximal activity, while autolyzed calpain-2 requires 50 to 150 μM calcium for half maximal activity (Goll et al., 2003). Initial postmortem free calcium concentrations are too low (~10 μM) to activate calpain-2 (Koohmaraie et al., 1987). Free calcium concentrations increase initially postmortem due to the loss of the sarcoplasmic reticulum's ability to uptake calcium (Greaser et al., 1969). However, even with longer postmortem storage, free calcium concentrations may not be ample to activate calpain-2

(Koohmaraie et al., 1987). Koohmaraie et al. (1987) noted free calcium concentrations were ~10µM early postmortem, while Ji and Takahashi (2006) found that initial postmortem free calcium concentration in beef *longissimus thoracis* muscle was 16 µM. By 4 days postmortem the free calcium reached maximum of 210 µM (Ji and Takahashi, 2006). Similarly, Geesink et al. (2001) found that the free calcium concentration in lamb *longissimus* muscle reached a maximum of 230 µM seven days postmortem. Meanwhile, Pomponio and Ertbjerg (2012) observed that calcium concentrations in porcine *longissimus* muscle remained constant at 440 µM from day 1 to 5 postmortem. Conversely, Parrish et al. (1981) found that free calcium concentrations ranged from 638.0 to 970.6 µM in tough and tender beef *longissimus* muscles aged for 10 to 14 days. Ji and Takahashi (2006) attributed the high free calcium concentrations reported by Parrish et al. (1981) to the release of bound calcium or the addition of calcium during preparation. Nevertheless, free calcium concentrations likely increase with longer aging due to the liberation of phospholipids from the sarcoplasmic reticulum allowing calcium to flow into the sarcoplasim (Ji and Takahashi, 2006). Since the free calcium is less than what is required to activate calpain-2, the calpain-2 activation may be due to calcium being concentrated near the calpain-2. Goll et al. (2003) noted that most of the calpain is located near the Z-line with less in the I-band and very small amounts in the A-band. Therefore, an increase in calcium concentration near the Z-line during aging may be enough to activate calpain-2.

Manipulation of calpains to improve tenderness

Calcium activated tenderization is one way to activate calpain-2 early postmortem (Koohmaraie, 1988; Pringle et al., 1999; Wheeler et al., 1992). Koohmaraie (1988) first suggested that infusing calcium chloride into carcasses would activate the calpains and

subsequently increase the rate of postmortem tenderization. Morgan et al. (1991) noted that calcium chloride injection increases the sarcoplasmic calcium concentration which then activates calpain and improves meat tenderness. Wheeler et al. (1992) found that calpain-2 activity was 47% less in calcium injected steaks than control steaks after 7 days of aging when beef LD was injected with a 0.3 M calcium chloride solution to 110% of the green weight on day 1 postmortem. The activation of calpain-2 by calcium chloride injection resulted in an improvement in LD tenderness (Wheeler et al., 1992). Likewise, Pringle et al. (1999) found that beef LD injected with a 2.2% calcium chloride solution to 105% of the green weight at 24 hours postmortem had 77% less calpain-2 activity than control steaks at 48 hours postmortem. Additionally, Lawrence et al. (2003) noted that calcium injection improved strip loin tenderness, while zinc chloride injection inhibited tenderization. Interestingly, Pringle et al. (1999) found calcium injection did not improve Warner-Bratzler shear force (WBSF) of the top round but did improve the tenderness of the strip loin and top sirloin. On the other hand, Wheeler et al. (1991) found hot-boned beef top round injected with a 0.3 M calcium chloride solution to 110% of the green weight had lower WBSF values than the control throughout aging. Interestingly, whether the postmortem calcium injection time was 2 or 14 days and the aging time after injection was 7 or 35 days, injected strip loins had greater sensory tenderness scores than control steaks aged for the same total time (Wheeler et al., 1997). Lawrence et al. (2003) showed that in calcium enhanced muscles calpains are the major contributor to the improved tenderness, while salting-in of calcium ions plays a minor role. Morgan et al. (1991) noted that calcium chloride injection eliminated the need for aging mature cow beef. Calcium injection is an effective strategy to activate calpains and thus improve tenderness earlier postmortem.

Freezing before aging may be another strategy to activate calpain-2 earlier postmortem. Wheeler et al. (1992) showed that freezing LD steaks for 7 days and then aging the steaks for 7 days did not change calpain-2 activity compared to steaks not frozen and aged for 7 days. These authors did however find that frozen steaks had a decreased WBSF value. Shanks et al. (2002) found that freezing steaks at -16°C resulted in a more tender product than steaks aged for the same time but never frozen. Crouse and Koohmaraie (1990) found that aging after being frozen at 30°C for 27 days led to improved WBSF values. Neither Shanks et al. (2002) or Crouse and Koohmaraie (1990) measured calpain activity. However, postmortem proteolysis may be enhanced by freezing meat before aging (Crouse and Koohmaraie (1990). Additionally, these authors attribute the increase in proteolysis to the fact that calpastatin activity but not calpain activity was lost during frozen storage. Aroeira et al. (2016) found that freezing did increase proteolysis and reduce shear in *Bos taurus* cattle on day 0 but not on days 2, 14, or 21 of aging. Freezing and thawing prior to calcium marination results in a greater improvement in tenderness than freezing or calcium injection alone (Whipple and Koohmaraie, 1992). Whipple and Koohmaraie (1992) note that freezing decreases calpastatin activity, while calcium activates calpains resulting in an additive effect on meat tenderness. Freezing may be an effective strategy to activate calpains and improve tenderness earlier postmortem.

Summary

Extended aging improves beef tenderness but decreases the shelf-life of the product. Determining a method to improve shelf-life of extended aged product, improving the understanding of the role of calpain in meat tenderization, and developing a method to

manipulate calpains to make meat more tender earlier postmortem are of utmost importance to improve beef quality.

Improving color stability, reducing lipid oxidation, and decreasing microbial growth would result in extended aged beef having increased shelf-life. Natural antioxidants are a potential solution to these issues. Improving the retail shelf-life of extended aged beef top round would provide the consumer with a very tender and extremely acceptable product that would cost much less than the strip loin. Additionally, this would provide retail stores more flexibility when selling product that been purposely or mistakenly aged for an extended period of time.

A better understanding of calpain activity during extended aging could result in a method that allowed meat to reach its maximum tenderness early postmortem. Activating calpain-2 earlier postmortem would result in a more rapid improvement in tenderness. Potential strategies to activate calpain-2 earlier include calcium chloride injection or freezing. Producing meat that would be tender early postmortem will prevent the need for using antioxidants in order to lengthen the shelf-life of extended aged beef. Additionally, this would result in more consistent and desirable beef eating experiences as well as increase the demand for beef.

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Figures

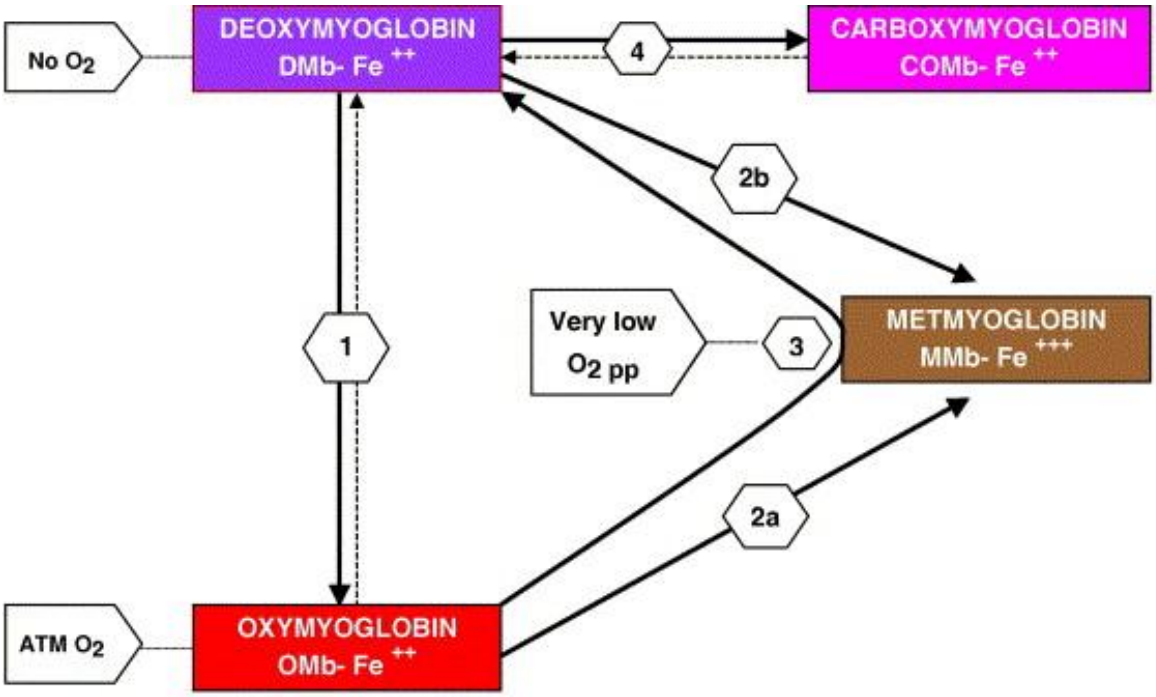

Figure 1.1: Meat color triangle. Myoglobin redox interconversions on the surface of fresh meat (Adapted from Mancini and Hunt, 2005).

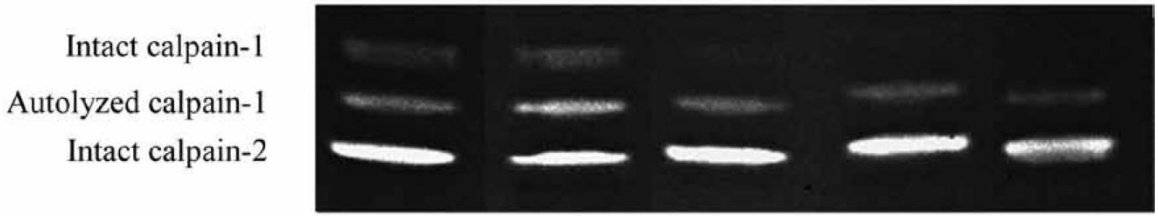

Figure 1.2: Casein zymogram example. The top, middle, and bottom rows of bands represent intact calpain-1, autolyzed calpain-1, and intact calpain-2, respectively (Adapted from Phelps et al., 2016).

CHAPTER 2

Effect of Ascorbic Acid and Rosemary Extract on Quality Characteristics and Sensory Perception of Extended Aged Beef

Abstract

Extended aging reduces shelf-life, but improves beef tenderness. This study analyzed the effect of ascorbic acid and rosemary extract on color, microbial growth, lipid oxidation, and sensory perception of beef *longissimus lumborum* (LL) (n=12) and *semimembranosus* (SM) (n=12) muscles wet aged at 0ºC for 14, 28, and 42 days. After aging, steaks were cut, sprayed with a 0.05% ascorbic acid + 0.1% rosemary extract solution (treated) or untreated (control), and subjected to retail display. Treated LL steaks had higher ($P < 0.05$) L* (lightness) values, but lower ($P < 0.05$) a* (redness) and b* (yellowness) values than control steaks. Additionally, treated LL steaks were less brown ($P < 0.05$) than control steaks on day 4 of retail display. Treatment did not affect microbial growth, lipid oxidation, or sensory flavor scores. As expected, aging reduced LL and SM color stability. The current study revealed slight improvements in color when using ascorbic acid and rosemary extract to improve aged beef shelf-life.

Introduction

The 2010/2011 National Beef Tenderness Survey revealed that aging times for beef retail subprimals in cold storage ranged from 1 to 358 days post-fabrication (Guelker et al., 2013). Aging beef improves tenderness (Bratcher et al., 2005; Colle et al., 2015; 2016; Dixon et al., 2012; Eilers et al., 1996; Gruber et al., 2006) but reduces color stability of the product (Colle et al., 2015; 2016). Color plays a critical role in a consumer's decision to

purchase a meat product (AMSA, 2012; Mancini and Hunt, 2005). Accordingly, discoloration causes meat to be perceived by consumers as old and less wholesome (Faustman and Cassens, 1990).

McKenna et al. (2005) categorized muscles as "high", "intermediate", "low", and "very low" color stability. These authors classified the *longissimus dorsi* (LD) and *semimembranosus* (SM) as "high" and "intermediate" color stability muscles, respectively. Our previous research demonstrated that these two muscles represent extremes for both tenderness and color stability in response to extended aging (> 28 days) (Colle et al., 2015; 2016).

The use of an antioxidant during retail display could improve color stability and therefore shelf-life of long aged product, while maintaining the positive sensory attributes associated with extended aged beef. Ascorbic acid is a reducing and chelating agent that prevents myoglobin oxidation (Ahn et al., 2004; Brewer, 2008). Djenane et al. (2003) found that LD steaks sprayed with a 0.1% rosemary/0.05% ascorbic acid solution significantly delayed the decrease in Commission International de l'Eclairage (CIE) a* values (redness) compared to untreated steaks. Furthermore, they observed a decrease in lipid oxidation, myoglobin oxidation, as well as less metmyoglobin formation in steaks treated with the antioxidant solution. Ahn et al. (2007) found that 1.0% rosemary lowered the thiobarbituric reactive substances (lipid oxidation) of cooked ground beef after 9 days of storage. SM steaks injected with a sodium ascorbate solution resulted in improved retail display lean color stability (Wheeler et al., 1996). Additionally, with longer retail display times the treated steaks were redder and had less surface discoloration (Wheeler et al., 1996).

The use of antioxidants, specifically ascorbic acid and rosemary extract, could increase the retail shelf-life of long aged *longissimus lumborum* (LL) and SM steaks by reducing lipid oxidation and improving color stability.

We examined the effects of these natural antioxidants on retail shelf-life of LL and SM steaks derived from USDA Choice carcasses. Our objectives were to 1) determine the effect of ascorbic acid and rosemary extract on color stability, microbial growth, and lipid oxidation during retail display of LL and SM steaks wet aged for 14, 28, and 42 days; and 2) examine the consumer acceptability of antioxidant treated LL and SM steaks subjected to extended aging. Antioxidant levels were determined from preliminary experiments using various levels of ascorbic acid and rosemary extract alone or in combination with one another.

Materials and Methods

Human subject participation in consumer panel

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

Product procurement

Carcasses were fabricated at 24 h postmortem. At 48 h postmortem, strip loin [Institutional Meat Purchase Specifications (IMPS) 180; NAMP, 2011] and top (inside) round (IMPS 168; NAMP, 2011) from USDA Choice carcasses (n = 12) were purchased from Washington Beef (Toppenish, WA) and transported to the University of Idaho Meat Science Laboratory at refrigeration temperatures. The drip and sales coolers at AB Foods

are set at 1.1 to 0°C and 2.8°C, respectively. Additionally, electrical stimulation occurs in two sections, the first is 26-29 volts and the second is 26 volts. It takes about one minute for a carcass side to go through the two sections.

Preparation of Product

The LL and SM were removed from their respective wholesale cuts for aging and subsequent analysis. The muscles were cut into three sections. Each section was assigned to one of the three aging periods (14, 28, and 42 days post-fabrication) so that muscle location was equally represented in each of the aging periods. Sections were vacuum shrink packaged (7 x 12 Durashrink bags, Winpak Films, Senoia, GA) and subsequently aged for the pre-determined time period at 0°C.

At the end of each aging period, designated sections were cut into four 2.54 cm-thick steaks. Two steaks were assigned to the untreated control group and the remaining two to the antioxidant treatment group. One steak in each group was assigned to shelf-life and tenderness analysis, while the other steak was designated for consumer acceptability. Preliminary experiments determined the optimum concentration of ascorbic acid (AA) and rosemary extract (RE) on retail color stability. Concentrations of 0.05%, 0.1%, and 0.4% AA; 0.05%, 0.1%, and 0.25% RE; 0.05% AA + 0.05% RE and 0.05% AA + 0.1% RE were utilized in the preliminary experiments. Steaks assigned to the treatment group were sprayed (~2ml) with the antioxidant (0.05% AA + 0.1% RE) solution, while control steaks were not sprayed. Retail display steaks were weighed, swabbed for microbial analysis, and then sampled for thiobarbituric acid reactive substances analysis, metmyoglobin reducing activity, and oxygen consumption. Steaks were then placed in white Styrofoam trays, and overwrapped with an oxygen permeable PVC film (#7500-3815, Koch Industries, Inc.,

Wichita, KS) with the freshly cut surface exposed to oxygen. Steaks were displayed in a glass-fronted retail display case (Model GDM-69, True Manufacturing Co., O'Fallon, MO) at 3°C for 4 days. The display case was equipped with natural white Hg 40W lights which were on throughout retail display, and the average light intensity was 408 lux.

Retail fluid loss

After steaks were treated and sampled, initial weights were recorded. Steaks were weighed following 4 days of retail display to determine percent retail fluid loss.

$$\text{Percent retail fluid loss} = \frac{\text{initial weight} - \text{final weight}}{\text{initial weight}} * 100$$

Retail color

Steaks were packaged and allowed to bloom for at least 60 min, then two objective color measurements per steak were taken using a spectrophotometer (MiniScan EZ, HunterLab, Restin, VA). This represented day 0 of retail display, and subsequent color measurements were taken on days 1, 2, 3, and 4. Large marbling flecks, connective tissue, and the product edge were avoided in the placement of the instrument. The spectrophotometer was equipped with a 25 mm-diameter measuring area and a 10° standard observer. The instrument was set to illuminant A and CIE L*, a*, and b* values were recorded. Calibration of the machine was carried out each day by measuring through the packaging film against black and white calibration tiles.

Steaks were evaluated daily during retail display for oxygenated lean color, amount of browning, discoloration, surface discoloration, and color uniformity by three evaluators following American Meat Science Association (AMSA) Meat Color Measurement

Guidelines (AMSA, 2012). To avoid potential effects due to display case location, steak positions were systematically rotated after each measurement.

Metmyoglobin reducing activity

Nitric oxide metmyoglobin reducing activity (MRA) was determined on days 0 and 4 of retail display for each of the three aging periods following the protocol provided in Section XI of the Meat Color Measurement Guidelines (AMSA, 2012) (Appendix B). The percentage of metmyoglobin (MMb) was calculated using the formula found in Tang et al. (2004). MRA was calculated as follows: $\text{MRA} = \left[\frac{\text{Initial \% MMb} - \text{Final \% MMb}}{\text{Initial \% MMb}}\right] \text{ x } 100$

Oxygen consumption

Oxygen consumption (OC) was determined on day 0 of retail display for each of the three aging periods following the protocol provided in Section XI of the Meat Color Measurement Guidelines (AMSA, 2012) (Appendix C). The percentage of oxymyoglobin (OMb) was calculated using the formula found in Tang et al. (2004).

$\text{OC} = \left[\frac{\text{Initial \% OMb} - \text{Ending \% OMb}}{\text{Initial \% OMb}}\right] \text{ x } 100$

Microbial growth

Each steak was dry swabbed (5 cm x 5 cm area) on days 0, 2, and 4 of retail display using 3M™ Quick Swabs (3M, St. Paul, MN). Letheen broth contained in the top of the swab was added. The samples were then diluted 1:100 using 3M Letheen broth (3M, St. Paul, MN), plated on 3M™ Petrifilm™ Aerobic Count Plates (3M, St. Paul, MN), and incubated at 35°C for 48 h for growth of mesophilic organisms. Colonies were counted

following the 3M Interpretation Guide (http://www.3m.com/intl/kr/microbiology/p_aerobic/use3.pdf; 3M, St. Paul, MN).

Lipid oxidation

Thiobarbituric acid reactive substances (TBARS) were analyzed on days 0, 2, and 4 of retail display following the protocol provided in Appendix O of the Meat Color Measurement Guidelines (AMSA, 2012) (Appendix D). The end (~1 cm) of the steak was discarded before samples were taken from the top half of the steak avoiding the steak edge. Samples were ~0.5 cm wide, ~2.0 cm long, and ~1.27 cm thick.

Cooking

Following retail display, steaks were cooked on open-hearth broilers (Model BG-16, DeLonghi, Upper Saddle River, NJ) to an internal temperature of 40°C, then flipped and cooked to a final internal temperature of 71°C. Temperature was monitored with hypodermic temperature probes (Omega Engineering Co., Stamford, CT) coupled with a 12-channel scanning thermocouple thermometer (Digi-Sense, Cole-Parmer Instrument Co., Vernon Hills, IL). Steaks were weighed before and after cooking to determine percent cook loss, and then refrigerated overnight for Warner-Bratzler shear force analysis (WBSF).

$$\text{Percent cook loss} = \frac{\text{precook weight} - \text{postcook weight}}{\text{precook weight}} * 100$$

Warner-Bratzler shear force

Following cooking, and refrigerated storage overnight at 3ºC, six cores (1.27-cm diameter) were mechanically removed parallel with the muscle fiber orientation using a drill press-mounted coring device, and shear force was determined by shearing each core (200

mm/min) perpendicular to the muscle fibers using a Warner-Bratzler shear machine (GR Manufacturing, Manhattan, KS).

Sensory analysis

Steaks designated for consumer acceptability were weighed and exposed to retail display conditions for 2 days, then reweighed, sampled for TBARS analysis, vacuum packaged and frozen at -20°C until completion of all aging periods. A separate consumer panel was conducted for each muscle. Consumer panels were conducted at the Washington State University Sensory Evaluation Facility. For consumer sensory analysis, steaks were thawed overnight at 4°C and subsequently cooked as described above. Samples were placed in insulated containers with hot packs to keep them warm until serving. Consumers (n = 60 per muscle) evaluated cooked steaks from designated treatments for overall acceptability, tenderness, juiciness, and flavor using a 9-point scale (9 = like extremely, extremely tender, extremely juicy, and like flavor extremely, respectively; 1= dislike extremely, not at all tender, extremely dry, and dislike flavor extremely, respectively) (Appendix G). Additionally, for each sample consumers were asked if they could detect an off flavor, if they would be willing to purchase the product, what trait (flavor, tenderness, juiciness, or texture/mouth feel) they liked the most, and what trait (flavor, tenderness, juiciness, or texture/mouth feel) they like the least. Five 1.27-cm x 1.27-cm x steak thickness cubes were obtained from each steak using a cutting die. Each consumer evaluated one cube from each of six steaks that represented all aging periods and treatments for a muscle.

Statistical analysis

A split-split-plot with repeated measures was utilized. Aging time was the whole plot factor, antioxidant treatment was the split-plot factor, and retail display time was the split-split-plot factor. Muscle served as a block. Data were analyzed using the Mixed Model procedure of the Statistical Analysis System (SAS Institute, Inc., Cary, NC). Muscle and muscle by age served as random variables, day of aging, day of retail display, treatment, and the interaction between day of aging, day of retail display, and treatment served as fixed variables. Color measurements, aerobic plate counts, and TBARS values were analyzed as repeated measures with a compound symmetry covariance structure. Aerobic Plate Counts were $\log_{10}$ transformed prior to analysis. Differences in least squares means (LSM) were compared by the DIFF option. P-values of ≤ 0.05 were considered statistically significant and P-values ≤ 0.10 were considered trends in the data.

Results

Antioxidant treatment did not affect LL or SM retail fluid loss ($P = 0.39$ and 0.16, respectively), cook time ($P = 0.74$ and 0.15, respectively), cooking loss ($P = 0.48$ and 0.23, respectively), or WBSF ($P = 0.71$ and 0.66, respectively) values (data not shown). Additionally, aging period did not affect LL or SM retail fluid loss, cook time, cooking loss, or WBSF values with the exception of SM retail fluid loss which decreased ($P < 0.05$) from day 14 to 28 of aging (Table 2.1).

Furthermore, aerobic plate counts for the LL ($P = 0.30$) and SM ($P = 0.17$) and lipid oxidation for the LL ($P = 0.15$) and SM ($P = 0.12$) were not affected by the antioxidant treatment (data not shown). However, an aging period by day of retail display interaction (*P*

< 0.05) was observed for microbial growth and lipid oxidation for both muscles (Table 2.2). Microbial growth and lipid oxidation of the LL and SM increased from days 14 to 28 of aging and then decreased from days 28 to 42 of aging, while both factors generally increased with retail display time.

Antioxidant treated LL steaks were lighter (higher L*), less red (lower a*), and less yellow (lower b*) than control steaks ($P < 0.05$) (Table 2.3). However, objective color measurements did not differ between SM treated and control steaks (data not shown). Longer aging periods and retail display times resulted in darker, less red, and less yellow LL and SM steaks ($P < 0.001$).

A treatment by day of retail display interaction ($P < 0.05$) was observed for LL amount of browning (Table 2.4). Antioxidant treated steaks were less brown than control steaks on day 4 of retail display. Additionally, antioxidant treated steaks tended to have brighter oxygenated lean color than control steaks for the LL ($P = 0.069$) and SM ($P = 0.061$) (Table 2.5). All other subjective color measurements did not differ between antioxidant treated and control steaks (data not shown). An aging period by day of retail display interaction ($P < 0.01$) was observed for all subjective color measurements for both muscles (data not shown). Steak color became less desirable with longer aging periods and retail display times.

A treatment by aging period interaction ($P < 0.05$) was observed for LL MRA (Figure 2.1). MRA of treated steaks was numerically less than control steaks on day 14 of aging, while after 28 and 42 days of aging treated steaks had a higher MRA than control steaks. Additionally, a day of retail display by aging period interaction ($P < 0.01$) was

observed for LL MRA (Figure 2.1). On day 0 of retail display, LL MRA decreased from day 28 to 42 of aging, while on day 4 of retail display MRA increased from day 14 to 28 of aging and then decreased from day 28 to 42 of aging.

A treatment by day of retail display interaction ($P < 0.01$) was also observed for SM MRA (Figure 2.2). MRA of antioxidant treated SM steaks was greater than control steaks on day 0 of retail display, while treated and control steaks did not differ on day 4 of retail display. Furthermore, SM MRA was higher ($P < 0.001$) following 28 days of aging than the other two aging periods.

Oxygen consumption was not affected by antioxidant treatment for either muscle (data not shown). However, LL OC decreased ($P < 0.05$) from 18.0% on day 14 of aging to 8.0% on day 42 of aging with OC on day 28 of aging intermediate at 12.3%. Additionally, SM OC remained constant (9.9% and 11.1%, respectively) from day 14 to 28 of aging and subsequently decreased ($P < 0.05$) to 5.2% on day 42 of aging.

Demographics of consumer panelists are shown in Table 2.6. An aging period by treatment interaction ($P < 0.05$) was observed for SM sensory tenderness and juiciness (Table 2.7). Additionally, there was a treatment by aging period interaction trend ($P = 0.052$) for SM acceptability as well as a day of aging trend ($P = 0.092$) for SM flavor (Table 2.7). For all traits antioxidant treatment was generally better for 14 day aged steaks and was the same or inferior at the remaining two aging times. The antioxidant treatment did not affect LL acceptability, flavor, tenderness, or juiciness (data not shown). Furthermore, aging period did not affect LL acceptability, flavor, tenderness, or juiciness (Table 2.7).

Consumer preferences can be found in Table 2.8. Interestingly, after 14 days of aging consumers' willingness to purchase SM antioxidant treated steaks was 14% greater than control steaks. Additionally, after 42 days of aging 23.7% of consumer panelists identified SM treated steaks as having an off flavor, while 43.1% of SM control steaks were identified as having an off flavor by panelists.

Discussion

Antioxidant treatment resulted in color improvements of LL steaks. On day 4 of retail display treated steaks had reduced browning compared to control steaks. Furthermore, LL treated steaks had higher L* values and tended to have brighter oxygenated lean color. SM treated steaks also tended to have brighter oxygenated lean color. The added water on the surface of the steak from the antioxidant treatment may have lead to increased light reflection and therefore a greater L* value and brighter oxygenated lean color. Unfortunately, the antioxidant treatment in the present study did not affect lipid oxidation or microbial growth of either muscle. The antioxidant treatment resulted in slight improvements in color stability of LL and SM steaks. Higher levels of the antioxidants and/or a different application method may result in larger improvements in color stability.

Ascorbic acid and rosemary extract have been shown by several studies to improve various shelf-life attributes of beef (Wheeler et al., 1996; Sánchez-Escalante et al., 2001; Djenane et al., 2003; Ahn et al., 2007). One potential reason for the minor improvements in the LL and SM shelf-life in the current study is that at low levels ascorbic acid acts as a prooxidant (Buettner and Jurkiewicz, 1996; Wheeler et al., 1996) by reducing ferric iron to ferrous iron, the latter being a pro-oxidant (Buettner and Jurkiewicz, 1996). At the same

levels of antioxidants used in this experiment, Djenane et al. (2003) found that antioxidant treated LD steaks significantly delayed the decrease in a* values (redness) compared untreated steaks. However, these authors used the antioxidant combination in meat packaged in modified atmospheric packaging that was already treated with lactic acid. Therefore, the antioxidant treatment likely had a synergistic affect with the lactic acid or packaging to improve redness during shelf-life.

Both MRA and OC generally decreased with longer aging periods and MRA decreased with retail display time. This was expected because metmyoglobin reductase decreases over time due to a lack of NADH being produced from the citric acid cycle (Sammel et al., 2002). English et al. (2016) also found that MRA and OC decreased with increased aging time. McKenna et al. (2005) similarly noted that nitric oxide MRA decreased over retail display time in beef muscles. The increased MRA in SM antioxidant treated steaks was intriguing since higher MRA values generally indicate improved color stability. Improved color stability should correspond with reduced lipid oxidation since myoglobin oxidation and lipid oxidation are closely related (Faustman and Cassens, 1990). Furthermore, lipid oxidation also affects product flavor (Dave and Ghaly, 2011).

The sensory panel results were critical to assessing the value of the treatment. SM antioxidant treated steaks were favored compared to control steaks on day 14 of aging, while control steaks were favored for the remaining aging periods. Fortunately, the antioxidant was effective in reducing off flavors in the SM after 42 days of aging. The increased MRA in SM antioxidant treated steaks could explain the reduced off flavors in the SM after 42 days of aging. However, this did not increase the willingness to purchase treated SM steaks aged for 42 days because tenderness and juiciness was not improved. However, for the LL

there was no antioxidant or aging period affect. An aging effect was not expected since a majority of USDA Choice LD aging response is completed by 14 days of aging (Gruber et al., 2006).

Conclusions

Although previous studies have shown substantial benefits of antioxidants on beef shelf-life, the current experiment show limited improvements in LL color attributes as well as SM flavor, and a few other effects of ascorbic acid and rosemary extract. Unfortunately, we do not know the background of these cattle including breed, gender, age, diet, and implant/growth promotant strategy, all of which could affect shelf-life characteristics. Additional research to find a more effective application strategy of these and other antioxidants is needed to improve the shelf-life of extended aged beef.

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Tables and Figures

Table 2.1: Characteristics of extended aged beef (n=12)

	Day of Aging			
	14	28	42	SEM
Percent retail fluid loss				
Longissimus lumborum	0.90	0.98	0.92	0.10
Semimembranosus	1.21[a]	0.90[b]	0.83[b]	0.06
Cook time[1]				
Longissimus lumborum	22	21	23	2
Semimembranosus	30	30	25	2
Percent cook loss				
Longissimus lumborum	22.7	23.1	24.7	1.1
Semimembranosus	28.8	27.8	25.9	1.3
WBSF (kg)				
Longissimus lumborum	2.25	2.28	2.12	0.08
Semimembranosus	3.16	3.16	2.73	0.15

[1]Minutes to 71°

[ab]Within a row, means without a common superscript differ ($P < 0.05$).

Table 2.2: Characteristics of extended aged beef (n=12)

	Day of Display	Day of Aging 14	28	42	SEM
Aerobic plate counts[1,2]					
Longissimus lumborum	0	2.8^{ax}	4.8^{by}	2.4^{x}	0.3
	2	2.4^{ax}	4.2^{bcy}	1.8^{x}	0.3
	4	3.6^{bx}	4.1^{cx}	2.1^{y}	0.3
Semimembranosus	0	1.9^{x}	2.6^{y}	2.5^{ay}	0.2
	2	1.8^{x}	2.8^{y}	0.3^{bz}	0.2
	4	1.5^{x}	3.1^{y}	0.8^{bz}	0.2
Lipid oxidation[3]					
Longissimus lumborum	0	0.73	0.85^{a}	0.78^{a}	0.12
	2	0.75^{x}	1.25^{by}	1.11^{by}	0.12
	4	0.89^{x}	1.40^{by}	1.16^{bz}	0.12
Semimembranosus	0	0.30^{a}	0.34^{a}	0.25^{a}	0.09
	2	0.29^{ax}	0.80^{by}	0.46^{bz}	0.09
	4	0.42^{bx}	0.72^{by}	0.74^{cy}	0.09

[1]Log_{10} colony-forming units/cm^2
[2]Samples were diluted 1:100 with 3M Letheen Broth and plates were estimated following the 3M Interpretation Guide
[3]mg malondialdehyde/kg meat
[ab]Within a column, trait, and muscle, means without a common superscript differ ($P < 0.05$).
[x-z]Within a row, means without a common superscript differ ($P < 0.05$).

Table 2.3: *Longissimus lumborum* objective color across all aging and retail display times (n=12)

	Antioxidant[1]	Control	SEM
L*	39.48[a]	38.48[b]	0.87
a*	30.25[a]	30.79[b]	0.24
b*	25.86[a]	26.37[b]	0.24

[1]0.05% ascorbic acid + 0.1% rosemary extract
[ab]Within a row, means without a common superscript differ ($P < 0.05$).

Table 2.4: *Longissimus lumborum* amount of browning[1] across all aging periods (n=12)

Day of display	Control	Antioxidant[2]	SEM
0	1.0[a]	1.0[a]	0.2
1	1.2[a]	1.2[a]	0.2
2	2.3[b]	2.4[b]	0.2
3	2.7[c]	2.8[c]	0.2
4	4.5[d]	3.9[e]	0.2

[1]1 = no evidence of browning, 2 = dull, 3 = grayish, 4 = brownish-gray, 5 = brown, 6 = dark brown

[2]0.05% ascorbic acid + 0.1% rosemary extract

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

Table 2.5: Oxygenated lean color[1] across all aging and retail display times (n=12)

	Antioxidant[2]	Control	SEM
Longissimus lumborum	3.64	3.76	0.17
Semimembranosus	4.43	4.52	0.23

[1]1 = extremely bright cherry-red, 2 = bright cherry-red, 3 = moderately bright cherry-red, 4 = slightly bright cherry-red, 5 = slightly dark cherry-red, 6 = moderately dark red, 7 = dark red, 8 = Extremely dark red

[2]0.05% ascorbic acid + 0.1% rosemary extract

Table 2.6: Demographics of consumer panelists (n=60/panel)

	Longissimus lumborum		*Semimembranosus*	
	n	%	*n*	%
Age				
18-19	2	3.4	2	3.4
20-29	27	45.8	36	61.1
30-39	10	16.8	8	13.5
40-49	7	11.8	4	6.8
50+	13	22.0	9	15.2
Gender				
Male	26	44.1	25	41.0
Female	33	55.9	36	59.0
Beef meals/wk[1]				
0 to 1	10	16.7	10	16.7
2 to 4	34	56.7	39	65.0
5 to 7	14	23.3	10	16.7
8+	2	3.3	1	1.6
Most consumed[2]				
Ground	40	61.5	38	60.3
Roast	5	7.7	3	4.8
Steak	19	29.3	22	34.9
Other	1	1.5	0	0.0

[1]Please indicate the number of meals a week in which you consume beef: 0-1, 2-4, 5-7, or 8+

[2]Please indicate the form in which you most commonly consume beef: ground, roast, steak, or other

Table 2.7: Sensory analysis by consumer panelists (n=60/panel)

	Day of Aging			
	14	28	42	SEM
Longissimus lumborum				
Acceptability[1]	6.1	6.2	5.8	0.2
Tenderness	5.9	6.2	6.4	0.2
Juiciness	5.3	5.6	5.5	0.2
Flavor	6.0	6.2	5.5	0.3
Semimembranosus				
Acceptability				
Control	5.0	5.5	5.1	0.3
Treatment[2]	5.6	5.1	4.9	0.3
Tenderness				
Control	4.6^{y}	5.5^{az}	5.4^{yz}	0.3
Treatment	5.2	4.6^{b}	5.0	0.3
Juiciness				
Control	4.4^{ay}	5.3^{az}	5.3^{az}	0.3
Treatment	5.3^{b}	4.6^{b}	4.6^{b}	0.3
Flavor	5.4	5.4	4.7	0.2

[1]Scale, 9 = like extremely, extremely tender, extremely juicy, and like flavor extremely, respectively; 1= dislike extremely, not at all tender, extremely dry, and dislike flavor extremely, respectively.

[2]0.05% ascorbic acid + 0.1% rosemary extract

[ab]Within a column and trait, means without a common superscript differ ($P < 0.05$).

[yz]Within a row, means without a common superscript differ ($P < 0.05$).

Table 2.8: Consumer panel preferences for the *longissimus lumborum* and *semimembranosus*

	Longissimus lumborum (n=12)						*Semimembranosus* (n=12)					
	Day of Aging						Day of Aging					
	14		28		42		14		28		42	
	Trt[1]	Control	Trt	Control	Trt	Control	Trt	Control	Trt	Control	Trt	Control
Like most[2]												
Flavor	43.6	28.3	33.3	33.9	32.7	22.4	36.9	50.0	49.0	47.1	34.0	22.0
Tenderness	23.6	23.9	37.0	32.1	40.0	34.5	29.2	18.5	17.6	18.9	34.0	30.0
Juiciness	16.4	32.6	22.2	17.9	14.5	29.3	26.2	20.4	19.6	18.9	19.1	42.0
Texture	16.4	15.2	7.4	16.1	12.7	13.8	7.7	11.1	13.7	15.1	12.8	6.0
Like least[3]												
Flavor	20.8	19.6	27.3	30.9	35.2	47.3	24.0	12.3	16.7	26.4	32.7	55.4
Tenderness	20.8	31.4	33.3	20.0	25.9	14.5	40.0	38.6	33.3	34.0	25.0	16.1
Juiciness	39.6	29.4	24.2	27.3	24.1	23.6	26.0	40.3	35.2	20.7	34.6	21.4
Texture	18.8	19.6	15.2	21.8	14.8	14.5	10.0	8.8	14.8	18.9	7.7	7.1
Off flavor[4]												
Yes	23.7	18.6	20.0	24.6	31.7	35.1	21.0	20.3	21.7	18.6	23.7	43.1
No	76.3	81.4	80.0	75.4	68.3	64.9	79.0	79.7	78.3	81.4	76.3	56.9
Purchase[5]												
Yes	69.0	74.1	63.3	69.5	60.7	72.4	62.1	48.3	45.9	58.6	48.3	44.8
No	31.0	25.9	36.7	30.5	39.3	27.6	37.9	51.7	54.1	41.4	51.7	55.2

[1]0.05% ascorbic acid + 0.1% rosemary extract

[2]Percentage of panelists that liked that attribute the most

[3]Percentage of panelists that liked that attribute the least

[4]Percentage of panelists that did or did not detect an off flavor

[5]Percentage of panelists willing to or not willing to purchase the product

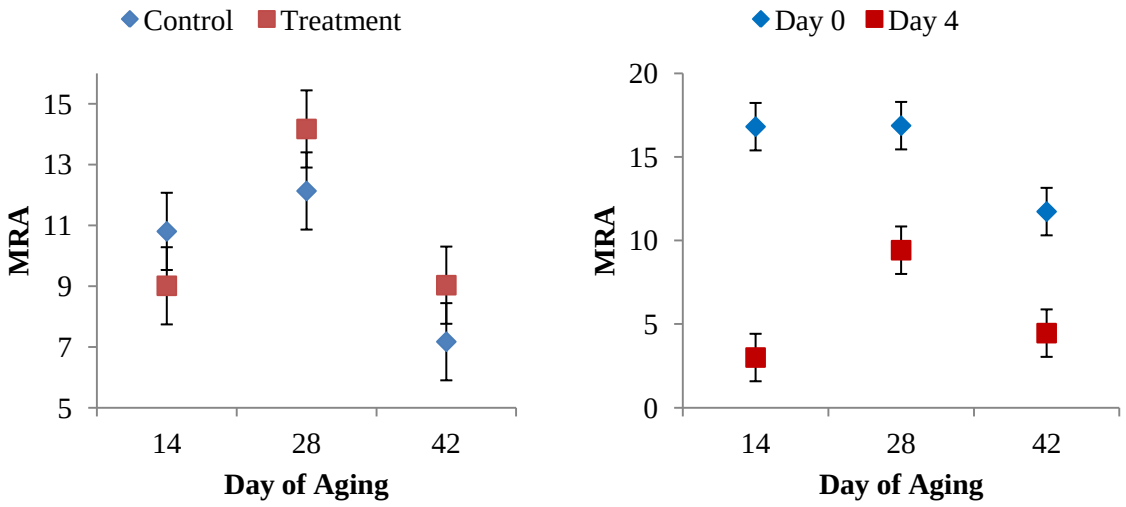

Figure 2.1: Metmyoglobin reducing activity (MRA) values for treatment x day of aging (left) and day of retail display x day of aging (right) for the *longissimus lumborum* (n=12). Product was aged for 14, 28, and 42 days post-fabrication. At the end of each aging period, 2.54 cm-thick steaks were cut from designated sections. One steak was assigned to the treatment group, while the other was assigned to the untreated control group. Steaks assigned to the treatment group were sprayed (~2ml) with the antioxidant (0.05% AA + 0.1% RE) solution, while control steaks were not sprayed. Steaks were then placed in white Styrofoam trays, overwrapped with an oxygen permeable PVC film, and displayed in a glass-fronted retail display case at 3°C for 4 days. MRA was determined on days 0 and 4 of retail display for each of the three aging periods with the following equation: MRA = [(Initial % metmyoglobin – Final % metmyoglobin) ÷ Initial % metmyoglobin] x 100. Values are shown as least square means ± SE.

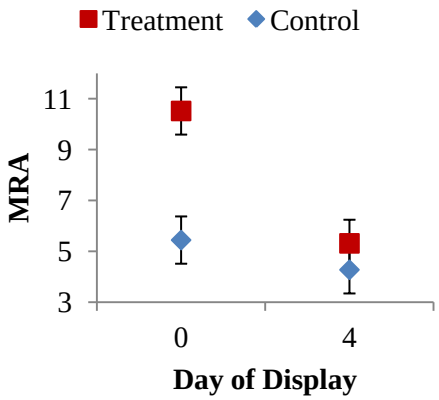

Figure 2.2: Metmyoglobin reducing activity (MRA) values for treatment by day of retail display for the *semimembranosus* (n=12). Data represented in the graph is combined from product aged for 14, 28, and 42 days post-fabrication. At the end of each aging period, 2.54 cm-thick steaks were cut from designated sections. One steak was assigned to the treatment group, while the other was assigned to the control group. Steaks assigned to the treatment group were sprayed (~2ml) with the antioxidant (0.05% AA + 0.1% RE) solution, while control steaks were not sprayed. Steaks were then placed in white Styrofoam trays, overwrapped with an oxygen permeable PVC film, and displayed in a glass-fronted retail display case at 3°C for 4 days. MRA was determined on days 0 and 4 of retail display for each of the three aging periods with the following equation: MRA = [(Initial % metmyoglobin – Final % metmyoglobin) ÷ Initial % metmyoglobin] x 100. Values are shown as least square means ± SE.

CHAPTER 3

Effect of Extended Aging on Calpain-1 and -2 in Beef *Longissimus Lumborum* and *Semimembranosus* Muscles

Abstract

Two experiments were conducted to determine the effect of extended aging on calpain activity in beef. In Exp. 1 our objective was to determine the effect of postmortem aging (2, 3, 4, 14, 28, and 42 days) on calpain-1 and -2 activities in beef *longissimus lumborum* (LL) and *semimembranosus* (SM) steaks. The objective of Exp. 2 was to determine the effect of postmortem aging (2, 3, 4, 14, 28, 42, 63, and 84) on calpain-2 activity in beef SM steaks. Calpain-1 was not active in either muscle following 14 days of aging. Native calpain-2 activity decreased ($P < 0.001$) with longer aging periods for both the LL and SM in Exp. 1 and for the SM in Exp. 2. Autolyzed calpain-2 activity increased ($P < 0.001$) with longer aging for the LL and SM in Exp. 1 and for the SM in Exp. 2. Our results indicate that calpain-1 is activated early postmortem, while calpain-2 is activated in most LL and SM by day 14 of aging and all muscles by day 28. Therefore, both calpain-1 and calpain-2 may contribute to the postmortem improvement of beef tenderness, with calpain-1 being responsible for the tenderness improvement early postmortem and calpain-2 responsible for additional tenderization during extended aging.

Introduction

The 2010/2011 National Beef Tenderness Survey revealed that post-fabrication aging times for subprimal cuts in cold storage facilities ranged from 1 to 358 days and 9 to 67 days for retail and foodservice subprimals, respectively (Guelker et al., 2013). We

recently found that consumer perception of *semimembranosus* (SM) tenderness was greater after 42 days of aging than after 14 days (Colle et al., 2016), while *longissimus lumborum* (LL) tenderness did not improve after 14 days of aging (Colle et al., 2015). Proteolysis of myofibrillar proteins during extended aging by calpain-2 may explain the improved tenderness of SM steaks.

Calpain proteases are enzymes that breakdown myofibrillar proteins. The calcium requirements for half maximal activity of calpain-1 and -2 are 3-50 µM and 400-800 µM, respectively (Goll et al., 2003). Both calpain-1 and -2 undergo autolysis which reduces the calcium requirement for half maximal activity of calpain-1 and -2 to 0.5-2.0 µM and 50-150 µM, respectively (Goll et al., 2003). Autolysis of calpains coincides with their activation, degradation of myofibrillar proteins, and subsequent improvement in tenderness (Geesink et al., 2006). Calpain-1 is believed to be largely if not solely responsible for the postmortem tenderization of beef (Koohmaraie et al., 1987; Geesink et al., 2006; Koohmaraie and Geesink, 2006). However, calpain-1 is activated early postmortem and by day 2 minimal calpain-1 activity is detected in beef LL and SM muscles (Camou et al., 2007).

Several studies have found that postmortem free calcium levels are too low to activate calpain-2 (Geesink and Koohmaraie, 1999; Veiseth et al., 2001; Ji and Takahashi, 2006). However, Parrish et al. (1981) found that in tough and tender beef *longissimus* muscles aged for 10 to 14 days, free calcium concentrations ranged from 638.0 to 970.6 µM. These concentrations would be high enough to activate calpain-2.

Our objectives were to (Exp 1) determine the effect of postmortem aging (2, 3, 4, 14, 28, and 42 days) on calpain-1 and -2 activity in beef *longissimus lumborum* (LL) and

semimembranosus (SM) steaks and (Exp 2) determine the effect of postmortem aging for two extended periods (63 and 84 days) on calpain-2 activity of beef *semimembranosus* steaks.

Materials and Methods

Product procurement

At 48 h postmortem, strip loin [Institutional Meat Purchase Specifications (IMPS) 180; NAMP, 2011] and top (inside) round (IMPS 168; NAMP, 2011) from USDA Choice carcasses (n = 12) were purchased from AB Foods (Toppenish, WA) and transported to the University of Idaho Meat Science Laboratory.

Preparation of product

The LL and SM were removed from their respective wholesale cuts and samples from the anterior portion of the LL and proximal portion of the SM were removed and vacuum packaged. Subsamples were removed and frozen in liquid nitrogen and stored at -75°C for calpain analysis on days 2, 3, 4, 14, 28, and 42 postmortem (Exp 1). In Exp 2 samples from the SM were frozen in liquid nitrogen and stored at -75°C for calpain analysis on days 2, 3, 4, 14, 28, 42, 63, and 84 days postmortem.

Calpain Extraction

One gram of each sample was placed in extraction buffer (3 mL)(100 mM Tris, 10 mM EDTA, 10 mM DTT, pH 8.3) and homogenized (POLYTRON® PT 10-35 GT; PT-DA 12/2EC-B154) at 12,000 rpm on ice 3 times for 15 sec with 15 sec cooling between bursts.

The homogenate was pipetted into two 2 mL microcentrifuge tubes. Samples were then centrifuged for 30 min at 8,800 x g at 4°C. The supernatant fluid was aliquoted and stored at -75°C until calpain analysis (Appendix H).

Casein Zymography

Calpain-1 and -2 activity was determined utilizing casein zymography as described by Pomponio et al. (2008) with minor modifications (Appendix I). A day 0 sample from the *sternocephalicus* was collected 10 minutes postmortem from a steer harvested at the University of Idaho Meat Science Lab. This sample served as the reference standard on each gel. Polyacrylamide gels (12.5%; 75:1 acrylamide to bisacrylamide) containing 0.2% casein were poured and overlaid with stacking gel (4%; 75:1 acrylamide to bisacrylamide) the day the gels were run. Gels (8 x 10 x 0.1 cm) were pre-run with running buffer (25 mM Tris, 1 mM DTT, 192 mM glycine, 1 mM EDTA, pH 8.3) at 100 V for 15 min in an ice bath before loading samples. Sample buffer (10 µL)(150 mM Tris, 20% glycerol, 10 mM DTT, 0.02% bromophenol blue, pH 6.8) was added to the supernatant fluid containing calpain extract (40 µL). Samples (20 µL) were loaded and the gels were run at 100 V for 6 hours in an ice water bath. Gels were then placed in incubation buffer (~60 mL; 50 mM Tris, 10 mM DTT, 4 mM calcium chloride, pH 7.5) at room temperature with slow shaking for 17 hours. Buffer was changed (~60 mL) at 30 minutes and (~130 mL) 60 minutes. Gels were stained in Coomassie Blue R-250 for 1 hour and destained in Coomassie Blue R-250 destaining solution for 3 hours. The clear bands indicating calpain activity were quantified by inverting the image and then comparing the density of each band to the reference standard on each gel utilizing a ChemiDoc MP^{TM} System (BioRad). Autolysis was used as an indicator of calpain activation (Geesink et al., 2006).

Statistical analysis

Before analysis, calpain band densities were taken as a percentage of a day 0 reference standard included on each gel. Data were analyzed using the Mixed Model procedure of the Statistical Analysis System (SAS Institute, Inc., Cary, NC). The LL and SM removed from the strip loins and top rounds, respectively, served as the experimental units (n = 12 of each subprimal). Day of aging was analyzed as a repeated measure. Differences in least squares means (LSM) were compared by the DIFF option. P-values of ≤ 0.05 were considered statistically significant. Calpain activity is reported as a percentage of the internal reference standard on each gel and therefore may be greater than 100 percent.

Results

Calpain activity was measured in beef LL and SM using the casein zymography method. An example of a casein zymogram from the SM is shown in Figure 3.1. The top row of bands is calpain-1, the middle row of bands is native calpain-2, and the bottom row of bands is autolyzed calpain-2 (Pomponio et al., 2008).

By day 2 of aging only 5.4% and 2.0% of the calpain activity present in the zero hour reference standard was detected in the SM and LL, respectively. Aging longer than 2 days led to decreased ($P < 0.05$) SM calpain-1 activity but did not affect LL calpain-1 activity, which was already low (Table 3.1). Calpain-1 activity in the SM decreased from day 2 to 3 and by day 14 there was no calpain-1 activity detected in any of the SM samples. Calpain-1 activity of the LL did not significantly decrease past 2 days of aging but like the SM by day 14 of aging no calpain-1 activity was detected in any of the samples.

Native calpain-2 activity decreased ($P < 0.001$) with longer aging periods for both the LL and SM in Exp 1 (Table 3.2) and the SM in Exp 2 (Table 3.3). Activity decreased from days 2 to 4, 4 to 14, and 14 to 28 of aging in the LL, while native calpain-2 activity in the SM remained constant from day 2 to 4 then decreased from days 4 to 14 and 14 to 28 of aging (Exp 1). Similar results were observed for the SM in Exp. 2, and native calpain-2 activity also decreased from day 42 to 63 of aging (Table 3.3).

Autolyzed calpain-2 activity increased ($P < 0.001$) with longer aging for the LL and SM in Exp 1 (Table 3.2) and the SM in Exp 2 (Table 3.3). Autolyzed calpain-2 activity increased from day 14 to 28 of aging in the LL, while the SM autolyzed calpain-2 activity increased from days 4 to 14, 14 to 28, and 28 to 42 days of aging in both experiments, and then decreased from days 42 to 63 and 63 to 84 in Exp. 2(Table 3.3). On day 2 of aging autolyzed calpain-2 was not detected in any of the LL or SM muscles. Autolyzed calpain-2 was first detected in LL at 14 days of aging and only 3 days of aging in SM. Autolyzed calpain-2 was detected in all of the LL and SM muscles by day 28 (Table 3.4). Exp. 2 revealed that all SM muscles had autolyzed calpain-2 activity until 63 days of aging, but calpain-2 activity was undetectable in one of the samples after 84 days of aging.

Discussion

Casein zymography was used in the current study since it is able to differentiate between calpain-1 and -2 as well as the native and autolyzed forms of these enzymes (Veiseth et al., 2001; Geesink et al., 2006). Previous research has measured calpain activity by using chromatography to separate calpains followed by casein hydrolysis in solution to determine activity (Dayton et al., 1975; Koohmaraie et al., 1990). Unfortunately, the

previously used methods do not allow for native and autolyzed calpain to be differentiated. In the current study, the reason for no change in LL calpain-1 activity and only minor changes in SM calpain-1 activity is that by day 2 of aging, only 2.0 and 5.4% of the initial calpain-1 activity was present in the LL and SM, respectively. Similarly, Boehm et al. (1998) found that on days 1 and 7 postmortem beef SM had 20% and less than 4% of its original calpain-1 activity, respectively. Furthermore, Camou et al. (2007) found that calpain-1 activity was less than 4% of its original activity 2 days postmortem in both the LL and SM. On the other hand, Koohmaraie et al. (1987) found that on days 1, 6, and 14 postmortem beef *longissimus* calpain-1 activity was approximately 45, 40, and 20% of its original activity, respectively. Meanwhile, Phelps et al. (2016) found that calpain-1 activity was detected through 42 days of aging the *semitendinosus* (ST). These authors did not note the percentage of ST muscles analyzed had calpain-1 through 42 days of aging and therefore a couple of outliers could skew the interpretation. On the contrary, in the present study, calpain-1 activity was not detected in any of the samples at 14 days postmortem.

In agreement with the present study, several studies have found that calpain-2 activity decreased with aging (Koohmaraie et al., 1987; Boehm et al., 1998; Camou et al., 2007; Pomponio et al. 2008). Boehm et al. (1998) found that by day 7 postmortem calpain-2 activity was 63% of its initial activity in beef SM, while Koohmaraie et al. (1987) found that 80.2% of calpain-2 activity remained after 14 days of aging beef *longissimus* muscle. Additionally, Phelps et al. (2016) noted ST calpain-2 activity decreased from day 42 to 70 of aging. Furthermore, Camou et al. (2007) found that at 6 days postmortem LL and SM calpain-2 activity was between 10 and 20% of its original activity. Likewise, Pomponio et al. (2008) showed that in hog *longissimus* muscle calpain-2 activity decreased from 100%

on day 1 postmortem to 61% on day 6 postmortem. On the other hand, Veiseth et al. (2001) found that in lamb LL aged for 15 days calpain-2 activity did not change. Additionally, Geesink and Koohmaraie (1999) found that lamb calpain-2 activity was not different between 0 and 56 days postmortem.

Interestingly, in the present study, calpain-2 autolysis was observed in 50% of LL muscles and 75% of SM by day 14 and all muscles by day 28 postmortem. Likewise, Pomponio et al. (2008) found that at 3 days postmortem calpain-2 autolysis was observed in 37% of 75 pig *longissimus dorsi* and 80% of SM muscles. In contrast, Boehm et al. (1998) noted that western blot analysis showed that calpain-2 did not autolyze during the first 7 days of aging. Camou et al. (2007) also found that in LL and SM no autolyzed calpain-2 was detected over 6 days of aging.

Koohmaraie et al. (1987) noted that even with longer postmortem aging, free calcium concentration may not be high enough to activate calpain-2. Veiseth et al. (2001) found that the calcium concentration in lamb 15 days postmortem is not high enough to activate calpain-2. However, Parrish et al. (1981) found that in tough and tender beef *longissimus* muscles aged for 10 to 14 days, free calcium concentrations ranged from 638.0 to 970.6 µM, which would be a sufficient amount to activate calpain-2.

Our previous research (Colle et al., 2015 and 2016) was the first to report that SM sensory tenderness improves up to 42 days of aging, while LL tenderness only improves up to 14 days of aging. This study shows that autolyzed calpain-2 activity increases up to 42 days in the SM. Since calpain-1 activity is nearly gone by day 4 postmortem, the activation of calpain-2 is likely responsible for the continued tenderness improvement in the top round.

The lack of significant improvement in strip loin tenderness after day 14 is probably because the strip loin is already very tender by day 14 when the calpain-2 is activated. Based on our research calpain-2 seems to be the likely candidate for improving SM tenderness during extended aging.

Conclusions

Our results indicate that calpain-1 is active within the first 14 days, while calpain-2 is activated in most LL and SM muscles by day 14 of aging and all muscles by day 28. Therefore, both calpain-1 and calpain-2 may be responsible for the postmortem improvement of beef tenderness, with calpain-1 being responsible for the tenderness improvement early postmortem and calpain-2 responsible for the improvement after 14 days of aging. Further studies need to be conducted to determine the mechanism of calpain-2 activation during extended aging as well as the role of calpain-2 in the improvement of tenderness of extended aged beef.

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Tables and Figures

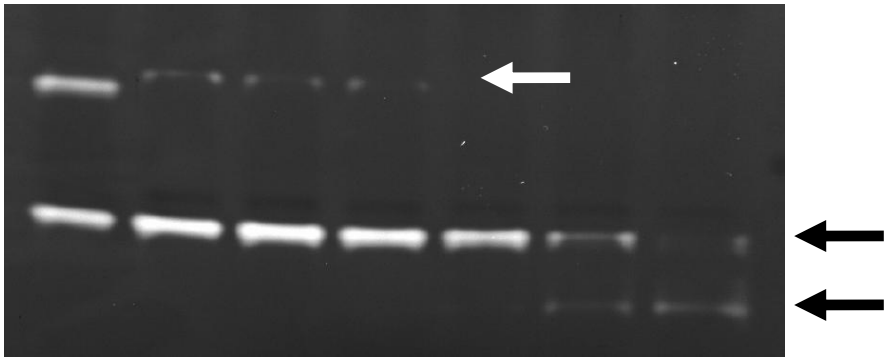

Figure 3.1: Casein zymogram showing native calpain-1 activity (top arrow), native calpain-2 activity (middle arrow), and autolyzed calpain-2 activity (bottom arrow) in the *semimembranosus* aged 2 to 42 days. The day 0 sample (left lane) was from the *sternocephalicus* of a different animal. This sample was frozen 10 min postmortem and was included on all gels as a standard. Samples were aged for up to 42 days postmortem, frozen in liquid nitrogen, and stored at -75ºC until the completion of all aging periods.

Table 3.1: Calpain-1 activity of extended aged beef (Exp. 1; n=12 per muscle)

	Day of Aging						
	2	3	4	14	28	42	SEM
Longissimus lumborum							
Calpain-1 activity[1]	2.0	1.5	0.8	NA[2]	NA	NA	0.7
Semimembranosus							
Calpain-1 activity	5.4[a]	1.2[b]	0.9[b]	NA	NA	NA	1.4

[1]Percentage of day 0 calpain-1 activity

[2]No activity detected

[ab]Within a row, means without a common letter differ ($P < 0.05$).

Table 3.2: Calpain-2 activity of extended aged beef (Exp. 1; n=12 per muscle)

	Day of Aging						
	2	3	4	14	28	42	SEM
Longissimus lumborum							
Native calpain-2 activity[1]	89.7[a]	82.1[ab]	77.9[b]	63.0[c]	29.6[d]	32.7[d]	6.3
Autolyzed calpain-2 activity[1]	NA[2]	NA	NA	1.9[a]	12.2[b]	11.1[b]	2.0
Semimembranosus							
Native calpain-2 activity	87.4[a]	82.7[a]	82.4[a]	56.2[b]	24.6[c]	17.8[c]	6.7
Autolyzed calpain-2 activity	NA	0.1[a]	0.3[a]	4.7[b]	8.8[c]	14.6[d]	3.4

[1]Percentage of day 0 native calpain-2 activity

[2]No activity detected

[a-d]Within a row, means without a common letter differ ($P < 0.05$).

Table 3.3: Calpain-2 activity of extended aged beef (Exp. 2; n=12 per muscle)

	Day of Aging								
	2	3	4	14	28	42	63	84	SEM
Semimembranosus									
Native calpain-2 activity[1]	113.7[a]	117.5[a]	105.7[a]	63.1[b]	43.6[c]	40.4[c]	20.1[d]	10.9[d]	7.6
Autolyzed calpain-2 activity[1]	NA[2]	0.6[e]	1.4[e]	8.4[d]	18.2[bc]	28.9[a]	18.5[b]	12.1[cd]	2.9

[1]Percentage of day 0 native calpain-2 activity

[2]No activity detected

[a-d]Within a row, means without a common letter differ ($P < 0.05$).

Table 3.4: Percentage of muscles with calpain-2 autolysis (n=12)

	Experiment 1		Experiment 2
Day of Aging	*Longissimus lumborum*	*Semimembranosus*	*Semimembranosus*
2	0.0	0.0	0.0
3	0.0	8.3	8.3
4	0.0	8.3	25.0
14	50.0	75.0	75.0
28	100.0	100.0	100.0
42	100.0	100.0	100.0
63	-	-	100.0
84	-	-	91.7

CHAPTER 4

Strategies to Improve Beef Tenderness by Activating Calpain-2 Earlier Postmortem

Abstract

Our objectives were to determine the effect of post rigor calcium chloride injection or freezing on 1) sarcoplasmic calcium concentration and calpain-2 activity of *beef longissimus lumborum* (LL) and *semimembranosus* (SM) steaks aged 1, 4, and 14 days post-treatment and on 2) Warner-Bratzler shear force, water holding capacity, and consumer acceptability of LL and SM steaks aged 4 and 14 days post-treatment. Free calcium levels in the calcium-injected, frozen, and control steaks averaged 1256, 127, and 121µM for the LL and 1520, 120, and 111µM for the SM, respectively. Measurable LL native calpain-2 activity was lower in calcium and frozen steaks than control steaks ($P < 0.01$), while SM native calpain-2 activity was lowest in calcium steaks and intermediate in frozen steaks ($P < 0.01$). LL calcium steaks were more tender ($P = 0.04$) than control steaks. In conclusion, calcium chloride injection and freezing activated calpain-2 earlier postmortem even though free calcium concentration never reached >300µM in the frozen steaks.

Introduction

We recently evaluated the effects of wet aging for 2, 14, 21, 42, and 63 days on retail color stability, microbial growth, Warner-Bratzler shear force, and consumer acceptability of strip loin (*longissimus lumborum*), top round (*semimembranosus*), top sirloin (*gluteus medius*), and bottom round (*biceps femoris*) steaks (Colle et al., 2015 and 2016). The *longissimus lumborum* (LL) showed improvement in tenderness from day 2 to 14, but

remained relatively consistent thereafter for all traits evaluated by the consumer panelists. Interestingly, our research showed that the *semimembranosus* (SM) continues to improve for 42 days after carcass fabrication. Panelist scores for overall acceptability, tenderness, and juiciness of the SM showed steady improvement up to 42 days of aging. The continuous improvement of tenderness and juiciness was unexpected. Proteolysis, or breakdown, of myofibrillar proteins during extended aging may explain the improved tenderness by causing less resistance to shearing or chewing.

Calpains are enzymes that breakdown proteins. Autolysis of calpains coincides with degradation of myofibrillar proteins and therefore improved tenderness (Geesink et al., 2006). It is widely believed that calpain-1 is largely if not solely responsible for the postmortem tenderization of beef (Koohmaraie et al., 1987; Geesink et al., 2006; Koohmaraie and Geesink, 2006). However, recently we have shown that calpain-2 is activated postmortem in both the strip loin and top round (Colle and Doumit, 2016). Since calpain-1 activity is nearly gone by day 4 postmortem, the activation of calpain-2 is likely responsible for the continued tenderness improvement in the top round. The lack of significant improvement in strip loin tenderness after day 14 is probably because the strip loin is already very tender by day 14 when the calpain-2 is activated. The 2010/2011 National Beef Tenderness Survey found the minimum post-fabrication aging time was 2 days for both the strip loin and top round, additionally the percentage of strip loins and top rounds aged less than 14 days was 36.2 and 46.6 percent, respectively (Guelker et al., 2013). Activation of calpain-2 early postmortem would ensure more tender product as early as 2 days post-fabrication.

Activating calpain-2 earlier will speed up the postmortem tenderization of beef. Additionally, activating calpain-2 will lead to greater overall tenderness. The faster and greater tenderness improvement would result in a reduction in tenderness variation. This will lead to a more consistent and desirable beef eating experience at the same time when the product also has exceptional color stability.

One strategy to activate calpain-2 early postmortem is calcium activated tenderization (CAT). Wheeler et al. (1992) found that in beef *longissimus dorsi* (LD) injected with a 0.3 M calcium chloride solution to 110% of the green weight on day 1 postmortem, calpain-2 activity was 47% less in the calcium injected steaks than uninjected steaks after 7 days of aging. These authors noted an improvement in tenderness in LD steaks injected with calcium chloride. Likewise, Pringle et al. (1999) showed that in beef LD injected with 2.2% calcium chloride to 105% of the green weight at 24 hours postmortem, calpain-2 activity was 77% less in injected LD than uninjected LD at 48 hours postmortem. Both Wheeler et al. (1992) and Pringle et al. (1999) determined calpain activity by using column chromatography to separate calpains followed by casein hydrolysis in solution to determine activity. This method allows for the quantification of calpain-2 activity by determining the caseinolytic units per 50 g of tissue, but is unable to distinguish between native and autolyzed calpain-2. Casein zymography is able to distinguish between native and autolyzed calpain-2 and would allow for a better understanding of the effect of CAT on calpain-2 activity (Veiseth et al., 2001; Geesink et al., 2006). Interestingly, Pringle et al. (1999) found CAT improved Warner-Bratzler shear force (WBSF) of the strip loin and top sirloin but not the top round. On the contrary, Wheeler et al. (1991) found hot-boned beef top round injected with a 0.3 M calcium chloride solution to 110% of the green weight

had lower WBSF values than control throughout aging. However, these authors did not evaluate calpain activity. Additionally, Wheeler et al. (1991 and 1992) and Pringle et al. (1999) did not use a sensory panel to determine tenderness of the calcium treated steaks.

Freezing is another potential strategy to activate calpain-2 earlier postmortem. Shanks et al. (2002) showed that freezing steaks at -16°C resulted in a more tender product than steaks aged for the same time but never frozen. Crouse and Koohmaraie (1990) found that aging after being frozen at 30°C for 27 days led to improved WBSF values. They note postmortem proteolysis may be enhanced by freezing meat before aging. They attribute the increase in proteolysis to the fact that calpastatin activity but not calpain activity was lost during frozen storage. Wheeler et al. (1992) found that freezing LD steaks for 7 days and then aging the steaks for 7 days did not significantly change the calpain-2 activity compared to steaks not frozen and aged for 7 days. However, frozen steaks did have lower WBSF values. Using casein zymography will allow for differences in native and/or autolyzed calpain-2 activity to be quantified in steaks that have been frozen. We anticipate that freezing will cause the sarcoplasmic reticulum to release enough calcium to active calpain-2.

We are not aware of published research that examines calpain-2 activity with casein zymography over time after CAT or short term frozen storage. Both the strip loin and top round are excellent candidates for accelerating tenderization because of the high percentage of these subprimals aged 14 days or less (Guelker et al., 2013). Additionally, the top round seems to be a logical candidate for CAT because of its high initial toughness and ability to tenderize over 42 days of aging (Colle et al., 2016). Our objectives were to determine the effect of post rigor calcium chloride injection or post rigor freezing on 1) sarcoplasmic calcium concentration and calpain-2 activity of beef strip loin and top round steaks aged 0,

1, 4, and 14 days post-treatment and on 2) Warner-Bratzler shear force, water holding capacity, and consumer acceptability of beef strip loin and top round steaks aged 4 and 14 days post-treatment.

Materials and Methods

Human Subject Participation in Consumer Panel

The University of Idaho Institutional Review Board certified this project as Exempt (Appendix J).

Product Procurement

At 48 hours postmortem strip loin (IMPS 180) (n=12) and top (inside) round (IMPS 168) (n=12) from the right side of USDA Select carcasses were purchased from a commercial slaughter facility and transported to the University of Idaho Meat Science Laboratory.

Preparation of Product

The *longissimus lumborum* and *semimembranosus* were removed from their respective wholesale cuts for treatment and subsequent analysis. These muscles (n=12 *longissimus lumborum* and n=12 *semimembranosus*) were cut into 3 sections. Each section was randomly assigned to one of the three treatments (control (CO), calcium chloride injection (CA), or freezing (FR)).

On day 2 postmortem the CO sections were cut into 4, 2.54 cm-thick steaks and vacuum sealed. The CA sections were injected to 105% of their green weight with a 2.2%

calcium chloride solution, vacuum tumbled (20 min), cut into 4 steaks and then vacuum sealed. The FR sections were cut into 4 steaks, vacuum sealed, and frozen (-20°C) on day 2 and on day 4 were thawed at 4°C. Two steaks from each treatment were designated for cooking and WBSF measurement. The other two steaks were designated for sensory analysis. Each steak was weighed prior to vacuum packaging and after aging to determine purge. Steaks were aged at 0°C until completion of their assigned aging time. Samples from each treatment were removed and frozen in liquid nitrogen on days 0, 1, 4, and 14 post-treatment and subsequently stored at -75°C until determination of calcium concentration and calpain-2 activity.

Warner-Bratzler Shear Force

On days 4 and 14 post-treatment, steaks designated for WBSF were cooked on open-hearth broilers to an internal temperature of 40°C, then turned and cooked to a final internal temperature of 71°C. Temperature was monitored with hypodermic temperature probes (Omega Engineering Co.) coupled with a 12-channel scanning thermocouple thermometer (Digi-Sense, Cole-Parmer Instrument Co.). Steaks were allowed to cool to room temperature, and re-weighed to determine cooking loss. Six cores (1.27-cm diameter) were mechanically removed parallel with the muscle fiber orientation using a drill press-mounted coring device, and shear force was determined by shearing each core perpendicular to the muscle fibers using a Warner-Bratzler shear machine (GR Manufacturing, Manhattan, KS).

pH

pH was measured before steaks were cooked for the sensory panel. A portable pH meter (Seven2Go pro, Mettler Toledo, Woburn, MA) equipped with a InLab SolidsPro

puncture-type electrode was used to measure pH. The pH meter was calibrated each day using standard pH 4.0 and 7.0 buffers.

Sensory Panel

On days 4 and 14 post-treatment, steaks designated for consumer acceptability were vacuum packaged and frozen at -20°C until the sensory panel was conducted. For consumer sensory analysis, steaks were thawed at 4°C for 24 hours and subsequently cooked as described above. Two consumer panels were conducted (n=72 panelists per muscle) to evaluate cooked steaks from the designated treatments for overall acceptability, tenderness, juiciness, and flavor using a 9-point scale (9 = like extremely, extremely tender, extremely juicy, and like flavor extremely, respectively; 1= dislike extremely, not at all tender, extremely dry, and dislike flavor extremely, respectively) (Appendix G). Four 1.27-cm x 1.27-cm x steak thickness cubes were obtained from each steak. Using an incomplete block design panelists sampled 4 samples from the 3 treatments and 2 aging periods.

Calcium Concentration

Calcium concentration was determined following a procedure by Hopkins and Thompson (2001) with minor modifications (Appendix L). Samples that had been stored at -80°C were weighed (2 g) and placed in a -20°C freezer at least 36 hours prior to calcium measurement. Following equilibration to -20°C samples were placed in a refrigerator (4°C) for 20 min before being finely diced, placed on ice, and then centrifuged (Sorvall RT1 Centrifuge, Thermo Scientific, Waltham, MA) at 30,000 g at 5°C for 40 min. 250 µl aliquots of the supernatant were mixed with 5 µl of Calcium Ion Strength Adjuster (Mettler Toledo, Woburn, MA). These samples were then incubated in a water bath at room

temperature for 5 min. Calcium concentration was determined using a calcium selective electrode (PerfectION combination Ca^{2+}, Mettler Toledo, Woburn, MA) attached to a portable ion meter (Seven2Go pro, Mettler Toledo, Woburn, MA). The electrode was allowed to soak in a 1 x 10^{-2} calcium solution at least 1 h prior to calibration and reading samples. A calibration curve was created prior to each run. Calcium standards containing 8000 µM, 2000 µM, 1000 µM, 500 µM, 100 µM, and 50 µM calcium were made from Calcium ISE standard 1000 mg/l as Ca^{2+} (Mettler Toledo, Woburn, MA).

Calpain Extraction

One gram of each sample in extraction buffer (3 mL)(100 mM Tris, 10 mM EDTA, 10 mM DTT, pH 8.3) was homogenized (POLYTRON® PT 10-35 GT; PT-DA 12/2EC-B154) at 18,000 rpm on ice 3 times for 15 sec with 15 sec cooling between bursts. The homogenate was pipetted into two 2 mL microcentrifuge tubes. Samples were then centrifuged for 30 min at 8,800 x g at 4°C. The supernatant fluid was aliquoted and stored at -75°C until calpain analysis (Appendix H).

Casein Zymography

Calpain activity was determined utilizing casein zymography as described by Pomponio et al. (2008) with minor modifications (Appendix I). Polyacrylamide gels (12.5%; 75:1 acrylamide to bisacrylamide) containing 0.2% casein were poured and overlaid with stacking gel (4%; 75:1 acrylamide to bisacrylamide) the day the gels were run. Gels (8 x 10 x 0.1 cm) were prerun with running buffer (25 mM Tris, 1 mM DTT, 192 mM glycine, 1 mM EDTA, pH 8.3) at 100 V for 15 min in an ice bath before loading samples. Sample buffer (10 µL)(150 mM Tris, 20% glycerol, 10 mM DTT, 0.02% bromophenol blue, pH 6.8)

was added to the supernatant (40 µL). Samples (20 µL) were added and the gels were run at 100 V for 6 hours in an ice water bath. Gels were then placed in incubation buffer (~60 mL; 50 mM Tris, 10 mM DTT, 4 mM calcium chloride, pH 7.5) at room temperature with slow shaking for 17 hours. Buffer was changed (~60 mL) at 30 minutes and (~130 mL) 60 minutes. Gels were stained in Coomassie Blue R-250 for 1 hour and destained in Coomassie Blue R-250 destaining solution for 3 hours. The clear bands indicating calpain activity were quantified by inverting the image and then comparing the density of each band to the day 0 post-treatment sample on each gel utilizing a ChemiDoc MP^{TM} System (BioRad). Autolysis was used as an indicator of calpain activation (Geesink et al., 2006).

Statistical Analysis

Before analysis, calpain band densities were taken as a percentage of the day 0 post-treatment sample on each gel. Data were analyzed using the Mixed Model procedure of the Statistical Analysis System (SAS Institute, Inc., Cary, NC). Individual muscle served as a random variable. Differences in least squares means (LSM) were compared by the DIFF option. P-values of ≤ 0.05 were considered statistically significant. All data are presented as least squares means ± SEM. Calpain activity is reported in relative and not absolute terms.

Results

Weights

A day of aging by treatment interaction for LL ($P = 0.01$) and SM ($P < 0.01$) percent storage loss was observed (Table 4.1). After 4 days of aging, storage loss from FR steaks

was greater than CA steaks, while after 14 days of aging they were not different. At both aging periods the CO steaks had the least storage loss. Additionally, FR steaks lost 2.15% and 3.15% for the LL and SM, respectively during the freezing and thawing process prior to post treatment aging.

Cooking

There was a treatment difference for SM cook time ($P < 0.01$) (Table 4.2). SM FR steaks took less time to cook than the CA steaks, while CO steaks were intermediate and not different from the others.

A day of aging by treatment interaction for LL ($P = 0.04$) (Table 4.1) but not SM ($P = 0.47$) percent cook loss was observed. Percent cook loss was the lowest for LL CO steaks aged for 4 days.

WBSF

There was a treatment difference for LL WBSF ($P = 0.04$) (Table 4.2). CA steaks were more tender than CO steaks. Additionally, LL steaks became more tender ($P < 0.01$) with aging (Table 4.3).

pH

There was a treatment difference for SM pH ($P < 0.01$) (Table 4.2). CA steaks had a lower pH than the CO or FR steaks. Additionally, LL pH increased ($P < 0.01$) with aging (Table 4.3).

Sensory

Demographics of consumer panelists are shown in Table 4.4, and consumer panel preferences are shown in Table 4.5. There was a day of aging by treatment interaction for LL sensory acceptability ($P < 0.01$), tenderness ($P = 0.01$), juiciness ($P < 0.01$), and flavor ($P < 0.01$) (Table 4.6). Generally, CA steaks aged for 4 days were comparable in acceptability and tenderness to all steaks that were aged for 14 days. On the other hand, CO and FR steaks aged for 4 days were less acceptable and less tender. Additionally, FR steaks aged for 4 days were the least juicy. However, FR steaks aged for 14 days were as juicy as the CO and CA steaks. Lastly, CA steaks aged for 4 days and control steaks aged for 14 days had the best flavor. SM sensory tenderness improved ($P < 0.01$) from day 4 to 14 post-treatment (Table 4.3). Longer aging periods resulted in increased tenderness of the SM. Treatment did not improve SM sensory tenderness ($P = 0.19$)

Calcium

A day of aging by treatment interaction was observed for LL ($P < 0.01$) and SM ($P = 0.05$) calcium concentration (Table 4.7). As expected, calcium levels in the CA steaks were higher than in the CO and FR steaks, while CO and FR steaks were not different. Calcium levels in the CO or FR steaks never reached 300µM.

Calpain

A treatment difference for LL ($P < 0.01$) and SM ($P < 0.01$) native calpain-2 activity was found (Table 4.2). LL native calpain-2 activity was lower in CA and FR steaks than CO

steaks, while SM native calpain-2 activity was lowest in CA steaks and intermediate in FR steaks. Additionally, LL native calpain-2 activity decreased ($P < 0.01$) from days 1 to 4 and 4 to 14 post-treatment (Table 4.3). A day of aging by treatment interaction for SM ($P = 0.01$) autolyzed calpain-2 activity was observed (Table 4.7). SM calpain-2 autolysis was first observed on day 1 post-treatment for CA steaks, day 4 post-treatment for FR steaks, and day 14 post-treatment for CO steaks. Additionally, by day 14 post-treatment no native or autolyzed calpain-2 activity was detected in SM CA steaks.

Discussion

Water holding capacity (WHC) is the ability of meat to hold its own or added water (Honikel and Hamm, 1994). Percent purge of 1 to 2 percent is considered acceptable (Johnson, 1974). The only treatment in the current study that was less than 2% was the CO aged for 4 days. We expected CO steaks to have the best WHC for two reasons. First, CA steaks were injected to 105% of the green weight resulting in those steaks having more free water to lose during aging. Secondly, freezing disrupts muscle structure allowing water to leave during thawing as well as during aging (Leygonie et al., 2012). Aroeira et al. (2016) noted that freezing for 40 days leads to increased purge and cook loss. Similarly, Crouse and Koohmaraie (1990) found that freezing increases the cook loss of beef loin steaks. The reduced WHC of FR steaks in the current study resulted in poorer sensory juiciness of LL frozen steaks aged for 4 days. Meanwhile, Wheeler et al. (1997) found that calcium injected steaks had greater juiciness scores than control steaks. In the present study, CA LL steaks aged for 4 days numerically had the highest juiciness scores. However, it was only significantly different from the FR steaks aged for 4 days. The lower SM pH in the CA steaks may have contributed to its reduced WHC, while the increase in LL pH from day 4 to

14 was not enough to prevent fluid loss during aging. Additionally, the reduced water in the FR steaks may have contributed to the shorter cook time of those steaks. The poor WHC of the CA and FR steaks reduces the amount of saleable product for the retailer. Additionally, the excess fluid is unsightly to the consumer.

Calcium injection improved LL WBSF and day 4 sensory tenderness scores, but did not affect SM tenderness. These results are consistent with those of Pringle et al. (1999), where they found CAT improved WBSF of the strip loin but not the top round. Sensory tenderness scores of LL CI steaks were as good at 4 days of aging as all of the steaks aged for 14 days. Wheeler et al. (1992) and Lawrence et al. (2003) also found calcium injection improves LD tenderness. Furthermore, regardless of injection or post injection aging time, calcium injected strip loin steaks had improved sensory tenderness scores when compared to control steaks aged for the same amount of time (Wheeler et al., 1997). Lawrence et al. (2003) showed that in calcium enhanced muscles the calpains are the major contributor to the improved tenderness, while salting-in of calcium ions plays a minor role. Interestingly, Wheeler et al. (1991) found that calcium injection improved WBSF of hot-boned beef top round. It is unclear why calcium injection did not improve SM tenderness in the present study.

Freezing did not improve WBSF values or sensory tenderness of either muscle. On the contrary, Wheeler et al. (1992) and Shanks et al. (2002) found that freezing resulted in more tender product. Similarly, Crouse and Koohmaraie (1990) found that aging meat after being frozen at -30°C for 27 days led to improved WBSF values. These authors note postmortem proteolysis may be enhanced by freezing meat before aging, since calpastatin activity but not calpain activity was lost during frozen storage. In the current study steaks

were only frozen for 2 days, which is not enough time for all of the calpastatin activity to be lost. This may be the reason for the lack of tenderness improvement in the FR steaks.

Casein zymography was used to measure calpain activity since it is able to detect both the native and autolyzed forms of calpain-1 and -2 (Veiseth et al., 2001; Geesink et al., 2006). Both LL and SM, CA and FR steaks had reduced native calpain-2 activity. We have seen that CA activates calpain-2 earlier postmortem in the beef *adductor* (Unpublished data). Additionally, by day 14 there was native or autolyzed calpain-2 activity in the calcium injected *adductors*. Likewise, Wheeler et al. (1992) found that in beef LD injected on day 1 postmortem, calpain-2 activity was 47% less in the calcium injected versus control steaks after 7 days of aging. Additionally, Pringle et al. (1999) showed that calpain-2 activity in beef LD injected at 24 hours postmortem was 77% less than control steaks at 48 hours postmortem. Unlike the current study, Wheeler et al. (1992) found that freezing LD steaks for 7 days and then aging for 7 days did not reduce the calpain-2 activity. Interestingly, Aroeira et al. (2016) noted that freezing did increase proteolysis and reduce shear of beef *longissimus thoracis* on day 0 but not on days 2, 14, or 21. In the present study had day 1 post treatment WBSF been measured the FR treatment may have had lower WBSF values than the control. Neither Wheeler et al. (1992) or Pringle et al. (1999) were able to determine autolyzed calpain-2 activity due to methodology. Calpain-2 is active earlier in the CA and FR steaks. We found that autolyzed calpain-2 activity is detected in CA steaks on day 1 of aging in the SM and day 4 in both the LL and SM. By day 14 postmortem no autolyzed calpain-2 activity is detected in the CA steaks of either muscle. Autolyzed calpain was detected in SM FR steaks on day 4 of aging and in both the LL and SM on day 14 of aging. Autolyzed calpain in the CO samples were only detected on day 14 in the SM.

These results are intriguing since calcium levels never reach 300 µM in frozen steaks. Additionally, there was no improvement in FR steak tenderness even though we detected autolyzed calpain-2 activity earlier postmortem in the FR steaks than CO steaks. Had tenderness been measured on day 1 post treatment we may have seen a tenderness improvement.

Conclusions

As expected calcium chloride injection provides enough calcium to activate calpain-2 early postmortem resulting in improved tenderness. Freezing also activated calpain-2 earlier postmortem. However, free calcium levels in FR steaks never reached the published level required to activate calpain-2. Further research needs to be conducted to determine what caused the calpain-2 to be activated earlier postmortem in steaks that had been frozen. The disruption of the muscle cell structure during freezing and thawing may cause an endogenous compound such as phospholipids to interact with calpain-2 which could potentially reduce the calcium requirement for calpain-2 activation. Additionally, further research is needed to develop a method that activates calpain-2 earlier postmortem, leading to improved tenderness.

Future Research

Understanding the role of calpain-2 in the tenderness improvement of beef during extended aging must remain a priority. Calpain-2 has been shown to be active in the soluble fraction but not the particulate fraction of the rat *soleus* muscle immobilized for 5 days (Vermaelen et al., 2007). In relation to the present research, this raises questions about the role of autolyzed calpain-2 in improving beef tenderness during extended aging. If the calpain-2 associated with myofibrillar proteins is not activated postmortem then calpain-2 would not contribute to the tenderness improvement during extended aging. However, if the calpain-2 that is associated with the insoluble portion is activated then determining which myofibrillar proteins are being degraded by calpain-2 would be of utmost importance. An understanding of the location of autolyzed calpain-2 during extended aging of top round and strip loin muscles will help clarify the role of calpain-2 in postmortem tenderization of beef.

Further research needs to be conducted to determine what caused the calpain-2 to be activated earlier postmortem in steaks that had been frozen even though the calcium concentration was not different than the control. The disruption of the muscle cell structure during freezing and thawing may cause an endogenous compound such as phospholipids to interact with calpain-2 which could potentially reduce the calcium requirement for calpain-2 activation.

Finally, determining a method to activate calpain-2 earlier postmortem to ensure a tender product by 4 days of aging must remain a priority. This would result in more consistent and desirable beef eating experiences and therefore a greater demand for beef.

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Tables

Table 4.1: Water holding capacity (n=12)

	Day of Aging							
	4			14				Age x Trt
	Control	Calcium	Frozen	Control	Calcium	Frozen	SEM	P-Value
Percent Storage Loss								
Longissimus lumborum	1.61^{d}	2.76^{c}	4.06^{b}	2.51^{c}	4.93^{a}	4.87^{a}	0.28	0.01
Semimembranosus	3.50^{e}	4.27^{de}	5.70^{bc}	4.96^{cd}	7.38^{a}	6.54^{b}	0.34	<0.01
Percent Cook Loss								
Longissimus lumborum	20.58^{b}	24.60^{a}	25.39^{a}	25.87^{a}	26.48^{a}	24.58^{a}	1.21	0.04
Semimembranosus	27.49	32.96	30.39	28.81	30.34	27.65	1.87	0.47

$^{a-d}$Within a row, means without a common letter differ ($P < 0.05$).

Table 4.2: Treatment effect on cook time, WBSF, pH, and calpain-2 activity (n=12)

	Treatment				Trt
	Control	Calcium	Frozen	SEM	P-Value
Cook time[1]					
Longissimus lumborum	21.04	22.08	20.67	1.29	0.72
Semimembranosus	22.75[ab]	26.21[a]	20.00[b]	1.27	<0.01
WBSF (kg)					
Longissimus lumborum	4.04[a]	3.57[b]	3.85[ab]	0.21	0.04
Semimembranosus	4.36	4.54	4.32	0.26	0.75
pH					
Longissimus lumborum	5.65	5.63	5.64	0.04	0.45
Semimembranosus	5.65[a]	5.60[b]	5.64[a]	0.03	<0.01
Native calpain-2 activity[2]					
Longissimus lumborum	85.8[a]	24.2[b]	32.8[b]	5.5	<0.01
Semimembranosus	72.3[a]	6.4[c]	48.0[b]	6.7	<0.01
Autolyzed calpain-2 activity[2]					
Longissimus lumborum	0.0	1.3	3.0	1.7	0.45
Semimembranosus	0.2	0.7	2.0	0.5	--

[1]Minutes to 71°C

[2]Percentage of day 0 native calpain-2 activity

[a-c]Within a row, means without a common letter differ ($P < 0.05$).

Table 4.3: Aging effect on tenderness, pH, and calpain-2 activity (n=12)

	Day of Aging				Trt
	1	4	14	SEM	P-Value
WBSF (kg)					
Longissimus lumborum		4.16[a]	3.48[b]	0.19	<0.01
Semimembranosus		4.50	4.31	0.23	0.44
pH					
Longissimus lumborum		5.61[b]	5.67[a]	0.04	<0.01
Semimembranosus		5.63	5.63	0.03	0.63
Sensory Tenderness[1]					
Longissimus lumborum		5.9	6.3	0.2	--
Semimembranosus		4.4[b]	5.4[a]	0.2	<0.01
Native calpain-2 activity[2]					
Longissimus lumborum	61.6[a]	48.1[b]	33.1[c]	5.5	<0.01
Semimembranosus	46.5	46.4	33.9	6.7	0.21
Autolyzed calpain-2 activity[2]					
Longissimus lumborum	0.0	1.3	3.0	1.7	0.45
Semimembranosus	0.5	0.7	1.8	0.5	--

[1]Scale, 9 = like extremely, extremely tender, extremely juicy, and like flavor extremely, respectively; 1= dislike extremely, not at all tender, extremely dry, and dislike flavor extremely, respectively.

[2]Percentage of day 0 native calpain-2 activity

[ab]Within a row, means without a common letter differ ($P < 0.05$).

Table 4.4: Demographics of consumer panelists (n=72/panel)

	Longissimus lumborum		*Semimembranosus*	
	n	%	*n*	%
Age				
18-19	2	2.8	2	2.8
20-29	49	68.1	44	61.1
30-39	11	15.3	9	12.5
40-49	1	1.4	1	1.4
50+	9	12.5	16	22.2
Gender				
Male	30	41.7	28	38.9
Female	42	58.3	44	61.1
Beef meals/wk[1]				
0-1	14	19.4	13	18.1
2-4	41	56.9	36	50.0
5-7	16	22.2	22	30.6
8+	1	1.4	1	1.4
Most consumed[2]				
Ground	45	56.3	44	57.9
Roast	7	8.8	5	6.6
Steak	25	31.3	23	30.3
Other	3	3.8	4	5.3

[1]Please indicate the number of meals a week in which you consume beef: 0-1, 2-4, 5-7, or 8+

[2]Please indicate the form in which you most commonly consume beef: ground, roast, steak, or other

Table 4.5: Consumer panel preferences for the *longissimus lumborum* and *semimembranosus*

	Longissimus lumborum (n=12)						*Semimembranosus* (n=12)					
	Day of Aging						Day of Aging					
	4			14			4			14		
	Control	Calcium	Frozen	Control	Calcium	Frozen	Control	Calcium	Frozen	Control	Calcium	Frozen
Like most[1]												
Flavor	27.9	32.6	47.6	39.1	26.2	34.8	65.7	48.8	52.6	30.0	38.1	50.0
Tenderness	27.9	34.9	28.6	32.6	31.0	47.8	5.7	24.4	7.9	32.5	31.0	17.5
Juiciness	34.9	20.9	7.1	13.0	28.6	15.2	22.9	7.3	21.1	22.5	14.3	15.0
Texture	9.3	11.6	16.7	15.2	14.3	2.2	5.7	19.5	18.4	15.0	16.7	17.5
Like least[2]												
Flavor	39.5	22.0	26.1	33.3	40.0	41.0	13.3	14.0	19.6	25.5	24.4	34.1
Tenderness	25.6	29.3	21.7	21.4	22.2	10.3	37.8	38.0	49.0	34.0	26.7	25.0
Juiciness	18.6	39.0	39.1	31.0	24.4	43.6	26.7	28.0	21.6	27.7	37.8	22.7
Texture	16.3	9.8	13.0	14.3	13.3	5.1	22.2	20.0	9.8	12.8	11.1	18.2
Off flavor[3]												
Yes	25.5	21.3	17.0	14.6	38.3	23.9	26.1	19.1	35.4	26.1	31.9	21.3
No	74.5	78.7	83.0	85.4	61.7	76.1	73.9	80.9	64.6	73.9	68.1	78.7
Purchase[4]												
Yes	58.3	89.4	56.3	79.6	68.8	83.0	59.6	47.8	45.8	59.1	59.6	56.3
No	41.7	10.6	43.8	20.4	31.3	17.0	40.4	52.2	54.2	40.9	40.4	43.8

[1]Percentage of panelists that liked that attribute the most

[2]Percentage of panelists that liked that attribute the least

[3]Percentage of panelists that did or did not detect an off flavor

[4]Percentage of panelists willing to or not willing to purchase the product

Table 4.6: Sensory analysis by consumer panelists[1] (n=72/panel)

	Day of Aging							
	4			14				Age x Trt
	Control	Calcium	Frozen	Control	Calcium	Frozen	SEM	P-Value
Longissimus lumborum								
Acceptability	5.7[bc]	6.5[a]	5.4[c]	6.4[a]	6.0[ab]	6.3[ab]	0.3	<0.01
Tenderness	5.5[b]	6.4[a]	5.5[b]	6.5[a]	6.3[a]	6.8[a]	0.3	<0.01
Juiciness	5.4[a]	6.1[a]	4.4[b]	5.6[a]	5.4[a]	5.6[a]	0.3	<0.01
Flavor	5.4[c]	6.5[a]	5.5[c]	6.3[ab]	5.5[c]	5.7[bc]	0.3	<0.01
Semimembranosus								
Acceptability	5.3	5.1	4.9	5.2	5.8	5.4	0.3	0.16
Tenderness	4.4	4.7	4.2	5.1	5.7	5.4	0.3	0.50
Juiciness	4.8	4.3	4.3	4.8	4.8	4.7	0.3	0.59
Flavor	5.3	5.5	5.2	5.4	5.5	5.2	0.3	0.95

[1]Scale, 9 = like extremely, extremely tender, extremely juicy, and like flavor extremely, respectively; 1= dislike extremely, not at all tender, extremely dry, and dislike flavor extremely, respectively.

[a-c]Within a row, means without a common letter differ ($P < 0.05$).

Table 4.7: Calcium concentration and autolyzed calpain-2 activity day of aging x treatment interactions (n=12)

	Day of Aging										
	1			4			14				Age x Trt
	Control	Calcium	Frozen	Control	Calcium	Frozen	Control	Calcium	Frozen	SEM	P-Value
Calcium Concentration (µM)											
Longissimus lumborum	121.6[c]	854.3[b]	114.5[c]	96.3[c]	1004.8[b]	118.7[c]	146.0[c]	1909.3[a]	148.0[c]	125.3	<0.01
Semimembranosus	107.1[c]	1701.5[a]	99.8[c]	107.5[c]	1150.5[b]	110.2[c]	117.2[c]	1708.6[a]	150.7[c]	127.3	0.05
Native calpain-2 activity[1]											
Longissimus lumborum	88.9	48.4	47.5	90.2	23.4	30.7	78.3	0.8	20.2	8.2	0.15
Semimembranosus	65.9	16.0	57.7	87.6	3.2	48.3	63.5	NA	38.1	10.5	0.44
Autolyzed calpain-2 activity[1]											
Longissimus lumborum	NA	NA	NA	NA	3.9	NA	NA	NA	9.1	2.9	0.21
Semimembranosus	NA[2]	1.4[a]	NA	NA	0.8[a]	1.4[a]	0.7[a]	NA	4.6[b]	0.8	0.01

[1]Percentage of day 0 native calpain-2 activity

[2]No activity detected

[a-c]Within a row, means without a common letter differ ($P < 0.05$).

Appendix A

Exempt certification for IRB project number 13-166

University of Idaho
Office of Research Assurances (ORA)
Institutional Review Board (IRB)
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Moscow ID 83844-3010

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July 17, 2013

To: Matthew Doumit
Cc: William Loucks, Michael Colle, Hailey Sutton

From: IRB, University of Idaho Institutional Review Board

Subject: Exempt Certification for IRB project number 13-166

Determination: July 17, 2013
Certified as Exempt under category 2 & 6 at 45 CFR 46.101(b)(2 & 6)
IRB project number 13-166: Consequences of Extended Aging on Retail Shelf-Life and Consumer Acceptability of Four Beef Muscles

Appendix B

Metmyoglobin reducing capacity of intact or ground meat

Adapted from Appendix J (AMSA, 2012)

Principle

Surface pigments are initially oxidized to MMb by soaking the sample slice in a dilute sodium nitrite solution for 20 minutes. The slice (1.27 cm thick) is vacuum packaged, and surface % MMb is monitored for 2 hours at 30°C by measuring reflectance *K*/*S* ratios (572/525 nm). Sample reducing ability is defined as the percentage decrease in surface MMb concentration during the incubation period. The decline in MMb is assumed to reflect the tissue's ability to reduce ferric heme iron.

Reagent

1. 0.3% (w/w) sodium nitrite solution: Tare a large beaker, and weigh 3.0 g NaNO2 into the beaker and add distilled water to 1000 g. Make fresh daily. Incubate at room temperature.

Procedure

1. Remove a 3 cm × 3 cm × 2 cm sample of muscle tissue with no visible fat or connective tissue. For ground meat, use a similar sized sample that has been uniformly packed together to help avoid crumbling when the sample is immersed.
2. Be sure to orient sample to identify which surface will be evaluated later. This surface may be fresh cut or the surface that was displayed.
3. Submerge sample in 0.3% NaNO2 solution for 20 minutes at room temperature to induce MMb formation. Ground samples can be placed on a small screen to help lower and raise the cube with minimal crumbling.
4. Remove sample from beaker, and blot to remove excess solution. Retain the 3-dimensional shape as much as possible and place the surface for evaluation up in an impermeable bag and vacuum package (a good, uniform vacuum). The vacuum may slightly flatten or round the samples.
5. Scan immediately for reflectance from 400 to 700 nm to determine the initial amount of MMb formed on the surface. Maintain surface integrity.
6. Place sample in an incubator at 30°C, and rescan after 2 hours to determine the remaining amount of MMb.

Calculations

%MMb = [*K*/*S*572 ÷ *K*/*S*525 (for 100% DMb)] − [*K*/*S*572 ÷ *K*/*S*525 (sample)] ÷ [*K*/*S*572 ÷ *K*/
*S*525 (for 100% DMb)] − [*K*/*S*572 ÷ *K*/*S*525 (for 100% MMb)][× 100].
MRA (% of MMb reduced) = [(Initial %MMb − Final %MMb) ÷ Initial %MMb] × 100
or
Use the initial MMb formed as an indicator of MRA (see note below).

Notes

Some authors (McKenna et al., 2005; Mancini et al., 2008) indicate that the initial amount of MMb formed by oxidation in sodium nitrite solution is a good indicator of sample MRA. However King et al. (2011) found that percentage reduction was better than the initial amount of MMb formed. Thus, it is best to collect and statistically analyze both the initial amount of MMb formed, and the percentage of MMb reduced over the incubation time.

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Appendix C

Oxygen consumption of intact muscle or ground meat

Adapted from Appendix I (AMSA, 2012)

Principle:
Freshly cut meat slices are oxygenated (allowed to bloom) for a standardized time and temperature and then vacuum packaged. The decline in OMb due to enzyme respiration is measured as an indicator of the tissue's ability to consume oxygen. Reflectance spectra over the range 400 to 700 nm are recorded immediately and a second time after 20 minutes in a water bath or incubator kept at 25°C. Oxymyoglobin levels are calculated using the ratio of the reflectance at 610 and 525 nm after *K*/*S* transformation as described in Section IX. Higher *K*/*S*610/*K*/*S*525 ratios indicate higher OMb levels. Oxygen consumption (OC) is reported as the difference in percentage from the first and last measurements.

Equipment and Supplies

1. Vacuum packaging machine
2. PVC film
3. Highly oxygen-impermeable vacuum bags (O2 permeability ≤0.6 g O2/625 cm2/24 hours
   at 0°C)
4. Spectrometer that can scan and record surface reflectance from 400 to 700 nm (see Section IX)

Procedure

1. All samples to be assayed must be the same temperature, 4°C, for instance. Otherwise
   oxygen consumption will be faster for samples at warmer temperatures and bloom development (oxygenation) will be less; it will be slower for those at colder temperatures and bloom development will be more.
2. Keep all samples at 2 to 4°C to help ensure uniform oxygenation. For intact, whole muscle, use a sharp knife to remove a 3 cm × 3 cm × 2 cm sample with minimal visible fat or connective tissue. For ground samples, prepare a comparable sized cube that has been uniformly packed. Again, the visible fat level should be typical of the lean portion of the sample. Avoid dull knives that disrupt surface structure. Also avoid excessive handling and pressing of the blooming surface of ground product (see Madhavi and
   Carpenter, 1993).
3. If the surface is not a fresh cut, then just before starting the bloom step, remove a thin surface layer to expose fresh tissue.
4. Cover the freshly cut surface with a small piece of oxygen-permeable film to avoid drying. Keep the film (polyvinyl chloride film is commonly used) in one, smooth layer to ensure uniform exposure of the surface to air. Make note of the film's oxygen permeability.

5. Bloom for 2 hours at 2 to 4°C (or some other standardized time). Take care to keep all samples at the same temperature during this step because blooming is very temperature dependent.
6. After bloom, remove the PVC film and place the sample in a pouch with very low oxygen permeability. Quickly vacuum package with high vacuum; keep the vacuum uniform from sample to sample.
7. IMMEDIATELY scan the surface of the sample for reflectance from 400 to 700 nm to determine the initial % OMb. The spectrophotometer must be calibrated through the vacuum bag film.
8. To speed up oxygen consumption, use an incubator or water bath at 25°C. Re-scan the same surface after 20 minutes (or some standardized time appropriate to the meat being used).

Calculations

%OMb = [*K/S*610 ÷ *K/S*525 (for 100% DMb)] − [*K/S*610 ÷ *K/S*525 (sample)] ÷ [*K/S*610 ÷ *K/*
*S*525 (for 100% DMb)] − [*K/S*610 ÷ *K/S*525 (for 100% OMb)][× 100].

Oxygen consumption = [(Initial % OMb − Ending % OMb) ÷ Initial % OMb] × 100.

Notes

Madhavi and Carpenter (1993) described a reflectance procedure for measuring oxygen consumption (OC), using a spectrophotometer with reflectance attachment to measure surface
OMb levels of vacuum packed samples initially, and at 5-minute intervals (20 minutes total) at 4°C. Samples were smaller (2.5 × 2.5 × 0.5 cm) to fit in the sample port of the reflectance unit.
Relative concentration of OMb was calculated using the method of Krzywicki (1979). However, that method was modified by Tang et al. (2004) and their revised wavelengths are recommended (see Section IX). OC was expressed as percentage of time-zero surface OMb consumed during
10 minutes in vacuum. Mancini, Hunt and Kropf (2003) reported a method using reflectance at 610 nm to directly determine OMb. This is possible because OMb has its unique reflectance at 610 while 610 is isobestic for both DMb and MMb (see Section IX for further discussion of meat surface reflectance measurements and calculation of *K/S* ratios). This method has been used successfully (see King et al., 2011). Some research has reported an actual "rate of oxygen consumption" using percentage changes of OMb per unit of time. This is more laborious and time consuming. With a large number of samples, "oxygen consumption" is often calculated as the "average percentage reduction of OMb" relative to the initial level of OMb formed on the sample. The time for deoxygenation of the sample must to be standardized. Usually, 20 minutes is sufficient to detect sample differences.

References

King, D. A., S. D. Shackelford, A. B. Rodriguez, and T. L. Wheeler. 2011. Effect of time of measurement on the relationship between metmyoglobin reducing activity and oxygen

consumption to instrumental measures of beef longissimus color stability. Meat Sci. 87:26–32.

Madhavi, D. L., and C. E. Carpenter. 1993. Aging and processing affect color, metmyoglobin reductase and oxygen consumption of beef muscles. J. Food Sci. 58:939–942.

Mancini, R. A., M. C. Hunt, and D. H. Kropf. 2003. Reflectance at 610 nanometers estimates oxymyoglobin content on the surface of ground beef. Meat Sci. 64:157–162.

Mohan, A., M. C. Hunt, T. J. Barstow, T. A. Houser, and D. M. Hueber. 2010a. Near-infrared oximetry of three postrigor skeletal muscles for following myoglobin redox forms. Food Chem. 123:456–464.

Tang, J., C. Faustman, and T. A. Hoagland. 2004. Krzywicki revisited, equations for spectrophotometric determination of myoglobin redox forms in aqueous meat extracts. J. Food Sci. 69:C717–C720.

Appendix D

TBARS for oxidative rancidity - rapid, wet method

Adapted from Appendix O (AMSA, 2012)

Principle:
In the presence of thiobarbituric acid (TBA), malonaldehyde and other aldehyde products of lipid oxidation (TBA reactive substances; TBARS) form pink chromogens with maximum absorbance at 532-535 nm. However, in the presence of interfering sugars, a yellow chromagen forms, which can be avoided using the distillation method (Tarladgis, 1960).

Reagents:

1. TBA stock solution - 0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25N HCl.
2. Stock solutions (100 mL) are sufficient for 20 individual tests. Stock solution may be stored at room temperature in the dark (foil-wrapped container).

Procedure:

1. Finely chop or mince a portion of the product of interest. Weigh out duplicate 0.25 g samples.
2. Add 1.25 ml TBA stock solution to each sample, giving a dilution factor of 6. Mix well.
3. Heat samples 10 min in boiling water in loosely capped 2.0 ml eppendorf tubes **Caution:** tightly capped tubes may burst during heating. Positive samples turn pink during heating.
4. Cool tubes in tap water.
5. Centrifuge at 5,000 × g for 10 min to obtain a clear supernatant.
6. Carefully pipette 200 µl of the supernatant to a 96 well plate. Take care that the solution remains clear.
7. Measure supernatant absorbance at 532 nm against a blank that contains all the reagents minus the meat.
8. Calculate the TBA value expressed as ppm malonaldehyde, using 1.56×10^5/M/cm as the extinction coefficient of the pink TBA chromogen (Sinnhuber and Yu, 1958), as follows:
   TBARS number (mg MDA/kg) = sample A_{532} × (1 M TBA chromagen/156,000) × [(1mole/L/M] × (0.003 L/0.5 g meat) × (72.07 g MDA/mole MDA) × 1000 mg/g) × 1000 g/kg)
   or
   TBARS value (ppm) = sample A_{532} × 2.77

References:

Buege, J.A. and Aust, S.D. 1978. Microsomal lipid peroxidation. Methods in Enzymology 52:302-304.

Sinnhuber, R.O. and Yu, T.C. 1958. 2-Thiobarbituric acid method for the measurement of rancidity in fishery products. II. The quantitative determination of malonaldehyde. Food Technology 12(1):9-12.

Appendix E

Sensory panel consent form

Sensory Panel Consent Form

1. The University of Idaho Human Assurance Committee has reviewed and found this study to be exempt.

2. The objective of this study was to evaluate the effects of extended aging and antioxidant treatment on beef. 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. This taste panel is part of research funded by the Idaho Beef Council.

3. You will be asked to evaluate 6 samples (approximately 1" x ½" x ½") per session for tenderness (1 = extremely tough to 9 = extremely tender), juiciness (1 = dry to 9 = juicy), and flavor (1 = bland to 9 = intense) using a 9 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. 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 aging beef.

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. Doumit.

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. Matthew E. Doumit
   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.

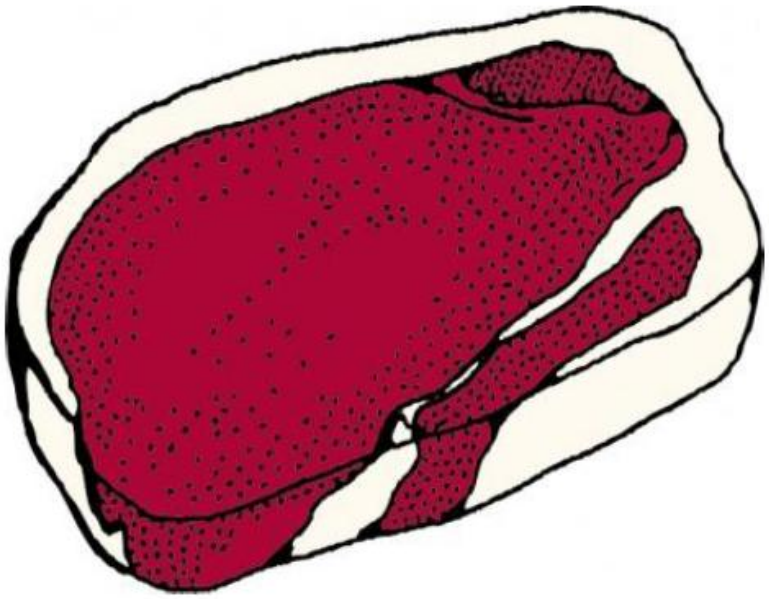
Participant Name: _____ Date: _____

Signature: _____ Date of Birth: _____

Appendix F

Sensory panel demographics questionnaire

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 G

Beef sensory panel questionnaire

BEEF 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:**

Appendix H

Calpain extraction

Solution:

Extraction buffer pH 8.3 (50ml)

Tris 0.6056g (100mM)
EDTA 0.1461g 10mM)
DTT 0.0772g (10mM)

Procedure:

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 2ml microcentrifuge tubes. 1.0 ml/tube, 2 tubes per sample and centrifuge at 8,800xg for 30 min @ 4ºC
3. Pour supernatant into 1.5ml microcentrifuge tubes and freeze in -80 freezer.

Appendix I

Casein zymography

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 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 (10ml)
trisHCL 0.1817g (150mM)
glycerol 2ml (20%)
DTT 0.01543g (10mM)
Bromophenol blue (0.02%) 0.25ml of 0.8% bromophenol blue

Running buffer pH 8.3 (1 liter)
trisHCL 3.0275g (25mM)
DTT 0.1543g (1mM)
Glycine 14.4g (192mM)
EDTA 0.2922 (1mM)

Incubation buffer pH 7.5 (250ml)
Tris HCL 1.5138g (50 mM)
DTT 0.3856g (10mM)
CaCl2 0.111g (4mM)

Separating gel 12.5%	Enough for 4 1 mm Gels**
1.5M Tris ph 8.8	6.25ml
30% Acrylamide	10.44ml
H2O	7.94mL
Casein	0.05g
APS	125µl
Temed	12.5µl

Stacking gel 4%	Enough for 4 1 mm Gels**
0.5M Tris pH 6.8	1.88ml
30% Acrylamide	1.0ml
H2O	4.55ml
APS	50µl
Temed	7.5µl

Procedure:

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 6 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. Stain for 1 hour in coomassie blue R250
5. Destain 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 J

Exempt certification for IRB project number 15-866

To: Matthew Doumit

From: Jennifer Walker
Chair, University of Idaho Institutional Review Board
University Research Office
Moscow, ID 83844-3010

Date: 8/31/2015 3:02:09 PM

Title: Strategies to improve beef tenderness by activating calpain-2 earlier postmortem

Project: 15-866

Certified: Certified as exempt under category 6 at 45 CFR 46.101(b)(6).

On behalf of the Institutional Review Board at the University of Idaho, I am pleased to inform you that the protocol for the above-named research project has been certified as exempt under category 6 at 45 CFR 46.101(b)(6).

This study may be conducted according to the protocol described in the Application without further review by the IRB. As specific instruments are developed, modify the protocol and upload the instruments in the portal. Every effort should be made to ensure that the project is conducted in a manner consistent with the three fundamental principles identified in the Belmont Report: respect for persons; beneficence; and justice.

It is important to note that certification of exemption is NOT approval by the IRB. Do not include the statement that the UI IRB has reviewed and approved the study for human subject participation. Remove all statements of IRB Approval and IRB contact information from study materials that will be disseminated to participants. Instead please indicate, 'The University of Idaho Institutional Review Board has Certified this project as Exempt.'

Certification of exemption is not to be construed as authorization to recruit participants or conduct research in schools or other institutions, including on Native Reserved lands or within Native Institutions, which have their own policies that require approvals before Human Subjects Research Projects can begin. This authorization must be obtained from the appropriate Tribal Government (or equivalent) and/or Institutional Administration. This may include independent review by a tribal or institutional IRB or equivalent. It is the investigator's responsibility to obtain all such necessary approvals and provide copies of these approvals to ORA, in order to allow the IRB to maintain current records.

As Principal Investigator, you are responsible for ensuring compliance with all applicable FERPA regulations, University of Idaho policies, state and federal regulations.

This certification is valid only for the study protocol as it was submitted to the ORA. Studies certified as Exempt are not subject to continuing review (this Certification does not expire). If any changes are made to the study protocol, you must submit the changes to the ORA for determination that the study remains Exempt before implementing the changes. Should there be significant changes in the protocol for this project, it will be necessary for you to submit an amendment to this protocol for review by the Committee using the Portal. If you have any additional questions about this process, please contact me through the portal's messaging system by clicking the 'Reply' button at either the top or bottom of this message.

Jennifer Walker

Jennifer Walker

Appendix K

Sensory panel consent form

Sensory Panel Consent Form

Cooking spray used contains soy

1. The University of Idaho Human Assurance Committee has reviewed and found this study to be exempt.

2. The objective of this study was to evaluate the effects of freezing and calcium addition on beef. 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. This taste panel is part of research funded by the Idaho Beef Council.

3. You will be asked to evaluate 4 samples (approximately 1" x ½" x ½") per session for tenderness (1 = extremely tough to 9 = extremely tender), juiciness (1 = dry to 9 = juicy), and flavor (1 = bland to 9 = intense) using a 9 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. 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 aging beef.

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. Doumit.

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. Matthew E. Doumit
   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: _____ Date of Birth: _____

Appendix L

Calcium concentration

Calcium concentration was determined following a procedure by Hopkins and Thompson (2001) with minor modifications.

Procedure:

1. Samples that had been stored at -80°C were weighed (2 g) and placed in a -20°C freezer at least 36 hours prior to calcium measurement.
2. Following equilibration to -20°C samples were placed in a refrigerator (4°C) for 20 min before being finely diced, placed on ice, and then centrifuged (Sorvall RT1 Centrifuge, Thermo Scientific, Waltham, MA) at 30,000 g at 5°C for 40 min.
3. 250 µl aliquots of the supernatant were mixed with 5 µl of Calcium Ion Strength Adjuster (Mettler Toledo, Woburn, MA).
4. These samples were then incubated in a water bath at room temperature for 5 min.
5. Calcium concentration was determined using a calcium selective electrode (PerfectION combination Ca^{2+}, Mettler Toledo, Woburn, MA) attached to a portable ion meter (Seven2Go pro, Mettler Toledo, Woburn, MA).
6. The electrode was allowed to soak in a 1×10^{-2} calcium solution at least 1 h prior to calibration and reading samples.
7. A calibration curve was created prior to each run. Calcium standards containing 8000 µM, 2000 µM, 1000 µM, 500 µM, 100 µM, and 50 µM calcium were made from Calcium ISE standard 1000 mg/l as Ca^{2+} (Mettler Toledo, Woburn, MA).