Meat and Muscle Biology™

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

B. J. Buseman[1], T. M. Weber[1], J. A. Nasados[1], P. D. Bass[1], J. B. Van Buren[1], J. M. Lancaster[1], J. H. Smart[1], M. E. Doumit[2], G. K. Murdoch[1], W. J. Price[2], K. Insausti[3], and M. J. Colle[1]*

[1]Department of Animal and Veterinary Sciences, University of Idaho, Moscow, Idaho 83844, United States
[2]College of Agriculture and Life Sciences, University of Idaho, Moscow, Idaho 83844, United States
[3]Agricultural Engineering School-IS FOOD, Public University of Navarra, Pamplona, Spain
*Corresponding author. Email: mjcolle@uidaho.edu (M.J. Colle)

Abstract: The objective of this study was to evaluate the effect of timing of electrical stimulation on free calcium concentration, calpain-2 activity, Warner-Bratzler shear force (WBSF), and consumer sensory analysis. Twenty-three beef steers were harvested and stimulated (S) using extra-low voltage or not stimulated (NS), at exsanguination and/or 1 h postmortem, resulting in 4 different stimulation treatments: NS-NS, NS-S, S-NS, or S-S. Samples were removed from the *longissimus lumborum* (LL) and *semimembranosus* (SM) for free calcium and calpain-2 analysis on days 1, 4, and 14 postmortem. WBSF and sensory analysis steaks were removed on day 4 and frozen (4 d) or aged to 14 d postmortem. Data were analyzed using the mixed model or generalized linear mixed model procedure of SAS (SAS Institute, Inc., Cary, NC), with significance determined at $P < 0.05$. There was a tendency for an aging-period-by-stimulation-treatment interaction for LL free calcium concentration ($P = 0.05$), and there was a significant difference between aging periods ($P < 0.01$). No difference was observed in free calcium concentration in the SM between stimulation treatments ($P = 0.44$); aging, however, significantly increased SM free calcium concentration ($P < 0.01$). Stimulation did not impact native calpain-2 activity in the LL ($P = 0.71$) or SM ($P = 0.89$). Stimulation treatment did not improve tenderness values for WBSF analysis for the LL ($P = 0.69$) or SM ($P = 0.61$) or consumer sensory analysis in the LL ($P = 0.56$) or SM ($P = 0.36$). A longer aging period tended to increase calpain-2 activity in the SM ($P = 0.08$), improve WBSF in the LL ($P = 0.09$), and significantly improve consumer tenderness scores in the SM ($P < 0.01$). In conclusion, the timing of electrical stimulation utilized in the current study tended to influence free calcium concentration in the LL but did not influence calpain-2 activity or beef tenderness. Aging, however, improved tenderness.

Key words: beef, electrical stimulation, calcium, calpain, tenderness
Meat and Muscle Biology 4(1): 24, 1–11 (2020) **doi:10.22175/mmb.10635**
Submitted 22 March 2020 Accepted 8 July 2020

Introduction

Consumers have consistently reported that tenderness is the most important quality trait they consider when consuming beef (Koohmaraie and Gesink, 2006). There are 3 primary factors that regulate meat tenderness: background toughness, the toughening phase, and the tenderization phase (Koohmaraie and Gesink, 2006; Veiseth-Kent et al., 2018). It is believed that proteases play a major role in the tenderization phase and determine the overall level of postmortem myofibrillar protein breakdown, including desmin, C-protein, tropomyosin, troponin T, troponin I, titin, nebulin, vimentin, gelsolin, vinculin, and α-actinin (Goll et al., 1991; Huang and Forsberg, 1998). Fragmentation of these proteins disrupts sarcomere integrity and influences postmortem tenderization.

Calpains are calcium-activated proteases that function to break down protein and are considered

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

Although multiple forms of calpain exist, calpain-1 and calpain-2 are most active in skeletal muscle (Aberle et al., 2012). Calpain-1 requires 3–50 μM of calcium to be activated, whereas calpain-2 requires 400–800 μM of calcium for half maximal activity (Goll et al., 1995; Goll et al., 2003). Due to its relatively low calcium requirement, calpain-1 is active early postmortem and is thought to be responsible for 95% of proteolytic activity in the first 7–14 d postmortem (Yang et al., 2018). Calpain-2 has been shown to activate after longer aging periods (Colle and Doumit, 2017). Calpain-2 requires increased levels of calcium to activate; however, extended aging results in calpain-2 activation and increases the likelihood of a more tender product (Goll et al., 1995; Goll et al., 2003; Colle and Doumit, 2017). Finding a method to increase free calcium concentration sooner postmortem should allow for earlier activation of calpain-2 and improved product tenderness.

Calcium is a positively charged ion. In theory, the use of an electrical current should pull enough free calcium out of the sarcoplasmic reticulum to activate calpain-2. Little research has been conducted analyzing the influence of electrical stimulation on free calcium concentration within muscle tissue. This, along with the lack of information available about the effect of extra-low voltage (ELV) stimulation on free calcium concentration, calpain-2 activity, and final product tenderness, provides an opportunity for further analysis.

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

Materials and Methods

Human subject participation in consumer panel

The University of Idaho Institutional Review Board certified this project as exempt.

Animal harvest and stimulation treatment

Twenty-three crossbred beef steers (Angus × Hereford × Simmental) were harvested at the University of Idaho Meat Laboratory under US Department of Agriculture (USDA) inspection. All steers were <30 mo of age based on dentition. Steers were harvested in 6 groups of 3 and 1 group of 5 over a 5-wk period. Carcasses were systematically assigned a stimulation treatment to account for harvest day. Following exsanguination, approximately half of the carcasses ($n = 11$) were electrically stimulated (21 volts for 20 s) with a Jarvis, Model ES-4, Low Voltage Beef Stimulator (Middletown, CT), and the remaining carcasses ($n = 12$) were not electrically stimulated. At exsanguination, the stimulator clamp was positioned on the nose of the carcass. At 1 h postmortem, immediately following the carcasses being split, one side of each carcass was stimulated, and the other side was not stimulated, resulting in 4 stimulation treatments: stimulated-stimulated (S-S; $n = 11$ sides), stimulated-not stimulated (S-NS; $n = 11$ sides), not stimulated-stimulated (NS-S; $n = 12$ sides), and not stimulated-not stimulated (NS-NS; $n = 12$ sides). At 1 h postmortem, the clamp was positioned on the *rhomboideus* of the stimulated side. The stimulator was grounded to the rail, which allowed the electrical current to travel through the entire carcass.

Carcass measurements

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

Calpain and free calcium analysis sampling procedures

On day 1 postmortem, samples for calpain and calcium analyses were collected from the SM and LL muscles. Samples were removed superficially from the proximal end of the SM, adjacent to the aitch bone. An approximately one-half-inch-thick outer crust was removed from the subprimal to ensure there was no outside influence from lactic acid spray or carcass dehydration. The LL samples were collected from the posterior surface of the 12th/13th rib interface following ribbing. The same sample was used to subsample on days 1, 4, and 14 to ensure consistency across sampling location. Between the aging periods, the remaining sample was vacuum packaged and stored at 0.5°C. On days 1, 4, and 14, samples were finely diced, snap frozen in liquid nitrogen, and then stored in 15 mL conical tubes at −76°C (Panasonic, MDF-C8V1-PA, Wood Dale, IL). A control sample to be analyzed on each calpain gel was obtained from the *sternocephalicus* muscle immediately following exsanguination on day 0 from the first non-stimulated carcass.

Fabrication

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

pH

The pH was recorded using a portable pH meter for food testing (SX811-SS, Apera Instruments, LLC, Columbus, OH) with a spear, puncture-type pH electrode (LabSen753, Apera Instruments, LLC, Columbus, OH). The meter was calibrated on a 3-point scale, using standards of 4.0, 7.0, and 10.0. Measurements were taken from the posterior end of the *longissimus thoracic*, at the 12th/13th rib interface of each side 24 h postmortem. Additionally, final pH was measured on the anterior end of the LL and the proximal end of the SM when steaks were cut on day 4 postmortem.

Calcium analysis

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

The calcium selective electrode (PerfectION combination Ca^{2+}, Mettler Toledo, Woburn, MA) was attached to a portable ion meter and used to measure the ionic strength of the solution. The electrode was soaked in a 1×10^{-2} calcium solution for approximately 1 h prior to calibration and reading samples. A calibration curve was formed prior to each run with calcium standards containing 10 µM, 50 µM, 100 µM, and 500 µM calcium, using the Calcium ISE standard 1,000 mg/l Ca^{2+} (Mettler Toledo, Woburn, MA). Samples were read and recorded along the calibration curve to determine total free calcium concentration (Hopkins and Thompson, 2001; Colle and Doumit, 2017).

Calpain extraction

On the day calpains were extracted, 1.0 g of muscle sample was combined with 3.0 mL of extraction buffer (100 mM Tris, 10 mM ethylenediaminetetraacetic acid [EDTA], 10 mM dithiothreitol [DTT] [pH 8.3]) and homogenized (POLYTRON® PT 10-35 GT; PT-DA 12/2EC-B154, Radnor, PA) on ice for three 15-s bursts, with a 15-s cooling period between each burst. One milliliter of the homogenate was then pipetted into 1.7 mL centrifuge tubes (SafeSeal Microcentrifuge Tubes, Sorenson BioScience, Inc., Salt Lake City, UT) and centrifuged (Sorvall RT1 Centrifuge, Thermo Scientific, Waltham, MA) at 8,800 × *g* for 30 min at 4°C. The remaining supernatant was placed in 1.7 mL microcentrifuge tubes and stored in a −76°C freezer for later analysis (Colle and Doumit, 2017).

Casein zymography

One-millimeter polyacrylamide gels were formed with a 12.5% separating gel containing 0.2% casein 25.2% 1.5 M Tris (pH 8.8), 42.1% of a 30% acrylamide solution, 32.0% millipore H_2O, 0.5% ammonium persulfate, and 0.1% tetramethylethylenediamine and were overlaid with a 4% stacking gel containing 25.1% 0.5 M Tris (pH 6.8), 13.4% of a 30% acrylamide solution, 60.8% Millipore H_2O, 0.7% ammonium persulfate, and 0.1% tetramethylethylenediamine. Casein gels were run at 100 V (Mini-PROTEAN® Tetra Vertical Eletrophoresis Cell) for 15 min with running buffer (25 mM Tris HCL, 1 mM DTT, 192 mM glycine, 1 mM EDTA) in an ice bath prior to loading samples. Frozen, homogenized samples were thawed at room temperature while gels were poured. Once thawed, 40 µL of supernatant and 10 µL of sample buffer (150 mM Tris HCL, 20% glycerol, 10 mM DTT, 0.02% of 0.8% bromophenol blue) were combined and mixed using a vortex mixer (VWR Vortexer 2, Scientific Industries, Inc., Bohemia, NY). Twenty microliters of sample and buffer combination were loaded into the casein minigels. One lane containing the D0 control sample was included on each gel. Gels were run at 100 V for a minimum of 3.5 h in an ice water bath. Gels were then incubated at room temperature in incubation buffer (50 mM Tris HCL, 10 mM DTT, 4 mM $CaCl_2$) for 17 h with slow shaking. Incubation buffer was replaced twice (30 min and 1 h). Incubation buffer was removed, and the gels were rinsed and stained for 1 h using Coomassie Blue R250 (BioRad). Following staining, gels were destained for 3 h with Coomassie Blue R250 Destaining Solution (BioRad). Gels were analyzed on a BioRad ChemiDoc MP_{TM} System (Hercules, CA). Images were inverted, and lanes and bands were detected on high sensitivity with manual adjustment to ensure that the entire sample was read. The volume of each band was recorded as a percentage of the control band run on each gel (Pomponio and Ertbjerg, 2012; Colle and Doumit, 2017).

Cooking

Steaks were thawed at 4°C for 24 h prior to cooking. Steaks were cooked on a clamshell-style countertop grill (Cuisinart Griddler Deluxe Model GR-150). Steaks were removed once an internal temperature of 66°C was reached and allowed to rest before a peak final temperature was recorded (32311-K Econo-$Temp_{TM}$ Thermocouple, Atkins, Middlefield, CT). Average final peak temperatures for the various analyses were as follows: LL consumer sensory analysis (72.2°C ± 0.2), SM consumer sensory analysis (69.7°C ± 0.3), LL WBSF (70.5°C ± 0.2), and SM WBSF (70.3°C ± 0.2). Raw steaks were weighed prior to grilling, and cooked steaks were weighed once they reached room temperature to determine percent cook loss.

$$\text{Percent cook loss} = \frac{\text{raw weight} - \text{cook weight}}{\text{raw weight}} \times 100.$$

WBSF

Following cooking, steaks were cooled to room temperature. Six 1.27-cm cores were removed using an oscillating drill press (Shop Fox, W1667 8-1/2", Bellingham, WA) with a 1.27-cm coring bit attachment) from both the superficial and deep portions of the SM, and 6 cores were removed from the LL, parallel to the muscle fibers avoiding connective tissue and fat. Cores were sheared (200 mm/min; Mecmesin BFG 1000N, Warner-Bratzler Meat Shear, G-R Manufacturing, Co., Manhattan, KS) perpendicular to the muscle fibers. Peak shear force was recorded for each sample and averaged to calculate a representative shear force for the whole steak.

Consumer sensory panel

Two consumer sensory panels, one for each muscle group, were implemented on the University of Idaho campus at the Margaret Ritchie School of Family and Consumer Sciences Mary Hall Niccolls Building Test Kitchen in accordance with the American Meat Science Association guidelines (AMSA, 2015) on 2 different days. Each panel consisted of 92 consumers, grouped into 20-min time slots with 4–8 per group. Steaks were cooked as previously described. Steaks were cooked in groups and kept in insulated bags with warming packs to ensure consistency in temperature for each panelist. Five, 1.27 cm × 1.27 cm × 2.54 cm cubes were cut from each steak, avoiding the edge of the steak and excessive connective tissue. Samples were assigned to panelists using an incomplete block design, with each panelist receiving 5 samples representing various stimulation treatment groups and aging periods, and panelists were asked to consume the samples in a predetermined order. Steaks were systematically assigned to panelists to ensure variation between treatments being sampled. Each steak was sampled by a minimum of 5 panelists. In addition to the sample, consumers were supplied with water and unsalted soda crackers as palate cleansers between

samples. Each sample was evaluated on a 10-point scale for overall acceptability, tenderness, juiciness, and flavor (1 = dislike extremely, 10 = like extremely).

Statistical analysis

Data were analyzed using a mixed model (carcass data, pH, calpain activity, WBSF, cook loss, and consumer sensory analysis) or a generalized linear mixed model for data that showed a non-normal distribution (free calcium concentration; Stroup, 2014). Within each model, carcass and side within carcass were random effects, while aging period, stimulation treatment, and their interaction were fixed effects. The relationship between calpain-2 activity and WBSF was assessed using Pearson correlation analysis. Significance was determined at ($P < 0.05$) and tendencies at ($P < 0.1$). For significant fixed effects, means were separated using pair-wise comparisons. All statistical analyses were carried out using SAS version 9.4 (SAS Institute, Inc., Cary, NC).

Results

Carcass data

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

pH

Stimulation treatment did not influence 24-h pH in the *longissimus thoracic* ($P = 0.49$), with an average final value of 5.50 ± 0.022. Additionally, significant differences were not seen between stimulation treatments on day 4 final pH in the LL ($P = 0.06$; Table 1) or SM ($P = 0.47$; Table 1).

Calcium analysis

There was a trend for an interaction between aging period and stimulation treatment for LL free calcium concentration ($P = 0.05$; Table 2). While all treatments had similar initial and final values, the NS-NS treatment showed a rapid increase relative to the other treatments at 4 d of aging. There was no interaction between aging and stimulation treatment for SM free calcium concentration ($P = 0.54$). Furthermore, free calcium concentration in the SM was not observed to be influenced by stimulation treatment ($P = 0.44$; Table 1). However, aging period did significantly increase SM free calcium concentration ($P < 0.01$; Table 3), with day 4 and day 14 having increased levels of free calcium concentration compared with day 1.

Table 1. Estimated mean values for calpain-2 activity, WBSF, percent cook loss, final pH, and *semimembranosus* free calcium concentration by stimulation treatment

	Stimulation Treatment					
	NS[1]-NS	NS-S[2]	S-NS	S-S	SEM	*P* Value
Longissimus lumborum						
Native calpain-2[3]	53.94	51.39	62.55	62.64	7.31	0.71
Autolyzed calpain-2[3]	0.06	0.06	0.00	0.00	0.38	0.67
WBSF[4]	3.12	3.11	2.88	3.03	0.20	0.69
Percent cook loss	16.56	16.74	19.15	17.58	1.44	0.58
Final pH	5.48	5.47	5.43	5.44	0.016	0.06
Semimembranosus						
Native calpain-2	63.82	65.95	66.02	63.50	6.80	0.89
Autolyzed calpain-2	1.01	0.17	4.89	5.60	3.66	0.76
WBSF	3.83	5.08	3.84	3.95	0.82	0.61
Percent cook loss	26.48	26.32	28.040	27.40	0.55	0.09
Final pH	5.47	5.46	5.42	5.45	0.022	0.47
Free calcium concentration[5]	102.51	113.30	121.51	113.30	1.08	0.44

[1]Not stimulated.

[2]Stimulated (21 V for 20 s).

[3]Values are percentages of native and autolyzed calpain-2 from day zero, *sternocephalicus* samples.

[4]Data reported in kg.

[5]Statistical inferences are based on log transformed data. Data reported in μM.

WBSF, Warner-Bratzler shear force.

Table 2. Trend for an interaction between stimulation treatment and aging period on estimated free calcium concentration in the *longissimus lumborum*

	Stimulation Treatment					
Aging Period (d)	NS[1]-NS	NS-S[2]	S-NS	S-S	SEM	*P* Value
1	23.10	22.87	25.03	29.37	1.18	0.05
4	56.83	42.95	25.53	30.88		
14	58.56	59.74	57.40	57.40		

[1]Not stimulated.

[2]Stimulated (210 V for 20 s).

All statistical inferences are based on log transformed data. Data are reported in μM.

Table 3. Estimated mean calpain activity and *semimembranosus* free calcium concentration by aging period

	Aging Period (d)				
	1	4	14	SEM	*P* Value
Longissimus lumborum					
Native calpain-2[1]	62.69	55.43	54.77	5.11	0.11
Autolyzed calpain-2	0.00	0.00	0.08	0.03	0.06
Semimembranosus					
Native calpain-2	63.62	62.68	68.17	4.72	0.08
Autolyzed calpain-2	0.00[b]	1.64[b]	7.72[a]	2.78	0.03
Free calcium concentration[2]	95.58[b]	116.75[a]	127.74[a]	1.06	<0.01

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

[2]Statistical inferences are based on log transformed data. Data reported in μM.

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

Calpain-2 analysis

There was no aging-period-by-stimulation-treatment interaction observed for native calpain-2 activity in the LL ($P = 0.57$) or the SM ($P = 0.70$). Furthermore, stimulation treatment did not significantly influence native calpain-2 activity in the LL ($P = 0.71$) or the SM ($P = 0.89$; Table 1). In the current study, aging period was not significant in increasing native calpain-2 activity in the LL ($P = 0.11$); however, aging did show a tendency for increased native calpain-2 activity in the SM on day 14 ($P = 0.08$; Table 3).

Stimulation treatment did not show an interaction with aging on autolyzed calpain-2 activity in the LL ($P = 0.46$) or the SM ($P = 0.44$). Stimulation treatment did not significantly influence autolyzed calpain-2 activity in the LL ($P = 0.67$) or the SM ($P = 0.76$; Table 1). Aging period tended to increase activity in the LL ($P = 0.06$) and significantly increased autolyzed calpain-2 activity in the SM ($P = 0.03$; Table 3).

WBSF

No differences in WBSF were seen between the deep and superficial portions of the SM ($P = 0.17$), so data were pooled. No aging-period-by-stimulation-treatment interaction was observed for WBSF in the LL ($P = 0.09$) or SM ($P = 0.40$). Additionally, stimulation treatment was not significant for WBSF in the LL ($P = 0.69$) or SM ($P = 0.61$; Table 1). There was a significant difference between aging period in the LL ($P < 0.01$), with steaks aged 14 d being more tender than those aged only 4 d (Table 4). Aging period was not observed to be significant in influencing WBSF values of the SM ($P = 0.61$; Table 4). Calpain-2 activity was not correlated with WBSF tenderness in the LL ($P = 0.66$) or SM ($P = 0.34$).

Cook loss

No interaction was observed between aging period and stimulation treatment on cook loss in the LL ($P = 0.77$) or the SM ($P = 0.91$). In the LL, cook loss was not observed to be influenced by stimulation treatment ($P = 0.58$; Table 1) or aging period ($P = 0.13$; Table 4). Stimulation treatment tended to influence percent cook loss in the SM ($P = 0.09$; Table 1). Additionally, no differences in percent cook loss were observed between aging periods in the SM ($P = 0.49$).

Consumer sensory panel

Demographics of the two consumer sensory panels are summarized in Table 5.

In the LL, no interaction was observed between aging and stimulation treatment in terms of consumer

Table 4. Estimated mean WBSF and percent cook loss by aging period

	Aging Period (d)			
	4	14	SEM	*P* Value
Longissimus lumborum				
WBSF[1]	3.29[a]	2.78[b]	0.12	<0.01
Percent cook loss	16.63	18.39	1.00	0.13
Semimembranosus				
WBSF	4.75	3.61	0.56	0.61
Percent cook loss	27.25	26.87	0.39	0.49

[1]Data reported in kg.

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

WBSF, Warner-Bratzler shear force.

Table 5. Consumer panel demographics ($n = 92$/panel)

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

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

[2]Please indicate the form in which you most commonly consume beef: Ground, Roast, Steak, Other.

Table 6. Estimated mean consumer sensory panel scores by stimulation treatment

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

[1]Not stimulated.

[2]Stimulated (21 V for 20 s).

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

Table 7. Estimated mean consumer sensory panel scores by aging period

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

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

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

sensory analysis for overall acceptability ($P = 0.65$), tenderness ($P = 0.60$), juiciness ($P = 0.78$), or flavor ($P = 0.56$). Additionally, no interaction was observed in the SM between aging and stimulation treatment in terms of consumer sensory analysis for overall acceptability ($P = 0.89$), tenderness ($P = 0.52$), juiciness ($P = 0.12$), or flavor ($P = 0.18$). No influence by stimulation treatment on consumer sensory analysis was observed within the LL or SM for overall acceptability ($P = 0.54$; $P = 0.53$), tenderness ($P = 0.56$; $P = 0.36$), or flavor ($P = 0.85$; $P = 0.82$), respectively (Table 6). Although there was no detectable stimulation treatment influence on consumer sensory analysis for juiciness in the LL ($P = 0.90$), there was a tendency for stimulation to decrease consumer acceptability for juiciness in the SM ($P = 0.08$). In the LL, aging did not show a significant influence on overall acceptability ($P = 0.35$), tenderness ($P = 0.71$), juiciness ($P = 0.86$), or flavor ($P = 0.90$; Table 7). Consumers preferred the tenderness of SM samples aged 14 d over those aged 4 d ($P < 0.01$) but did not distinguish differences in overall acceptability ($P = 0.43$), juiciness ($P = 0.73$), or flavor ($P = 0.29$).

Discussion

Electrical stimulation is commonly used in packing plants prior to carcasses entering the cooler to improve final product tenderness by causing extreme muscle contractions and subsequent muscle fiber tearing (Luo et al., 2008). There are 3 types of electrical stimulation utilized in beef processing: ELV (<100 V), low voltage

(LV) (100–110 V), and high voltage (HV) (>110 V, often 500–1,000+ V) (Adeyemi and Sazili, 2014). Although most studies analyzing the effect of electrical stimulation have seen an improvement in product tenderness regardless of stimulation type (Powell et al., 1984; Eilers et al., 1996; Hwang and Thompson, 2001), traditionally, LV and HV are the most common forms researched. A lack of research is available analyzing the effects of ELV stimulation. Great variability exists between methods of stimulation, including voltage, impulse, duration, frequency, timing, and location of electrode (Adeyemi and Sazili, 2014).

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

Hwang and Thompson (2001) speculated that massive catabolism caused by muscle contraction during stimulation can lead to increased levels of free calcium. Based on this hypothesis, it was anticipated that samples from S-NS and S-S treatment groups would show elevated levels of free calcium concentration compared to samples from NS-NS and NS-S treatment groups. In the present study, however, day 4 LL samples from S-NS and S-S treatment groups showed decreased levels of free calcium compared to samples from the NS-NS and NS-S treatment group. Samples from the S-NS and S-S treatment groups did not show increases in free calcium concentration until 14 d of aging. These results were contrary to the hypothesis of this study.

During the conversion of muscle to meat, adenosine triphosphate (ATP) is depleted, and therefore calcium reuptake into the sarcoplasmic reticulum eventually subsides (Aberle et al., 2012). Stimulation at exsanguination may have caused an increase in free calcium concentration; however, there was likely enough ATP available in the system to uptake the freely available calcium. Stimulation at 1 h postmortem, or following a further delay after exsanguination, may allow time for ATP to begin to deplete and the reuptake of calcium to slow, displaying increased levels of free calcium concentration.

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

Calpains are considered the leading protease in postmortem tenderization (Koohmaraie et al., 1995). Free calcium must be available in order to activate calpains (Goll et al., 2003). Due to a relatively low free calcium concentration requirement for activation (3–50 μM), calpain-1 is active early postmortem and is thought to be responsible for 95% of proteolytic activity in the first 7–14 d postmortem (Goll et al., 2003; Yang et al., 2018). Calpain-2 requires increased levels of free calcium (400–800 μM) for activation (Goll et al., 2003); therefore, extended aging (>28 d) results in calpain-2 activation and increases the likelihood of producing a more tender product (Colle and Doumit, 2017). In this study, calcium levels were highest on day 4 and 14 in the SM (116.75 ± 1.06 and 127.74 ± 1.06 μM, respectively). Although free calcium concentration never reached the required levels for activation, as described by Goll et al. (2003), calpain-2 was still observed in all samples across stimulation treatments and aging periods. No increase in native or autolyzed calpain-2 activity was observed in the LL or SM when electrical stimulation was used. This observation is supported by the work of Li et al. (2012), who found that calpain-2 activity did not change with LV stimulation (80 V, 35 s) 30 min postmortem. In the present study, there were significantly increased levels of autolyzed calpain-2 in SM samples aged 14 d rather than those aged 1 or 4 d. Additionally, the LL tended to have increased levels of autolyzed calpain-2 in samples aged 14 d compared with those only aged 1 or 4 d. The observations of this study are supported by studies showing increased levels of autolyzed calpain-2 as the product is aged (Goll et al., 2003; Colle and Doumit, 2017). This was also supported by Hwang and Thompson (2001), who evaluated the effects of various voltage levels at different time periods (HV/LV, 3 and 40 min postmortem; HV, 40 and 60 min postmortem; LV, 40 min postmortem). Activity level of calpain-2 remained consistent irrespective of stimulation type or time. However, the study by Hwang and Thompson (2001) only evaluated calpain activity prior to stimulation, post stimulation, and at 24 h postmortem.

No significant interaction was observed between stimulation treatment and aging period on WBSF values of the LL or SM. This differs from the observations of Razminowicz et al. (2008), who found that the *longissimus dorsi* from electrically stimulated sides showed decreased WBSF values at days 2 and 15 postmortem compared with unstimulated sides. However,

Razminowicz et al. (2008) only looked at one HV (230 V, 60 Hz, 30 s) stimulation at 30 min postmortem. Additionally, Li et al. (2012) found that electrical stimulation (80 V, 35 s, 30 min postmortem) accelerated meat tenderization specifically at 24 and 48 h postmortem and tended to improve tenderness up to 7 d postmortem. Hwang and Thompson (2001) noted that electrical stimulation increased meat tenderness, but early application of stimulation (3 min postmortem) had increased WBSF values compared with stimulation at 40 or 60 min postmortem. While little research is available specifically evaluating the effects of ELV stimulation, Powell et al. (1984) did see improvements in WBSF values when analyzing 3 different ELV stimulation treatments (45 V, 40 Hz, 90 s continuous; 60 s, 2 s on and 1 s off; or 40 s, 3 s on and 1 s off), irrespective of treatment type. Although an improvement in WBSF was seen when ELV stimulation was used, the WBSF value for the SM and *longissimus dorsi* was 8 kg, depicting a very tough product (Powell et al., 1984). Additionally, Eikelenboom et al. (1985) observed no difference in overall tenderness between type of stimulation administered at exsanguination, with consumers preferring both LV and HV treated samples over the non-stimulated control. Based on previous research and observations of this study, utilizing LV or HV stimulation treatments following a post-exsanguination delay may be the most effective method to improve tenderness. More research needs to be conducted to identify the ideal voltage and timing of stimulation to maximize final product tenderness.

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

Aging treatment in this study improved LL WBSF, with steaks aged 14 d being more tender (decreased WBSF value) than steaks aged 4 d, similar to what Hwang and Thompson found (2001). This is likely because LL tenderness has been found to improve during aging for up to 14 d (Eilers et al., 1996; Bratcher et al., 2005; Colle et al., 2016). Another study showed lower WBSF values in LL steaks aged 12 d than those aged 6, but no difference between 12 and 18 d of aging (Eilers et al., 1996). Interestingly, consumers in this study were not able to detect differences in tenderness in the LL. This was surprising, considering that there was an improvement of 0.51 kg of WBSF in steaks aged 14 d compared to those aged only 4 d. Though there was no observable difference detected, it is important to note that consumers consider 4.3 kg WBSF acceptable for beef tenderness (Miller et al., 1995). All LL steaks in the current study, regardless of stimulation treatment or aging period, fell below the threshold to qualify to be marketed as "Certified Very Tender" (WBSF < 3.9 kg; ASTM, 2011). Improvements in livestock genetics, animal handling, and processing is likely leading to improved product tenderness prior to stimulation.

Electrical stimulation can disrupt muscle fibers, leading to poorer structural integrity (Savell et al., 1978). In addition to impacting tenderness, muscle fiber disruption as well as accelerated pH decline may allow for increased levels of purge cook loss. In this study, there was a tendency observed for stimulation treatment to influence consumer perception of juiciness, with S-S steaks tending to be less juicy than NS-NS steaks. Interestingly, in the SM there was a tendency for S-NS steaks to have an increase in percent cook loss when compared to NS-NS and NS-S steaks. Other researchers have observed varied responses when evaluating the influence of electrical stimulation on juiciness; some found no influence (Lee et al., 2000; Hwang and Thompson, 2001), while others observed a decrease in juiciness when stimulation was used (Savell et al., 1978). Inconsistencies such as freezing and thawing techniques and cooking methods can be implicating factors outside of the stimulation treatment.

Conclusions

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

Acknowledgments

The researchers would like to acknowledge Vandal Brand Meats, the Margaret Ritchie School of Family and Consumer Sciences, and the AVS Meat Science Laboratory undergraduate employees for their assistance with this project. This work is/was supported by the USDA National Institute of Food and Agriculture, Hatch Project W4177 Enhancing the Competitiveness and Value of U.S. Beef. Support was also received from the Idaho Experiment Station.

Literature Cited

Aberle, E., J. Forrest, D. Gerrard, and E. Mills. 2012. Principles of meat science. 5th edition. W.H. Freeman and Company, Dubuque, IA. p. 89–91.

Adeyemi, K. D., and A. Q. Sazili. 2014. Efficacy of carcass electrical stimulation in meat quality enhancement: A review. Asian Austral. J. Anim. 27:447–456. https://doi.org/10.5713/ajas.2013.13463.

AMSA. 2015. Research guidelines for cookery, sensory evaluation, and instrumental tenderness measurements of meat. American Meat Science Association. https://meatscience.org/docs/default-source/publications-resources/amsa-sensory-and-tenderness-evaluation-guidelines/research-guide/amsa-research-guidelines-for-cookery-and-evaluation-1-01.pdf?sfvrsn=8ff581b3_8. (Accessed 13 March 2020).

ASTM. 2011. Standard specification for tenderness marketing claims associated with meat cuts derived from beef. Annual Book of ASTM Standards. https://www.ams.usda.gov/sites/default/files/media/F2925-11%20Standard%20Specification%20for%20Tenderness.pdf. (Accessed).

Bertelsen, B. N.D. USDA quality grades and yield grades. U.S. Premium Beef. http://www.uspb.com/DocumentItem.aspx?ID=21#:~:text=Yield%20grades%20can%20be%20determined,ribeye%20area%20in%20square%20inches. (Accessed 12 March 2020).

Bratcher, C. L., D. D. Johnson, R. C. Littell, and B. L. Gwartney. 2005. The effects of quality grade, aging, and location within muscle on Warner-Bratzler shear force in beef muscles of locomotion. Meat Sci. 70:279–284. https://doi.org/10.1016/j.meatsci.2005.01.013.

Chrystall, B. B., and C. J. Hagyard. 1976. Electrical stimulation and lamb tenderness. New Zeal. J. Agr. Res. 19:7–11. https://doi.org/10.1080/00288233.1976.10421039.

Colle, M. J., and M. E. Doumit. 2017. Effect of extended aging on calpain-1 and -2 activity in beef longissimus lumborum and semimembranosus muscles. Meat Sci. 131:142–145. https://doi.org/10.1016/j.meatsci.2017.05.014.

Colle, M. J., J. A. Nasados, J. M. Rogers, D. M. Kerby, M. M. Colle, J. B. Van Buren, R. P. Richard, G. K. Murdoch, C. J. Williams, and M. E. Doumit. 2018. Strategies to improve beef tenderness by activating calpain-2 earlier postmortem. J. Anim. Sci. 135:36–41. https://doi.org/10.1016/j.meatsci.2017.08.008.

Colle, M. J., R. P. Richard, K. M. Killinger, J. C. Bohlscheid, A. R. Gray, W. I. Loucks, R. N. Day, A. S. Cochran, J. A. Nasados, and M. E. Doumit. 2016. Influence of extended aging on beef quality characteristics and sensory perception of steaks from the biceps femoris and semimembranosus. Meat Sci. 119:110–117. https://doi.org/10.1016/j.meatsci.2016.04.028.

Eikelenboom, G., F. J. M. Smulders, and H. Ruderus. 1985. The effect of high and low voltage electrical stimulation on beef quality. Meat Sci. 15:247–254.

Eilers, J. D., J. D. Tatum, J. B. Morgan, and G. C. Smith. 1996. Modification of early-postmortem muscle Ph and use of postmortem aging to improve beef tenderness. J. Anim Sci. 74:790–798. https://doi.org/10.2527/1996.744790x.

Goll, D. E., W. R. Dayton, I. Singh, and R. M. Robson. 1991. Studies of the α-actinin/actin interaction in the z-disk by using calpain. J. Biol. Chem. 266:8501–8510.

Goll, D. E., V. F. Thompson, H. Li, W. Wei, and J. Cong. 2003. The calpain system. Am. J. Phys. 83:731–801. https://doi.org/10.1152/physrev.00029.2002.

Goll, D. E., V. F. Thompson, R. G. Taylor, T. Edmunds, and J. Cong. 1995. Properties and biological regulation of the calpain system. EC\CE/AMST, the Netherlands. p. 47–68.

Goll, D. E., V. F. Thompson, R. G. Taylor, and A. Ouali. 1998. The calpain system and skeletal muscle growth. Can. J. Anim. Sci. 78:503–512. https://doi.org/10.4141/A98-081.

Hopkins, D. L., and J. M. Thompson. 2001. Inhibition of protease activity 2. Degradation of myofibrillar proteins, myofibril examination and determination of free calcium levels. Meat Sci. 59:199–209. https://doi.org/10.1016%2FS0309-1740(01)00071-7.

Huang, J., and N. Forsberg. 1998. Role of calpain in skeletal muscle protein degradation. P. Natl. Acad. Sci. USA. 95:12100–12105. https://doi.org/10.1073/pnas.95.21.12100.

Hwang, I. H., and J.M. Thompson. 2001. The effect of time and type of electrical stimulation on the calpain system and meat tenderness in beef longissimus dorsi muscle. Meat Sci. 58:135–144. https://doi.org/10.1016%2FS0309-1740(00)00141-8.

Koohmaraie, M., and G. H. Geesink. 2006. Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. Meat Sci. 74:34–43. https://doi.org/10.1016/j.meatsi.2006.04.025.

Koohmaraie, M., T. L. Wheeler, and S. D. Shackelford. 1995. Beef tenderness: Regulation and Prediction. Proceedings, CSIRO Meat'94 A. 4:1–20.

Lee, S., P. Polidori, R. G. Kauffman, and B. C. Kim. 2000. Low-voltage electrical stimulation effects on proteolysis and lamb tenderness. J. Food Sci. 65:786–790. https://doi.org/10.1016/s0309-1740(99)00044-3.

Li, C. B., J. Li, G. H. Zhou, R. Lametsch, P. Ertbjerg, D. A. Brüggemann, H. G. Huang, A. H. Karlsson, M. Hviid, and K. Lundström. 2012. Electrical stimulation affects metabolic enzyme phosphorylation, protease activation, and meat tenderization in beef. J. Anim. Sci. 90:1638–1649. https://doi.org/10.2527/jas2011-4514.

Luo, X, Y. Zhu, and G. Zhou. 2008. Electron microscopy of contractile bands in low voltage electrical stimulation beef.

Meat Sci. 80:948–951. https://doi.org/10.1016/j.meatsci.2008.03.017.

Miller, M. F., L. C. Hoover, K. D. Cook, A. L. Guerra, K. L. Huffman, K. S. Tinney, C. B. Ramsey, H. C. Brittin, and L. M. Huffman. 1995. Consumer acceptability of beef steak tenderness in the home and restaurant. J. Food Sci. 60:963–965. https://doi.org/10.1111/j.1365-2621.1995.tb06271.x.

Pomponio, L., and P. Ertbjerg. 2012. The effect of temperature on the activity of μ- and m-calpain and calpastatin during post-mortem storage of porcine longissimus muscle. Meat Sci. 91:50–55. https://doi.org/10.1016/j.meatsci.2011.12.005.

Powell, V. H., R. F. Dickinson, N. G. McPhail, P. E. Bouton, and P. V. Harris. 1984. Evaluation of extra low voltage electrical stimulation systems for bovine carcasses. J. Food Sci. 49:363–365. https://doi.org/10.1111/j.1365-2621.1984.tb12422.x.

Razminowicz, R. H., M. Kreuzer, and M. R. Scheeder. 2008. Effect of electrical stimulation, delayed chilling and post-mortem aging on the quality of M. longissimus dorsi and M. biceps femoris of grass-fed steers. J. Sci. Food Agr. 88:1344–1353. https://doi.org/10.1002/jsfa.3222.

Savell, J. W., T. R. Dutson, G. C. Smith, and Z. L. Carpenter. 1978. Structural changes in electrically stimulated beef muscle. J. Food Sci. 43:1606–1607. https://doi.org/10.1111/j.1365-2621.1978.tb02553.x.

Stroup, W. W. 2014. Rethinking the analysis of non-normal data in plant and soil science. Agron. J. 106:1–17. https://doi.org/10.2134/agronj2013.0342.

USDA. 2020. Beef grading shields. Agricultural Marketing Service, United States Department of Agriculture. https://www.ams.usda.gov/grades-standards/beef/shields-and-marbling-pictures. (Accessed 12 March 2020).

Uytterhaegen, L., E. Claeys, and D. Demeyer. 1992. The effect of electrical stimulation on beef tenderness, protease activity and myofibrillar protein fragmentation. Biochimie. 74:275–281. https://doi.org/10.1016/0300-9084(92)90126-Y.

Veiseth-Kent, E., M. E. Pedersen, S. B. Ronning, R. Rodbotten. 2018. Can postmortem proteolysis explain tenderness differences in various bovine muscles? Meat Sci. 137:114–122. https://doi.org/10.1016/j.meatsci.2017.11.011.

Yang, J., D. Dashdorj, and I. Hwang. 2018. Effect of the calpain system on volatile flavor compounds in the beef longissimus lumborum muscle. Korean J. Food. An. 38:515–529. https://doi.org/10.5851/kosfa.2018.38.3.515.