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Metabolomic Investigation of Tenderness and Aging Response in Beef Longissimus Steaks

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Abstract: A study was conducted to identify molecular changes reflective of beef tenderness variation and tenderization during postmortem aging. Carcasses (U.S. Select) were selected to represent extremes in tenderness (n = 20; 10 per class). Two pairs of adjacent *longissimus lumborum* steaks from each strip loin were blocked by location and assigned to each aging time (2, 7, 14, or 28 d postmortem). One steak from each pair was designated for slice shear force determination and the other was used for sarcomere length, western blotting for desmin, and non-targeted LC- and GC–MS metabolite profiling. Tough steaks had higher ($P < 0.001$) slice shear force values than tender steaks, and increasing aging time decreased ($P < 0.001$) slice shear force values. Tender steaks had a greater ($P < 10^{-}4$) proportion of desmin degraded than tough steaks, and increasing aging time increased ($P < 10^{-22}$) desmin degradation in steaks from both classes. From 2,562 profiled metabolites, 102 metabolites were included in the final analysis after statistical screening. Twenty-eight metabolites could be annotated and loosely categorized into amino acids/peptides (n = 16), metabolism intermediates (n = 7), glycosides (n = 4), and fatty acids and phospholipids (n = 3). Amino acids were primarily associated with desmin degradation. Increased glucose levels were strongly associated to the tender classification and moderately associated to increased proteolysis, while increased glucose-6-phosphate was strongly related to the tender class but was related to decreased proteolysis. Increased malic acid was strongly associated to the tough classification, increased slice shear force, and decreased proteolysis. Increased levels of 3-phosphoglyceric acid and glycerol-3-phosphate was moderately associated with increased slice shear force and decreased proteolysis. These data indicate that accumulation of amino acids during aging is strongly related to postmortem proteolysis and may provide evidence of the fate of proteins degraded postmortem. Measures of glucose, glucose-6-phosphate, and malic acid concentrations may provide a metabolic fingerprint indicative of tenderness differences in beef longissimus

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Introduction

Tenderness is a primary driver of consumer satisfaction of beef products (Lorenzen et al., 1996; Boleman et al., 1997; Lusk et al., 2001). Thus, mechanisms regulating meat tenderness are of great interest and have been the subject of considerable study (Koohmaraie, 1996; Geesink and Koohmaraie, 1999; Huff Lonergan et al., 2010). Two component traits, sarcomere length (Herring et al., 1965; Hostetler et al., 1972; Smulders et al., 1990) and postmortem degradation of cytoskeletal proteins (Huff-Lonergan et al., 1996; Robson et

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al., 1997; Geesink and Koohmaraie, 1999) have been consistently implicated as affecting meat tenderness. These component traits have been studied to further the understanding of meat tenderness and its regulation (Rhee et al., 2004; King et al., 2009c; Grayson et al., 2014) although in general, these traits explain a relatively small part of the variation in tenderness. In some cases, these traits fail to provide insight into observed treatment differences in tenderness (Shackelford et al., 2012). Moreover, the measurement of sarcomere length and postmortem proteolysis are not conducive to high-throughput processing of large numbers of samples.

Numerous processes in ante-, peri-, and postmortem muscle impact meat tenderness, either directly or indirectly. However, many of the factors affecting meat tenderness are not well understood, and likely many remain unknown. Further identification and characterization of these factors is needed to explain a greater proportion of the variation in meat tenderness. Non-targeted molecular profiling techniques using mass spectrometry enable broad characterization of metabolites in meat that are reflective of differences in tenderness and tenderization during aging. Here, we have applied non-targeted molecular profiling using a combination of liquid and gas chromatography coupled to mass spectrometry to the analysis of meat with the goal of improving our understanding of the mechanisms regulating beef tenderness and identifying biomarkers that could be used as a tool to facilitate future studies of beef tenderness.

Materials and Methods

Carcasses were selected for inclusion in the present experiment as they were presented for grading at a large-scale, USDA inspected facility. Thus, Animal Care and Use approval was not sought.

Sample selection and handling

As carcasses were presented for grading (approximately 24 h postmortem), U.S. Select carcasses were evaluated using the tenderness prediction model in the VBG 2000 GigE beef grading system (Shackelford et al., 2003). Carcasses (n = 40) were selected from commercial production lots to have either tender (predicted slice shear force < 19 kg; n = 10) or tough (predicted slice shear force > 19.5 kg; n = 30) predicted slice shear force values. Our experience with the tenderness prediction model is that some carcasses predicted to be tough have low slice shear force values. Thus, we selected a greater number of carcasses predicted to be tough to ensure that a class of carcasses with adequately high slice hear force values were obtained. A total of 93 U.S. Select carcasses were screened to identify the 40 carcasses included in the experiment. As carcasses were fabricated, beef, loin, strip loin subprimals (similar to IMPS# 180; USDA, 2014) were obtained from the left side of each carcass, vacuum packaged, and transported under refrigeration to the U.S. Meat Animal Research Center. The next day, (2 d postmortem) subprimals were unpackaged and trimmed free of subcutaneous fat and accessory muscles. The *longissimus lumborum* muscle was cut into 28-mm-thick steaks perpendicular to the long axis of the muscle using a Graselli NSL400 slicer (Grasselli-SSI, Throop, PA). Identity of steak location was maintained. From each of the anterior and posterior halves of the *longissimus lumborum*, 4 pairs of adjacent steaks were blocked by location and assigned to one of 4 aging times (2, 7, 14, and 28 d postmortem) at 1°C. Thus, there were 2 pairs of adjacent steaks from each strip loin assigned to each aging time. Because vacuum packaging steaks can compress steaks, reducing the repeatability of belt grill cookery, and consequently, slice shear force determination, pairs of steaks were placed on plastic trays and packaged in oxygen impermeable bags (Clarity 3 mil standard barrier; Prime Source, St. Louis, MO; oxygen transmission rate = 0.01 mL/cm^2 per 24 h × 23°C and 1 atm) flushed with 75% N_2, 25% CO^2 (Linweld, Hastings, NE) to prevent spoilage during aging. One steak from each pair was designated for slice shear force determination and the other was designated for metabolite profiling.

After each aging time, packages of steaks assigned to that aging time were opened and steaks destined for slice shear force were transferred to a 5°C refrigerator and allowed to equilibrate before they were cooked on an electric belt grill as described by Wheeler et al. (1998). Slice shear force was measured using the protocol described by Shackelford et al. (1999). Steaks designated for metabolite profiling were trimmed free of epimysium and visible fat, diced, frozen in liquid nitrogen, and stored at –80°C until further analysis.

Slice shear force values from all aging times were utilized to identify the carcasses with the lowest (most tender) and highest (toughest) slice shear force values to create two tenderness classes (n = 10 for both the tough and tender class). Aliquots of diced, frozen samples from the steaks adjacent to the slice shear force steak at each aging time were transported to the Colorado State University Proteomic and Metabolomics laboratory. Additional diced and frozen aliquots were pulverized in liquid nitrogen into a ho-

mogenous powder and used for sarcomere length and desmin degradation determination.

Sarcomere length and mmunoblotting of desmin

Sarcomere length determination was made using the helium-neon laser diffraction method as described by Cross et al. (1981). Small aliquots of powdered tissue (approximately 0.5g; $n = 6$ per sample) were placed on microscope slides. Approximately 150 µL of 0.2 M sucrose in 0.1 M NaHPO4 buffer was added to each aliquot prior to determining sarcomere length. Six sarcomere laser diffraction patterns were recorded per aliquot on paper, for a total of 36 sarcomere lengths per sample. Diffraction patterns were scanned into JPEG (Joint Photographic Experts Group) images, and Image Pro (Media Cybernetics, Inc., Rockville, MD) software was used to measure the distance between primary diffraction bands and calculate sarcomere length using the equation reported by Cross et al. (1981).

Immuno-blotting for desmin was performed on a whole muscle extract of each sample to determine the extent of postmortem proteolysis (Wheeler et al., 2002). Each combination of tenderness class and aging time was represented on each gel. Potential lane effects were mitigated by blocking lane assignment of each treatment across gels. At-death standards from *longissimus lumborum* (collected within 40 min of exsanguination) were run in the center and two outermost lanes on each gel. The chemi-luminescent intensity of the entire desmin band of each sample was compared to the mean chemi-luminescent intensity of the entire desmin band of the at-death standards within each blot to calculate the percentage of desmin degraded in each sample.

Metabolomic analysis

Diced, frozen samples were lyophilized at –80°C and 0.008 mBar for 72 h before being homogenized with liquid nitrogen. Homogenized tissue (30 mg) was weighed into 2 mL glass vials and extracted in 1.5 mL 80% methanol 20% water (v/v) and vortexed at 4°C for 2 h. After centrifugation (3000 × *g*) for 20 min, the supernatant was transferred to a new 2 mL glass vial.

UPLC-MS Analysis

Extracts (300 µL) were dried under nitrogen gas and resuspended in 100 µL of 80% methanol 20% water (v/v). Samples (2 µL) were injected in duplicate in randomized order and separated using a Waters Acquity UPLC CSH Phenyl Hexyl column (1.7 µM, 1.0 × 100 mm), employing a gradient from solvent A (2 mM ammonium hydroxide, 0.1% formic acid) to solvent B (Acetonitrile, 0.1% formic acid). A pooled quality control (QC) sample comprised of equal amounts of all samples was injected after every 10 experimental samples. Injections were made in 100% A, held at 100% A for 1 min, ramped to 98% B over 12 min, held at 98% B for 3 min, and then returned to starting conditions over 0.05 min and allowed to re-equilibrate for 3.95 min, with a 200 µL/min constant flow rate. The column and samples were held at 65°C and 6°C, respectively. The column eluent was infused into a Waters Xevo G2 Q-TOF-MS with an electrospray source operating in positive mode, scanning 50 to 2000 m/z at 0.2 s per scan, alternating between MS (6V collision energy) and MSE mode (15 to 30V ramp). Calibration was performed using sodium iodide with 1 ppm mass accuracy. The capillary voltage was held at 2200 V, source temp at 150°C, and nitrogen desolvation temp a 350°C with a flow rate of 800 L/h.

Gas Chromatography-Mass Spectrometry Analysis

Extracts (400 µL) were dried under nitrogen, resuspended in 50 µL of pyridine containing 25 mg/mL of methoxyaminehydrochloride, incubated at 60°C for 45 min, sonicated for 10 min, and incubated for an additional 45 min at 60°C. Next, 50 µL of N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS, Thermo Scientific) was added and samples were incubated at 60°C for 30 min, centrifuged at 3,000 × *g* for 5 min, cooled to room temperature, and 80 µL of the supernatant was transferred to a 150 µL glass insert in a GC–MS auto sampler vial. Metabolites were detected using a Trace 1310 GC coupled to a Thermo ISQ-MS. Samples were injected in duplicate using a 10:1 split ratio. The inlet was held at 285°C, and transfer line and ions source were held at 300 and 260°C, respectively. Separation was achieved on a 30m TG-5 M S column (Thermo Scientific, 0.25 mm i.d., 0.25 µm film thickness) with a 1.2 mL/min helium gas flow rate, and the program consisted of 80°C for 30 sec, a ramp of 15°C per min to 330°C, and an 8 min hold. Masses between 50 and 650 m/z were scanned at 5 scans/sec using electron impact ionization. Pooled QC samples were injected after every 12 experimental samples.

Statistical analysis

For each sample, raw data files were converted to.cdf format, and a matrix of molecular features as

defined by retention time and mass (m/z) was generated using XCMS (Smith et al., 2006) software in R (R Development Core Team, 2008) for feature detection and alignment. Raw peak areas were normalized to total ion signal in R, outlier injections were detected based on total signal and PC1 of principle component analysis, and the mean area of the chromatographic peak was calculated among replicate injections ($n = 2$). Features were grouped based on a novel clustering tool, RAMClustR (Broeckling et al., 2014), which groups features into spectra based on co-elution and co-variance across the full data set. Compounds were annotated based on spectral matching to in-house, NISTv12, predicted MS1 and retention times spectral data bases using RAM search (Broeckling et al., 2016) and manual interrogation of the Metlin compound and spectral database. The peak areas for each feature in a spectrum were condensed as the weighted mean of all features in the spectrum to generate a single quantitative value for each metabolite.

Data were analyzed as a split-plot design with the PROC GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC). Tenderness classification (tough or tender) was the whole-plot treatment with carcass being the experimental unit. Aging time (2, 7, 14, 28) was the sub-plot treatment and steak was the experimental unit. Orthogonal polynomial contrasts were used to determine trends in each metabolite regarding aging time. Least-squares means were generated for main-effects and the tenderness class × aging time interaction. When appropriate, means were separated using the DIFF and LINES options.

From a total of 2,562, metabolites ($n = 651$) with significant F-tests for at-least one main effect that exceeded Bonferroni correction of multiple testing ($P \leq 0.00002$) and nominal ($P < 0.05$) for the remaining main-effect were included in further analysis. Pearson correlation coefficients and partial correlation coefficients (removing aging time effects) were generated between each metabolite and slice shear force, desmin degradation, and sarcomere length values across all aging times. Metabolites with the greatest partial correlation (significance levels < 0.00002) to slice shear force, desmin degradation, or sarcomere length were included in partial least-squares (PLS) regression, using the PROC PLS procedure of SAS, with slice shear force, desmin degradation, and sarcomere length as dependent variables and fixed effects of tenderness class and aging time. Bonferroni correction for multiple testing ($P < 0.00002$) was used for screening metabolites for inclusion in analyses. However, a predetermined significance level of (0.05) was used for judgments of statistical significance for discussion.

Results and Discussion

Slice shear force, desmin degradation, and sarcomere length change with aging time

Least-squares means for slice shear force values of *longissimus lumborum* steaks from carcasses classified as tender or tough after aging for 2, 7, 14, or 28 d are presented in Fig. 1. The carcasses were classified based on slice shear force values across aging times, and thus, the difference between tenderness classes was by design (i.e., carcasses classified as tender had to have consistently low slice shear force values at all aging times). At all aging times tested, steaks from carcasses classified as tough had higher ($P < 0.001$) slice shear force values than steaks from carcasses classified as tender. Each progressive increase in aging time resulted in lesser ($P < 0.001$) slice shear force values in steaks from carcasses classified as tough. In steaks from carcasses classified as tender, increasing aging time from 2 to 7 and from 14 to 28 d postmortem decreased ($P < 0.001$ and 0.02, respectively) slice shear force values. Steaks from carcasses classified as tender on d 2 postmortem had slice shear force values similar ($P = 0.42$) to those of steaks from carcasses classified as tough on d 28 postmortem. Increasing aging time resulted in substantial tenderization and was consistent with *longissimus* tenderization detected in previous reports (Gruber et al., 2006; King et al., 2009c).

Least-squares means for desmin degradation are presented in Fig. 2. Steaks classified as tender had a greater proportion of desmin degraded at 2 ($P = 0.01$), 7 ($P < 0.001$), 14 ($P < 0.001$), and 28 d ($P = 0.03$) than steaks from carcasses classified as tough. Each increase in aging time increased ($P < 0.04$) the amount of desmin degraded in steaks from both tenderness classes. However, the magnitude of the desmin degradation difference was not consistent with each incremental increase in aging time. Previous research from our laboratory has found increases in desmin degradation in various beef muscles through 42 d of aging (King et al., 2009a; King et al., 2009b; King et al., 2009c) which were associated with decreases in slice shear force, although statistical differences were not always detected with each incremental increase in aging time. By 28 d postmortem, desmin degradation in steaks classified as tough was similar ($P = 0.38$) to the desmin degradation at d 7 postmortem in steaks classified as tender. Sarcomere length did not differ across tenderness classes ($P = 0.42$) or aging times ($P = 0.40$) in the present experiment (Fig. 3).

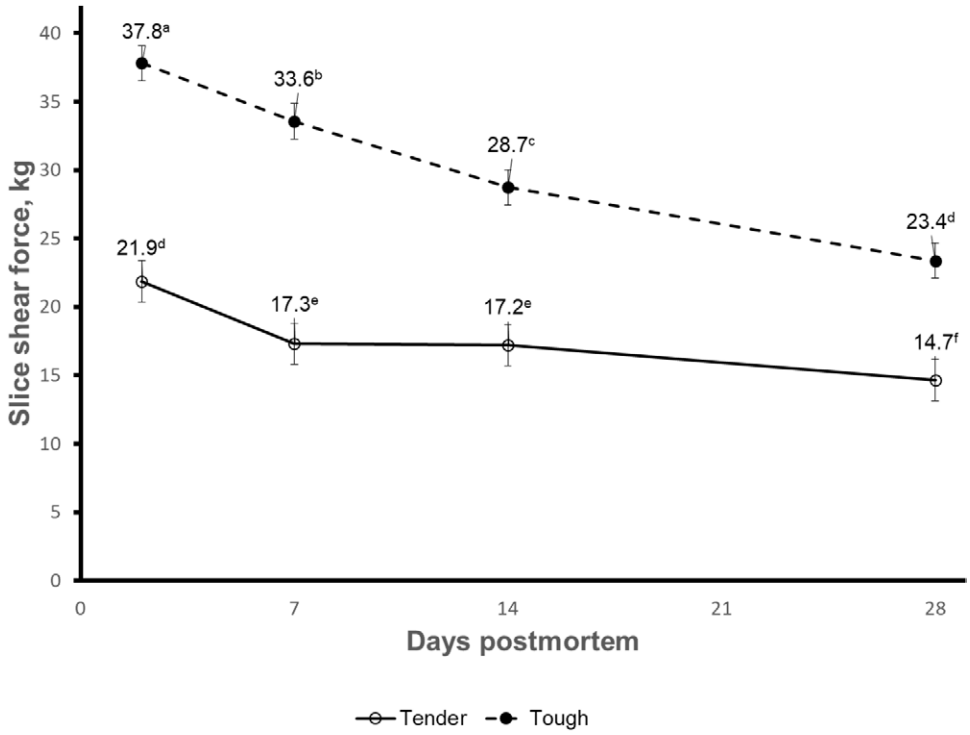

Figure 1. Least squares means for slice shear force values of *longissimus lumborum* steaks from carcasses classified as tender or tough after aging for 2, 7, 14, and 28 d. [a–f]LS means lacking common superscripts differ ($P < 0.05$).

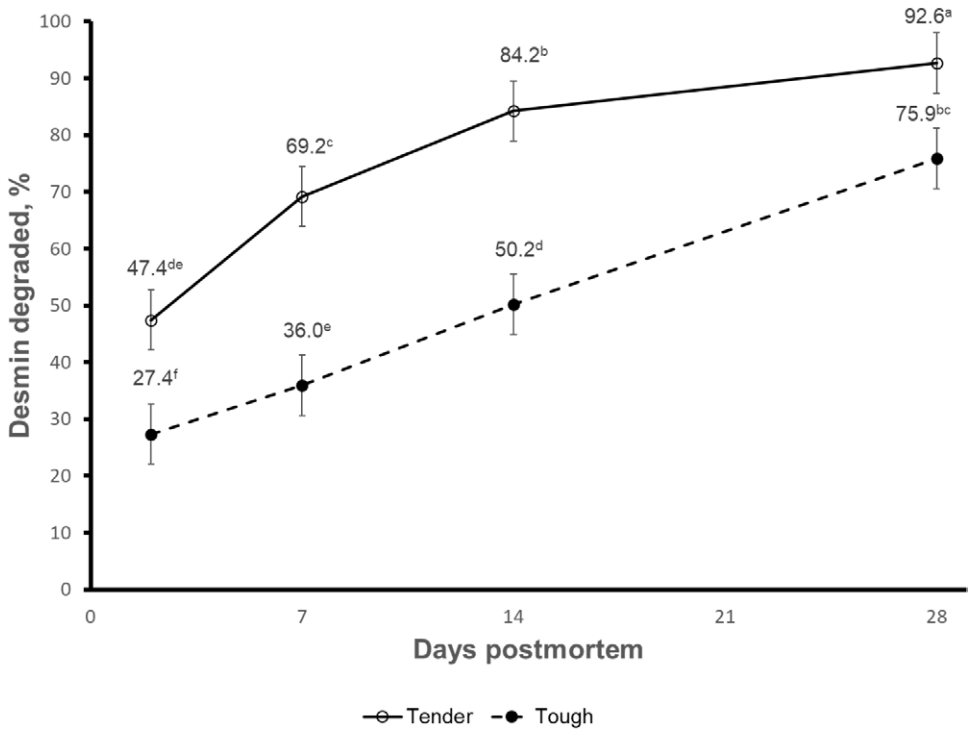

Figure 2. Least squares means for desmin degradation of *longissimus lumborum* steaks from carcasses classified as tender or tough after aging for 2, 7, 14, and 28 d. [a–f]LS means lacking common superscripts differ ($P < 0.05$).

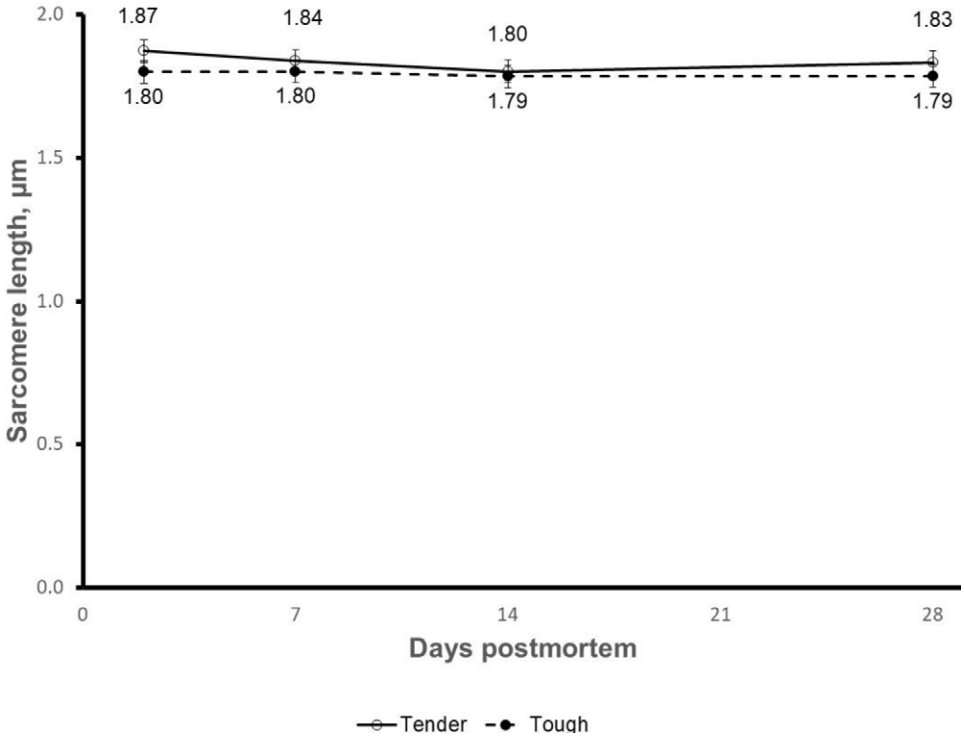

Figure 3. Least squares means for sarcomere length of *longissimus lumborum* steaks from carcasses classified as tender or tough after aging for 2, 7, 14, and 28 d.

Metabolites (n = 2562) were profiled in the beef samples using both GC–MS and UPLC-MS analytical platforms. Initial statistical screening of the data was accomplished by ANOVA F-tests for the fixed effects of tenderness class and aging time. A total of 651 metabolites had F-tests significant at the Bonferroni correction level (all for the aging time effect; Supplementary Table 1). For the tenderness class main effect, 3 metabolites met the Bonferroni correction criterion, although 254 were significant at a nominal significance level ($P < 0.05$). Additionally, 173 metabolites displayed an interaction ($P < 0.05$) between tenderness class and aging time.

Correlation analysis was used to examine the relationship of the remaining 254 metabolites to slice shear force, desmin degradation, and sarcomere length (Supplementary Table 2). Because these metabolites were selected, in part, based on postmortem changes, partial correlations were generated to remove the postmortem aging time effect. In some cases, partial correlations were much lower than those including the aging effect, suggesting that changes in the abundance of the metabolite were not related to changes in tenderness. Metabolites were selected for discussion and further analysis if the level of significance of the partial correlation to slice shear force, desmin degradation, or sarcomere length exceeded 0.00002. No metabolite had this level of correlation to sarcomere length. A total of 102 metabolites had highly significant ($P < 0.00002$) partial correlation to either slice shear force or desmin degradation. Of these, 61 met this criterion for slice shear force and 61 met this criterion for desmin degradation (19 were correlated to both slice shear force and desmin degradation). At a nominal significance level ($P < 0.05$), all 102 metabolites were correlated (partial) to slice shear force, 79 were correlated (partial) to desmin degradation, and 19 were correlated (partial) to sarcomere length.

The process of assigning a molecular identity to a detected mass spectrometry signal (metabolite annotation) is challenging and represents a current constraint in the field of metabolomics (Broeckling et al., 2016). Thus, in this study of the 102 metabolites included in the final analysis, 28 were annotated. As the primary objective of the present experiment was to identify molecular changes reflective of beef longissimus tenderness and the aging response in the longissimus, unknown metabolites were retained in the final analyses and the complete results are presented in Supplementary Tables 1 and 2. However, the discussion below is focused on the 28 annotated metabolites.

Table 1. Least square means for relative abundance of metabolites in *longissimus lumborum* steaks from carcasses classified as tender or tough and aged for 2, 7, 14, or 28 d

ID	Phenylalanine	Phenylalanine	Isoluecine	Isoleucine	Methionine	Methionine	Valine
Tenderness class effect							
Tender	144,180,000	71,396	458,030,000	182,050,000	75,961,339	10,225	293,810,000
Tough	104,320,000	44,687	344,600,000	142,390,000	56,747,892	6,891	239,970,000
SEM	377,658	4,105	20,698,878.	9,131,582	4,166,222	944	13,129,778
P > F	1.06×10^{-03}	2.22×10^{-04}	1.12×10^{-03}	6.60×10^{-03}	4.33×10^{-03}	2.25×10^{-02}	9.59×10^{-03}
Aging time effect							
2	53,322,746^{d}	27,244^{d}	217,480,000^{d}	100,100,000^{d}	21,442,613^{d}	1,819^{d}	172,690,000^{d}
7	81,544,105^{c}	46,816^{c}	305,470,000^{c}	127,130,000^{c}	39,841,207^{c}	5,748^{c}	209,700,000^{c}
14	121,640,000^{b}	61,182^{b}	430,390,000^{b}	165,290,000^{b}	68,222,205^{b}	9,687^{b}	270,030,000^{b}
28	240,490,000^{a}	96,925^{a}	651,940,000^{a}	256,350,000^{a}	135,910,000^{a}	16,978^{a}	415,140,000^{a}
S×10M	359,597	4208	18,237,625	7,607,292	4,250,803	974	11,572,078
P > F	8.05×10^{-33}	1.34×10^{-18}	4.15×10^{-30}	1.14×10^{-29}	5.13×10^{-29}	1.52×10^{-17}	1.07×10^{-27}
Linear	2.24×10^{-34}	3.67×10^{-20}	8.20×10^{-32}	2.53×10^{-31}	1.16×10^{-30}	4.06×10^{-19}	2.60×10^{-29}
Quadratic	4.33×10^{-03}	4.63×10^{-01}	4.41×10^{-01}	2.36×10^{-01}	1.57×10^{-01}	3.10×10^{-01}	1.26×10^{-01}
Cubic	5.70×10^{-01}	2.64×10^{-01}	8.36×10^{-01}	8.25×10^{-01}	9.70×10^{-01}	6.55×10^{-01}	9.60×10^{-01}
Tenderness class × Aging time interaction effect							
Tender2	57,628,949^{gf}	29,458^{de}	240,660,000^{de}	107,630,000^{ef}	22,578,954^{f}	2,033	183,010,000^{f}
Tender7	93,793,853^{de}	58,263^{c}	356,800,000^{c}	142,860,000^{d}	45,744,196^{de}	7,796	231,930,000^{de}
Tender14	145,620,000^{c}	79,700^{b}	516,780,000^{b}	190,330,000^{c}	80,680,770^{c}	12,358	305,110,000^{c}
Tender28	279,680,000^{a}	118,162^{a}	717,900,000^{a}	287,360,000^{a}	154,840,000^{a}	18,715	455,200,000^{a}
Tough2	49,016,543^{g}	25,030^{e}	194,300,000^{e}	92,565,443^{f}	20,306,271^{f}	1,606	162,370,000^{f}
Tough7	69,294,357^{ef}	35,368^{de}	254,130,000^{d}	111,400,000^{e}	33,938,218^{d}	3,701	187,470,000^{ef}
Tough14	97,652,670^{d}	42,665^{cd}	344,000,000^{c}	140,250,000^{d}	55,763,640^{ef}	7,017	234,950,000^{d}
Tough28	201,300,000^{b}	75,688^{b}	585,980,000^{b}	225,340,000^{b}	116,980,000^{b}	15,240	375,070,000^{b}
SEM	508,522	5,952	25,791,045	10,758,110	6011159	1,377	16,364,848
P > F	3.41×10^{-05}	1.58×10^{-03}	7.82×10^{-03}	4.56×10^{-03}	5.17×10^{-03}	1.95×10^{-01}	5.09×10^{-02}

ID	Tyrosine	Threonine	Methyl-aspartic acid	Mely-acetyl-lysinate	L-alpha-Aspartyl-L-valylgycyl-L-proline	Glucose	Glucose-6-phosphate
Tenderness class effect							
Tender	171,690,000	76,035,230	4,207	2,120	1,957	320,340,000	207,900,000
Tough	128,810,000	63,209,743	2,796	1,102	1,181	265,530,000	146,850,000
SEM	291,189	103,320	295	136	139	16,646,355	17,488,636
P > F	1.33×10^{-02}	4.63×10^{-02}	3.31×10^{-03}	5.01×10^{-05}	9.86×10^{-04}	3.18×10^{-02}	2.38×10^{-02}
Aging time Effect							
2	77,382,543^{d}	46,975,871^{c}	1,439^{d}	604^{d}	1,517^{b}	259,970,000^{c}	210,670,000^{a}
7	107,400,000^{c}	52,008,849^{c}	2,521^{c}	1,281^{c}	2,115^{a}	286,790,000^{b}	201,280,000^{a}
14	154,640,000^{b}	66,957,629^{b}	3,642^{b}	1,643^{b}	1,575^{b}	307,810,000^{a}	173,230,000^{b}
28	261,570,000^{a}	112,550,000^{a}	6,404^{a}	2,914^{a}	1,069^{c}	317,160,000^{a}	124,310,000^{c}
SEM	343,354	80,243	286	139	137	12,526,750	12,900,835
P > F	4.26×10^{-21}	4.90×10^{-26}	7.07×10^{-21}	1.22×10^{-18}	2.83×10^{-07}	2.06×10^{-10}	4.23×10^{-20}
Linear	1.02×10^{-22}	2.50×10^{-27}	1.59×10^{-22}	3.75×10^{-20}	1.64×10^{-05}	1.04×10^{-10}	1.21×10^{-21}
Quadratic	4.30×10^{-01}	5.43×10^{-04}	8.76×10^{-01}	7.89×10^{-01}	3.44×10^{-03}	4.19×10^{-04}	4.43×10^{-01}
Cubic	9.59×10^{-01}	7.10×10^{-01}	5.29×10^{-01}	9.69×10^{-02}	5.07×10^{-04}	7.01×10^{-01}	2.94×10^{-01}
Tenderness class × Aging time interaction effect							
Tender 2	82,229,910	49,174,256^{de}	1,549^{d}	685^{dc}	2,158^{ab}	285,390,000^{bc}	255,690,000^{a}
Tender 7	127,210,000	56,204,651^{de}	3,237^{c}	1,795^{b}	2,473^{a}	309,800,000^{b}	228,460,000^{b}
Tender 14	183,870,000	75,641,696^{c}	4,377^{b}	2,155^{b}	1,984^{b}	335,820,000^{a}	205,080,000^{c}
Tender 28	293,440,000	123,120,000^{a}	7,667^{a}	3,843^{a}	1,214^{cd}	350,330,000^{a}	142,380,000^{de}
Tough 2	72,535,176	44,777,486^{e}	1,330^{d}	524^{d}	876^{d}	234,560,000^{c}	165,640,000^{cd}
Tough 7	87,597,040	47,813,047^{e}	1,806^{d}	766^{cd}	1,758^{bc}	263,780,000^{bc}	174,110,000^{cd}
Tough 14	125,410,000	58,273,562^{d}	2,907^{c}	1,131^{c}	1,166^{d}	279,800,000^{bc}	141,390,000^{e}
Tough 28	229,700,000	101,970,000^{b}	5,140^{b}	1,985^{b}	923^{d}	283,990,000^{bc}	106,250,000^{e}
S×10M	485,534	113,479	404	196	194	17,715,430	18,244,502
P > F	1.15×10^{-01}	6.67×10^{-02}	8.16×10^{-03}	6.47×10^{-05}	2.39×10^{-02}	5.08×10^{-01}	5.47×10^{-04}

ID	Glycerol-3-phosphate	Glyceric acid-3-phosphate	Malic acid	Elaeodendroside V	Dictamnoside G	Glucopyranoside[1]	AnguiviosideXVI
Tenderness class effect							
Tender	46,195,230	4878,900	33,211,154	633	893	44,579	506

Continued

Table 1. (cont.)

ID	Glycerol-3-phosphate	Glyceric acid-3-phosphate	Malic acid	Elaeodendroside V	Dictamnoside G	Glucopyranoside[1]	AnguiviosideXVI
Tough	68,163,347	10,637,303	65,041,516	211	311	24,349	152
SEM	4,139,162	1,150,733	8,694,256	66	97	3,103	61
P > F	1.43×10^{-03}	2.30×10^{-03}	1.85×10^{-02}	2.93×10^{-04}	5.06×10^{-04}	2.17×10^{-04}	7.03×10^{-04}
Aging time effect							
2	112,890,000^{a}	19,386,579^{a}	58,330,335^{a}	38^{d}	73^{d}	22,633^{c}	25^{c}
7	37,187,501^{b}	4,473,635^{b}	64,623,181^{a}	201^{c}	308^{c}	33,692^{b}	173^{b}
14	38,198,723^{b}	3,507,347^{b}	41,635,653^{b}	557^{b}	786^{b}	36,271^{b}	506^{a}
28	40,437,890^{b}	3,664,846^{b}	31,916,169^{b}	893^{a}	1,240^{a}	45,260^{a}	613^{a}
SEM	4,649,439	1,503,459	72,176,23	62	87	2,816	62
P > F	2.15×10^{-19}	6.74×10^{-11}	4.32×10^{-06}	3.51×10^{-18}	1.19×10^{-18}	4.13×10^{-09}	3.28×10^{-11}
Linear	8.07×10^{-13}	1.30×10^{-07}	1.75×10^{-06}	1.43×10^{-19}	4.87×10^{-20}	6.76×10^{-10}	9.68×10^{-12}
Quadratic	3.49×10^{-14}	2.06×10^{-07}	8.67×10^{-01}	6.44×10^{-02}	4.62×10^{-02}	7.32×10^{-02}	3.65×10^{-03}
Cubic	9.21×10^{-09}	9.23×10^{-04}	1.33×10^{-02}	1.87×10^{-01}	2.19×10^{-01}	7.52×10^{-02}	1.48×10^{-01}
Tenderness class × Aging time interaction effect							
Tender2	75,952,210^{b}	9694,190^{b}	35,002,610^{bc}	61^{e}	122^{ef}	25,461^{de}	29^{d}
Tender7	33,189,101^{c}	3440,764^{c}	45,242,871^{b}	379^{cd}	557^{cd}	44,525^{bc}	301^{bc}
Tender14	35,655,608^{c}	3186,622^{c}	25,090,755^{c}	927^{b}	1,244^{b}	51,000^{ab}	858^{a}
Tender28	39,984,003^{c}	3194,024^{c}	27,508,378^{c}	1165^{a}	1,649^{a}	57,332^{a}	837^{a}
Tough2	149,830,000^{a}	29,078,967^{a}	81,658,061^{a}	15^{e}	24^{f}	19,805^{e}	20^{d}
Tough7	41,185,902^{c}	5506,505^{bc}	84,003,490^{a}	23^{e}	59^{f}	22,860^{e}	45^{d}
Tough14	40,741,837^{c}	3828,071^{bc}	58,180,551^{b}	187de	328de	21,542^{e}	154^{cd}
Tough28	40,891,778^{c}	4135,668^{bc}	36,323,960^{bc}	621^{c}	832^{c}	33,187^{cd}	388^{b}
SEM	6,574,773	2,126,002	10,207,052	88	123	3,983	88
P > F	8.25×10^{-08}	4.14×10^{-05}	2.19×10^{-02}	2.32×10^{-05}	7.17×10^{-05}	9.47×10^{-04}	1.46×10^{-04}

ID	Hexanoic acid[2]	Butyrate[3]	PC(32:1)	Beta-caraten[4]	Panclicin-D	Ethynylbenzene	Methoxyphenol[5]
Tenderness class effect							
Tender	1,254	8,716	1,123,146	498	630	95,795	1,060
Tough	679	3,975	779,602	118	2,690	72,771	630
SEM	124	581	57,466	60	302	6,143	71
P > F	4.19×10^{-03}	1.84×10^{-05}	5.07×10^{-04}	3.04×10^{-04}	1.39×10^{-04}	1.63×10^{-02}	4.43×10^{-04}
Aging time effect							
2	183^{c}	1,549^{d}	1,048,570^{a}	39^{c}	2,466^{a}	39,981^{d}	463^{c}
7	392^{c}	4,080^{c}	952,258^{b}	161^{c}	2,421^{a}	64,176^{c}	768^{b}
14	917^{b}	7,964^{b}	910,412^{b}c	432^{b}	1,258^{b}	92,260^{b}	930^{b}
28	2,375^{a}	11,788^{a}	894,255^{c}	602^{a}	494^{c}	140,715^{a}	1,219^{a}
SEM	121	611	44,087	63	305	6,735	76
P > F	1.10×10^{-22}	4.22×10^{-19}	3.04×10^{-06}	1.59×10^{-09}	4.95×10^{-07}	4.20×10^{-16}	1.28×10^{-09}
Linear	3.75×10^{-24}	1.90×10^{-20}	4.42×10^{-06}	1.37×10^{-10}	4.51×10^{-08}	1.12×10^{-17}	1.06×10^{-10}
Quadratic	1.37×10^{-02}	1.43×10^{-02}	2.73×10^{-03}	7.70×10^{-02}	5.82×10^{-01}	3.89×10^{-01}	7.99×10^{-02}
Cubic	7.49×10^{-01}	4.61×10^{-01}	3.28×10^{-01}	3.14×10^{-01}	1.11×10^{-01}	8.74×10^{-01}	3.17×10^{-01}
Tenderness class × Aging time interaction effect							
Tender2	200^{c}	2,011^{e}	1,206,444^{a}	65^{c}	953^{bc}	42,539^{f}	514^{c}
Tender7	537^{c}	5,835^{cd}	1,142,641^{ab}	295^{b}	971^{bc}	75,475^{de}	928^{b}
Tender14	1,309^{b}	11,052^{b}	1,094,580^{bc}	753^{a}	360^{c}	103,462^{bc}	1,327^{a}
Tender28	2,971^{a}	15,966^{a}	1,048,918^{cd}	881^{a}	235^{c}	161,703^{a}	1,470^{a}
Tough2	165^{c}	1,087^{e}	890,697^{d}	13^{c}	3,980^{a}	37,422^{f}	411^{c}
Tough7	246^{c}	2,326^{e}	761,874^{e}	26^{c}	3,871^{a}	52,877^{ef}	607^{c}
Tough14	525^{c}	4,876^{d}	726,244^{e}	111^{bc}	2,156^{b}	81,058^{cd}	533^{c}
Tough28	1,780^{b}	7,609^{c}	739,592^{e}	323^{b}	753^{c}	119,726^{b}	968^{b}
SEM	171	864	62,349	89	432	9,524	107
P > F	3.57×10^{-04}	3.72×10^{-05}	4.76×10^{-01}	1.00×10^{-03}	2.61×10^{-03}	1.99×10^{-01}	4.63×10^{-03}

$^{a–f}$Least squares means within a column and within and effect lacking common super scripts differ ($P < 0.05$).

[1][(2R,3S,4S,5R,6R)-6-Ethyl-3,4,5-trihydroxy-2-piperidinyl]methyl beta-D-glucopyranoside

[2] L-alpha-Amino-1H-pyrrole-1-hexanoic acid.

[3](3R,6aS,7S,8S,9R,10R,10aS)-1,3-Diacetoxy-10-hydroxy-7,8-dimethyl-7-[(2Z)-3-methyl-2,4-pentadien-1-yl]-5-oxo-3,5,6,6a,7,8,9,10-octahydronaphtho[1,8a-c]furan-9-yl butyrate.

[4](2R,2'R,3R,3'R)-2,2',3-Trihydroxy-beta,beta-caroten-3'-yl hydrogen sulfate.

[5]2-[3-(4-Hydroxy-2-methoxyphenyl)-1-(4-methoxyphenyl)propyl]-3-[(E)-2-(4-hydroxyphenyl)vinyl]-5-methoxyphenol.

Table 2. Pearson and partial correlation coefficients of metabolites to slice shear force, desmin degradation, and sarcomere length of longissimus steaks

	Pearson correlation coefficient			Partial correlation coefficient[1]		
Metabolite	Slice shear force	Desmin degradation	Sarcomere length	Slice shear force	Desmin degradation	Sarcomere length
Phenylalanine	-0.55[a]	0.77[a]	-0.14	-0.38[a]	0.58[a]	-0.19
Phenylalanine	-0.62[a]	0.73[a]	-0.10	-0.50[a]	0.51[a]	-0.06
Isoleucine	-0.57[a]	0.82[a]	-0.09	-0.47[a]	0.71[a]	-0.06
Isoleucine	-0.60[a]	0.81[a]	-0.10	-0.41[a]	0.67[a]	-0.08
Methionine	-0.53[a]	0.76[a]	-0.14	-0.32[b]	0.55[a]	-0.19
Methionine	-0.56[a]	0.77[a]	-0.13	-0.40[a]	0.58[a]	-0.12
Valine	-0.53[a]	0.80[a]	-0.10	-0.33[b]	0.63[a]	-0.09
Tyrosine	-0.56[a]	0.79[a]	-0.06	-0.37[a]	0.62[a]	-0.01
Threonine	-0.50[a]	0.76[a]	-0.13	-0.26[c]	0.55[a]	-0.14
3-Methylaspartic acid	-0.59[a]	0.81[a]	-0.10	-0.44[a]	0.66[a]	-0.08
Methyl N~2~-acetyl-L-lysinate	-0.63[a]	0.77[a]	-0.10	-0.51[a]	0.61[a]	-0.07
L-alpha-Aspartyl-L-valylglycyl-L-proline	-0.38[a]	0.05	0.21	-0.60[a]	0.33[b]	0.19
Glucose	-0.55[a]	0.28[c]	0.14	-0.48[a]	0.10	0.17
Glucose-6-phosphate	-0.22[c]	-0.20	0.27 [c]	-0.54[a]	0.14	0.27 [c]
Glycerol-3-phosphate	0.59[a]	-0.48[a]	-0.01	0.48[a]	-0.27[c]	-0.04
Glyceric acid-3-phosphate	0.56 [a]	-0.42[a]	0.03	0.46[a]	-0.22	0.00
Malic acid	0.56[a]	-0.28[c]	-0.13	0.49[a]	-0.10	-0.16
elaeodendroside V	-0.64[a]	0.82[a]	-0.14	-0.52[a]	0.71[a]	-0.12
Dictamnoside G	-0.64[a]	0.82[a]	-0.15	-0.53[a]	0.71[a]	-0.13
Glucopyranoside[2]	-0.66[a]	0.72[a]	-0.04	-0.58[a]	0.64[a]	-0.02
Anguivioside XVI	-0.57[a]	0.72[a]	-0.21	-0.45[a]	0.59[a]	-0.20
Hexanoic acid[3]	-0.54[a]	0.75[a]	-0.13 [a]	-0.35[b]	0.53[a]	-0.11
Butyrate[4]	-0.64[a]	0.77[a]	-0.16	-0.52[a]	0.59[a]	-0.16
PC(32:1)	-0.38[a]	0.22	0.16	-0.53[a]	0.44[a]	0.14
Beta-caroten[5]	-0.59[a]	0.65[a]	-0.18	-0.48[a]	0.50[a]	-0.17
Panclicin-D	0.62[a]	-0.42[a]	-0.04	0.53[a]	-0.22	-0.07
Ethynylbenzene	-0.53[a]	0.71[a]	-0.11	-0.34[b]	0.47[a]	-0.08
Methoxyphenol[6]	-0.61[a]	0.72[a]	0.02	-0.49[a]	0.59[a]	0.08

[a] $P < 0.001$.

[b] $P < 0.01$.

[c] $P < 0.05$.

[1]Partial correlation coefficient with aging time effect removed.

[2][(2R,3S,4S,5R,6R)-6-Ethyl-3,4,5-trihydroxy-2-piperidinyl]methyl beta-D-glucopyranoside.

[3](3R,6aS,7S,8S,9R,10R,10aS)-1,3-Diacetoxy-10-hydroxy-7,8-dimethyl-7-[(2Z)-3-methyl-2,4-pentadien-1-yl]-5-oxo-3,5,6,6a,7,8,9,10-octahydronaphtho[1,8a-c]furan-9-yl butyrate.

[4]L-alpha-Amino-1H-pyrrole-1-hexanoic acid.

[5](2R,2'R,3R,3'R)-2,2',3-Trihydroxy-beta,beta-caroten-3'-yl hydrogen sulfate.

[6]2-[3-(4-Hydroxy-2-methoxyphenyl)-1-(4-methoxyphenyl)propyl]-3-[(E)-2-(4-hydroxyphenyl)vinyl]-5-methoxyphenol.

Metabolite profiles differ across tenderness classes and change with aging time

Least-squares means for the tenderness class and aging time effects and the tenderness class × aging time interaction for annotated metabolites are presented in Table 1. Some of the annotated metabolites could be loosely grouped into classes of similar compounds.

Amino acids. Twelve metabolites were classified as amino acids or small peptides. Ten of these were affected ($P \leq 0.05$) by a tenderness class × aging time interaction. Generally, the abundance of amino acids was similar ($P > 0.05$) between tenderness classes on d 2 postmortem. The abundance of amino acid and small peptide metabolites were greater in steaks from carcasses that were classified as tender than in steaks from carcasses classified as tough on d 7, 14, and 28 of aging. Moreover, abundance of these metabolites increased with increased ($P < 0.05$) aging time. The abundance of the amino acids at each later aging time was greater

($P < 0.05$) in steaks from carcasses classified as tender compared to steaks from carcasses classified as tough. The 2 metabolites classified as amino acids (methionine and tyrosine) not affected by a tenderness class × aging time interaction ($P = 0.10$), were detected in steaks from carcasses classified as tender at greater abundance ($P \leq 0.02$) than in steaks from carcasses classified as tough. Both methionine and tyrosine increased in abundance with each increase in aging time. Increases in amino acids, including alanine, aspartate, glutamine, glutamate, isoleucine leucine, methionine, proline, threonine, and valine with increased postmortem aging time has been reported by Graham et al. (2010). The peptide L-α-Aspartyl-L-valylglycyl-L-proline was present at the highest abundance in steaks from carcasses classified as tender on d 2 and 7 of aging. Abundance of this compound decreased in steaks from carcasses classified as tender between d 7 and 14 and again between Day 14 and 28 ($P = 0.03$ and 0.001, respectively).

Energy pathway intermediates. Four metabolites (glucose, glucose-6-phosphate, glycerol-3-phosphate, and malic acid) were grouped as intermediates of glycolysis and/or the Krebs cycle. Abundance of free glucose was much greater ($P \leq 0.03$) in steaks from carcasses classified as tender than in steaks from carcasses classified as tough regardless of aging time. Glucose abundance increased with increased aging time between d 2 and 7 ($P = 0.0004$) and between d 7 and 14 ($P = 0.004$) of aging. Glucose abundance did not differ ($P = 0.18$) between d 14 and 28 of aging. Glucose-6-phosphate abundance was impacted ($P < 5 \times 10^{-4}$) by a tenderness class × aging time interaction. Steaks from carcasses classified as tender had greater ($P \leq 0.04$) abundance of glucose-6-phosphate than steaks from carcasses classified as tough on d 2, 7, and 14 of aging. Abundance of glucose-6-phosphate decreased ($P \leq 0.008$) with each increased aging time in steaks from carcasses classified as tender. Glucose-6-phosphate abundance decreased ($P = 0.0003$) in steaks from carcasses classified as tough between d 7 and 14 of aging. Glucose-6-phosphate abundance was not different between tenderness classes on d 28. These findings are in partial agreement with the previous study by Rhoades et al. (2005) which reported increases in both glucose and glucose-6-phosphate in beef muscle between death and 4 d postmortem. Glycerol-3-phosphate and 3-phosphoglyceric acid were both affected ($P < 8 \times 10^{-8}$ and 4×10^{-5}, respectively) by a tenderness class × aging time interaction and displayed similar differences across tenderness classes and aging times. Abundance of both compounds was much greater in steaks from carcasses classified as tough than in steaks from carcasses classified as tender on d 2 postmortem. A decrease in abundance of both compounds was observed between d 2 and 7 postmortem. Abundance of both compounds were similar between tenderness classes and aging times of 7, 14, or 28 d. In the present experiment, malic acid abundance was impacted by a tenderness class × aging time interaction ($P = 0.02$). Malic acid levels were detected at the greatest abundance in steaks classified as tough on d 2 and 7 of aging and decreased ($P = 0.004$) in steaks from this group between d 7 and d 14 postmortem. On d 14 and 28 of aging, malic acid abundance in steaks from carcasses classified as tough were similar ($P \leq 0.12$) to the abundance in steaks from carcasses classified as tender on d 2 and 7 postmortem. Malic acid abundance decreased ($P = 0.02$) in steaks classified as tender between d 7 and 14 of aging. Similarly, Santos et al. (2016) reported that at 14 d postmortem, the proteome of beef longissimus steaks with high shear force values (tough) had greater malate dehydrogenase abundance than beef longissimus steaks with low shear force (tender).

Plant glycosides. Four metabolites tentatively annotated as plant glycosides (Elaeodendroside V, Dictamnoside G, Glucopuranoside, and Angiovioside XVI), based on accurate mass and predicted molecular formula, were impacted ($P \leq 1 \times 10^{-4}$) by a tenderness class × aging time interaction. Generally, abundance of these compounds was similar across tenderness classes on d 2 of aging and abundance increased in both tenderness classes during increased postmortem aging times. The extent of these increases was greater in steaks from carcasses classified as tender than in steaks from carcasses classified as tough.

Fatty acids and bacterial products. A tenderness class × aging time interaction affected ($P = 3 \times 10^{-5}$ and 3×10^{-4}, respectively) two compounds that were identified as fatty acids (hexanoic acid and butyrate). Abundance of both fatty acids was similar between tenderness classes on d 2 of aging and generally increased in steaks from both classes with longer aging times. The extent of the increase was greater in steaks from carcasses classified as tender, and so steaks from this class had greater levels of these fatty acids on d 7, 14, and 28 of aging. A compound with a mass suggestive of the Streptomyces product panclicin-D was detected at much greater ($P \leq 0.005$) abundance in steaks from carcasses classified as tough at 2, 7, and 14 d of aging than in steaks from carcasses classified as tender. Abundance of panclicin-D declined dramatically between d 7 and 14 ($P = 0.001$) and between d 14 and 28 ($P = 0.007$) of aging in steaks from carcasses classified as tough. On d 28 of aging, panclicin-D abundance in steaks from carcasses classified as tough had declined to a point similar ($P \geq 0.40$) to the abundance

detected in steaks from carcasses classified as tender at all the aging times studied.

Metabolite profiles correlate with slice shear force, desmin degradation, and sarcomere length

Pearson correlation and partial correlation coefficients between metabolites and slice shear force, desmin degradation, and sarcomere length are presented in Table 2. Partial correlation coefficients represent relationships to variation in slice shear force, desmin degradation, and sarcomere length within aging times and removes correlation simply due to changes during aging that may be coincidental.

Amino acids. Amino acid metabolites had strong positive correlation (r ranged from 0.73 to.82; $P \leq 2 \times 10^{-14}$) to desmin degradation and strong negative correlation to slice shear force values (r ranged from –0.53 to –0.62; $P \leq 3 \times 10^{-13}$). Removing the effect of aging time reduced the magnitude of these correlations somewhat, but partial correlations to desmin degradation (r ranged from 0.58 to 0.71: $P \leq 0.1 \times 10^{-6}$) and slice shear force (r ranged from –0.26 to –0.51; $P \leq 0.02$) suggest that levels of these amino acids were indicative of variation in slice shear force and desmin degradation not just postmortem aging time unrelated to tenderness traits. One metabolite identified as the peptide L-α-Aspartyl-L-valylglycyl-L-proline was moderately correlated to slice shear force but was not correlated to desmin degradation. However, when the aging effect was removed, this compound was strongly correlated to slice shear force ($P = 5 \times 10^{-9}$) and was moderately correlated ($P = 0.002$) to desmin degradation.

Energy pathway intermediates. Glucose levels were highly negatively correlated ($P = 1 \times 10^{-7}$) to slice shear force and this relationship was of similar magnitude ($P = 8 \times 10^{-6}$) when the effects of aging time were removed. However, glucose levels were only moderately correlated ($r = 0.28$; $P = 0.01$) to desmin degradation and this relationship was not significant when the aging time effect was removed. Levels of glucose-6-phosphate had a moderate, inverse relationship to slice shear force ($P = 0.04$) and a moderate positive relationship to sarcomere length ($P = 0.01$). Interestingly, removing the aging time effect from the partial correlation did not impact the magnitude of the correlation between glucose-6-phosphate and sarcomere length and increased the relationship to slice shear force ($P = 4 \times 10^{-7}$). Glycerol-3-phosphate and 3-phosphoglyceric acid had very similar relationships to slice shear force and desmin degradation. Both compounds had strong, positive correlations to slice shear force ($P = 1 \times 10^{-8}$ and 1×10^{-4}, respectively) and strong, negative correlations to desmin degradation ($P = 1 \times 10^{-6}$ and 6×10^{-8}). When the aging time effect was removed, these compounds were still strongly correlated to slice shear force ($P = 0.01$ and 0.05, respectively) and moderately correlated to desmin degradation. Malic acid abundance had a strong, positive relationship to slice shear force ($P = 7 \times 10^{-8}$) and low, inverse relationship to desmin degradation ($P = 0.01$). After removing aging time effects, malic acid abundance was still strongly correlated to slice shear force values ($P = 4 \times 10^{-6}$) but was not correlated ($P = 0.30$) to desmin degradation.

Plant glycosides. Levels of compounds presumptively identified as plant glycosides were negatively correlated ($P \leq 3 \times 10^{-8}$) to slice shear force and strongly, positively correlated to desmin degradation ($P \leq 8 \times 10^{-14}$). These relationships were only slightly weakened by removal of aging time effects ($P \leq 4 \times 10^{-5}$ and 1×10^{-8} for slice shear force and desmin degradation, respectively). These relationships were very similar to those exhibited by fatty acid metabolites. Panclicin-D abundance was positively correlated to slice shear force values ($P = 7 \times 10^{-10}$) and negatively correlated to desmin degradation ($P = 1 \times 10^{-4}$). The relationship between panclicin-D and slice shear force was only slightly diminished by removing the effect of aging time ($P = 4 \times 10^{-7}$), but the relationship of this compound to desmin degradation was much lower ($P = 0.06$).

Partial-least squares regression of metabolite profiles to slice shear force, desmin degradation, and sarcomere length reveal additional relationships

The loadings plot of the metabolites included in the PLS analysis are presented in Fig. 4. All 102 metabolites meeting the previously described criteria were included in the PLS model. However, only those that could be annotated are shown in Fig. 4. The loadings for all 102 included metabolites are available in Supplementary Table 3. The first 2 factors derived in the PLS analysis explained 41 and 9% of the variance in the dependent variables, respectively. The first factor was highly related to both increased slice shear force (positive values) and increased desmin degradation (negative values). Aging time was strongly associated with Factor 1 with d 2 postmortem associated with positive values and increased aging times being associated with lower and negative values of Factor 1. The tender class of steaks was associated with negative values for Factor 1, while the tough class of steaks was associated with positive values. The second

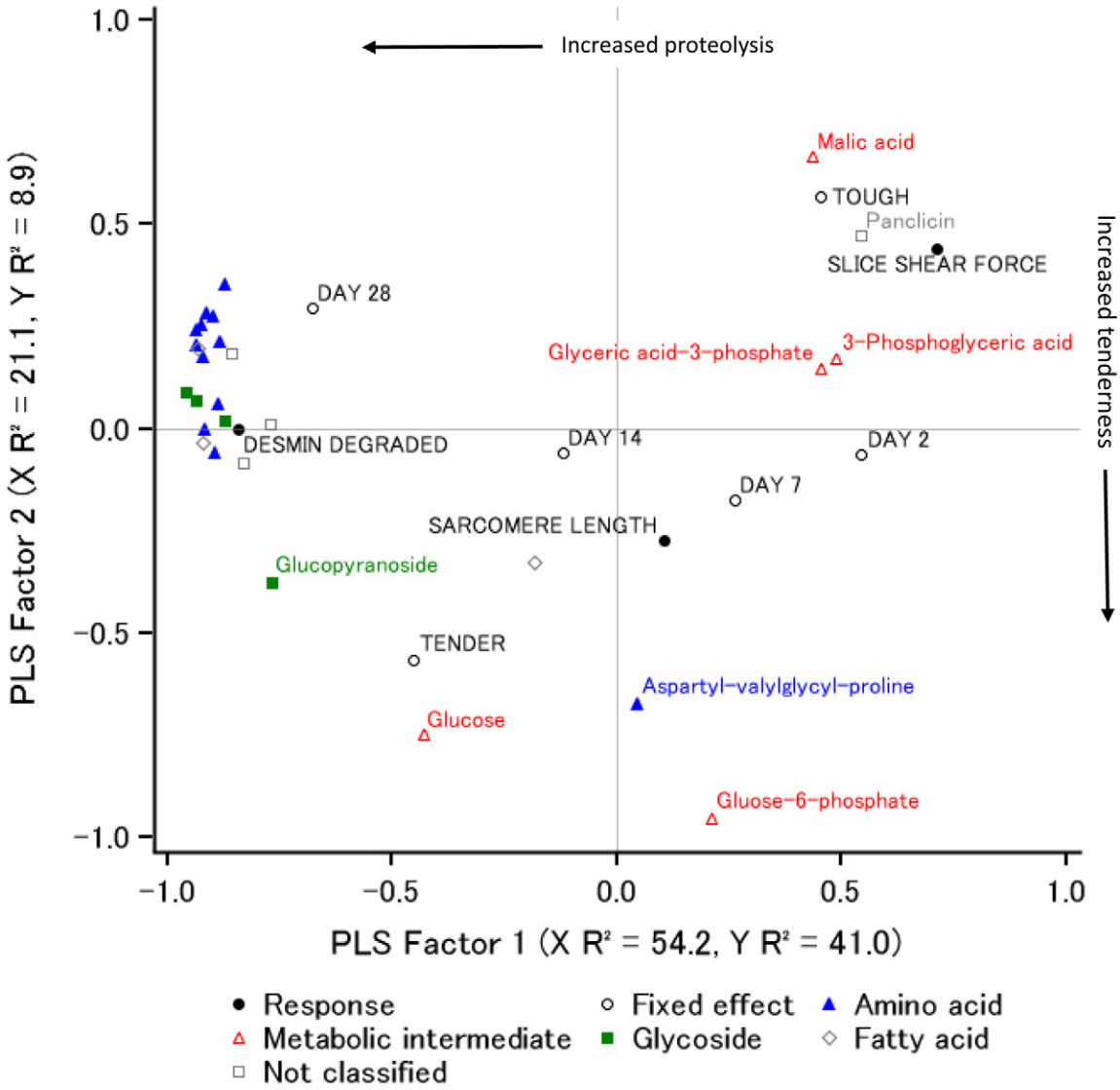

Figure 4. Loadings plot for factors 1 and 2 of partial least-squares regression using metabolites and fixed effects of tenderness class and aging time to predict slice shear force, desmin degradation, and sarcomere length.

factor was highly associated with the tenderness classes with the tough class and increased slice shear force associated with positive values and the tender class being associated with negative values. Amino acids were primarily related to negative values for factor 1, and clustered around the loadings for desmin degradation. The exception to this was the peptide L-α-aspartyly-L-valylglycyl-L-proline, which was strongly associated with Factor 2, and had loadings very close to the tender class of samples.

Amino acids. It is notable that several compounds identified as amino acids were strongly related to desmin degradation. Tenderization associated with postmortem storage has been attributed to degradation of cytoskeletal proteins by the calpain enzyme system (Koohmaraie, 1992a, 1994; Huff-Lonergan et al., 1996). However, changes observed in postmortem muscle involve the fragmentation of myofibrillar proteins rather than the complete hydrolysis of the protein to amino acids (Koohmaraie, 1988; Koohmaraie et al., 1991; Koohmaraie, 1992b). Thus, the observation of several amino acids so strongly associated with postmortem proteolysis is somewhat surprising. However, the mass spectrometry methodology used in the present experiment is more sensitive to smaller compounds than the SDS PAGE techniques (which do not resolve free amino acids) that are more typically used in investigations of changes in postmortem muscle. Generally, the accumulation of these amino acids during aging is consistent with the detected changes in desmin degradation. L-α-aspartl-L-valylgycyl-L-proline abundance differed between tenderness classes and generally increased between d 2 and 7 of aging before declining through the rest of the studied aging period. We speculate that this peptide may be an intermediate of protein degradation.

Energy pathway intermediates. Increased glucose abundance was strongly related to the tender classification and moderately related to increased desmin degradation. Glucose-6-phosphate was the metabolite most strongly and positively associated with the tender class, while negatively associated with desmin degradation. Malic acid was strongly inversely associated with the tough classification, increased slice shear force, and decreased desmin degradation. Glycerol-3-phosphate and 3-phosphoglyceric acid had very similar loadings to one another. Increased levels of these compounds were moderately associated with increased slice shear force and decreased desmin degradation. Muscle metabolism, usually measured by muscle pH, (Eilers et al., 1996; Wulf and Page, 2000; Grayson et al., 2014) has a direct impact on meat quality attributes, including tenderness. Several of the metabolites found to be associated with tenderness in the present experiment are intermediates of glycolysis, the Krebs cycle, and are part of energy-producing shuttles (glycerol phosphate shuttle and malate-aspartate shuttle) associated with these pathways. These findings emphasize the importance of muscle metabolism in regulating meat tenderness and highlight a needed area of further investigation. Importantly, these compounds contribute to energy metabolism via multiple pathways and were found to differ in their association with initial tenderness and tenderization during postmortem aging. Thus, these compounds represent valuable markers for future studies to further elucidate the molecular mechanisms associated beef tenderness.

Bacterial products and plant glycosides. The compound tentatively annotated as the Streptomyces product panclicin-D had loadings very similar to the loadings for slice shear force values and the loadings for steaks classified as tough. Overall, glycoside abundance was strongly associated with negative values for Factor 1 and primarily clustered around the loading for desmin degradation. Interestingly, one of the glycoside compounds, [(2R,3S,4S,5R,6R)-6-Ethyl-3,4,5-trihydroxy-2-piperidinyl] methylbeta-D-glucopyranoside, had a strong, positive association with desmin degradation, but was also moderately associated with Factor 2, which indicates that it is more associated with steaks from carcasses classified as tender than the other glycosides. The role these compounds play in postmortem muscle is not clear as they are not compounds that would typically be expected in muscle tissue. However, these results highlight the need for further studies using advanced molecular tools to increase our understanding of the postmortem changes in muscle tissue.

Conclusions

The results presented here provide new insights on factors regulating initial beef tenderness and tenderization during postmortem aging. Moreover, candidate biomarkers, which may be useful in future investigations, were identified. Further study is needed to refine the current understanding of the mechanisms by which these metabolites influence postmortem changes in the protein structure of meat. Taken together, the results provide new insights into the complexity of the postmortem tenderization process and lay the groundwork for future studies.

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