Meat and Muscle Biology™

Multivariate Examination of Metabolic Contributions to Beef *Longissimus Lumborum* Flavor

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Abstract: We examined the impact of muscle metabolic capacity on beef *longissimus lumborum* flavor. Beef carcasses were selected to have normal or dark cutting lean color ($n = 160$, each) and aged until 13 d postmortem. Muscle pH, glycolytic potential, mitochondrial DNA copy number, instrumental lean color, myoglobin concentration, carbonyls on sarcoplasmic proteins, initial metmyoglobin formation, bloom, proximate composition, slice shear force, sarcomere length, desmin degradation, overall tenderness, juiciness, and flavor profile were determined. Carcasses were clustered based on metabolic characteristics into dark cutting classes (Control, Shady, Moderate, and Severe), which were compared using analysis of variance and multiple factor analysis. Clusters were in general, but not complete, agreement with classifications based on muscle pH. Multiple factor analysis produced 2 dimensions that explained 30.8% and 13.8% of the variation, respectively. Dimension 1 had strong negative loadings for muscle pH and strong positive loadings for glycolytic potential, L^*, a^*, b^*, initial metmyoglobin formation, and bloom. Ratings for fat-like, overall sweet, sweet, and musty/earthy/humus had relatively weak positive loadings for dimension 1, whereas salt, sour, and metallic ratings had weak negative loadings for dimension 1. Overall tenderness and juiciness ratings, marbling score, intramuscular lipid content, carbonyls on sarcoplasmic proteins, and L^* had positive loadings for dimension 2. Ratings for fat-like, beef flavor identity, and brown/roasted had positive loadings for dimension 2, and intramuscular moisture content, slice shear force, and mitochondrial copy number had negative loadings. Sample scores stratified dark cutting clusters along dimension 1, which agreed with univariate comparisons for these traits. Sample scores for dimension 2 were greater for Moderate and Control steaks than for Shady steaks. These data indicate that clustering was effective in segmenting them into groupings more indicative of the metabolic machinery than pH alone. Moreover, these metabolic differences influenced animal variation in beef flavor profile.

Keywords: beef, flavor, metabolism, multivariate, pH
Meat and Muscle Biology 8(1): 17055, 1–14 (2024) **doi:10.22175/mmb.17055**
Submitted 18 November 2023 Accepted 16 February 2024

Introduction

Beef palatability is a multifaceted experience, and tenderness, juiciness, and flavor must all meet consumer expectations for that experience to be satisfactory. Thus, much effort has been expended in understanding contributing factors to variation in these traits. Moreover, meat scientists have increasingly understood that flavor is a multi-dimensional trait. Although flavor is experienced holistically, scientists have developed tools (Adhikari et al., 2011) to segment flavor attributes via highly trained panelists into components for detailed study.

Similarly, a host of physical, physiological, and chemical factors directly and indirectly impact each trait contributing to the overall concept of beef palatability. These factors interact in a multitude of ways to

determine the levels of tenderness, juiciness, and flavor attributes experienced by consumers. Despite the interrelated nature of both the contributing factors and resulting palatability traits, meat scientists generally consider these attributes individually utilizing univariate statistical tools. In Grayson et al. (2016), we have previously contrasted flavor attributes, tenderness, and juiciness of beef *longissimus* steaks across carcasses exhibiting differing levels of the dark cutting condition. In a companion study (McKeith et al., 2016), we also characterized metabolic factors contributing to the dark cutting condition and their impact on color traits of beef *longissimus*. In both reports, we utilized univariate statistical approaches to effectively characterize aspects of the dark cutting condition. However, additional analyses may add understanding to the relationships among traits included in both reports. Moreover, we have not previously related the metabolic data reported in McKeith et al. (2016) to the palatability ratings reported in Grayson et al. (2016).

Despite the effectiveness of those reports, the limitation of relying solely on univariate approaches was apparent. Even the assignment of experimental units to dark cutter classes was problematic because of the imperfect relationship between visual color appraisal, Commission Internationale de l'Eclairage (CIE) color space values, and muscle pH. Attempts to sort carcasses into groups based on any one of these logical criteria yielded substantially different groups, which was problematic for later analyses and interpretation. Univariate analysis of variance (ANOVA) was extremely useful in contrasting both palatability traits and biochemical measures across dark cutter classes. Correlations were also useful in examining pairwise associations to further understand the effects revealed by ANOVA. However, those analyses were not able to fully identify groups of variables moving in concert as part of multidimensional cause–effect relationships.

Multivariate statistical approaches offer the advantage of "reconstructing" the holistic nature of multifaceted data structures. While multivariate approaches will not replace univariate approaches, these statistical tools offer substantial augmentation of those approaches. The specific objectives of the present study were as follows: (1) develop groupings of lean color and metabolic traits and examine their contributions to groupings of beef flavor traits; (2) construct groupings of beef *longissimus lumborum* samples exhibiting differing levels of the dark cutting condition based on multivariate assessment of lean color, metabolic status, and muscle pH; (3) evaluate the effectiveness of those groupings utilizing univariate ANOVA techniques; and (4) evaluate relationships of lean color and metabolic traits to variation in beef flavor traits in a multivariate fashion.

Materials and Methods

Animal care and use approval was not sought because samples for this study were obtained from a USDA-inspected establishment after harvest.

Sample handling and preparation

Beef carcasses ($n = 320$) were selected as they were graded at approximately 36 h postmortem on each of 5 d over the course of 2 mo. Trained evaluators observed the ribbed surface of the *longissimus thoracis* at the 12th–13th rib interface to identify dark cutting carcasses ($n = 160$) as well as cohorts with normal lean color from the same production lot with similar marbling scores. Marbling scores of carcasses included in the study ranged from Traces[70] to Slightly Abundant[50] (USDA, 2017). *Longissimus* muscle pH was measured with a Reed SD-230 handheld pH meter (Reed Instruments, Wilmington, NC) in the anterior face (loin eye) of the *longissimus* exposed by ribbing. The VBG2000LED grading system (Shackelford et al., 2003) was used to quantify carcass grade factors.

Commission Internationale de l'Eclairage (CIE) L^*, a^*, and b^* color space values were determined with a Hunter Miniscan XE Plus Colorimeter (Hunter-Lab, Reston, VA) with a 25-mm port set to use Illuminant A and a 10° observer (King et al., 2023). Duplicate readings were taken on the *longissimus thoracis* muscle exposed by ribbing on the left side of each carcass. The beef, loin, strip loin, similar to IMPS #180 (NAMP, 2003; USDA, 2014), was obtained and aged until 13 d postmortem.

After aging, accessory muscles and fat exceeding 0.6 cm were removed from strip loins. One 2.54-cm-thick steak was removed from the anterior end for fresh slice shear force. Two 6.35-cm-thick sections were vacuum packaged and stored at −30°C for trained sensory panel evaluation. The first section was used for descriptive attribute evaluation, while the second was used for panel training. The rest of the strip loin was cut into 2.54-cm-thick steaks. The first steak from this portion was used for simulated retail display. Oxygen consumption and metmyoglobin reducing ability were determined using the second. The third was pulverized in liquid nitrogen and stored (−80°C) for laboratory analyses.

Slice shear force

Slice shear force steaks were cooked on a conveyorized electric belt grill to an ultimate temperature of 71°C as described by Wheeler et al. (1998). Immediately after maximum temperature was reached, the protocol outlined by Shackelford et al. (1999) was followed to sample steaks for slice shear force. Slice shear force was quantified, in kilograms, with an Instron electronic testing machine (Model 4411, Instron Corporation, Canton, MA) set to a 500 mm/min crosshead speed and fitted with a slice shear force blade.

Sarcomere length

Cooked slices sheared during slice shear force measurement were sampled for sarcomere length measurement. Six small cores were removed from each slice and placed in 0.2 M sucrose in 0.1 M $NaHPO_4$ buffer. Muscle fibers were teased from each core and placed on glass microscope slides. The method prescribed by Cross et al. (1981) was used to obtain 6 diffraction patterns from each core, which were traced onto white paper. These patterns ($n = 36$ total) were scanned into JPEG (Joint Photographic Experts Group) images; the distance between diffraction bands was measured using image analysis software (ImagePro, Media Cybernetics, Inc., Rockville, MD) and used to calculate sarcomere length.

Immunoblotting

Postmortem proteolytic degradation of the cytoskeletal protein desmin was quantified using Western blotting. A whole muscle extract was prepared from the cooked slice after slice shear force and sarcomere length determination and resolved with Western blots as described by Wheeler et al. (2002). The extent to which desmin had degraded was calculated by comparing the desmin band intensity of each sample to *longissimus lumborum* samples collected at the time of harvest.

Trained sensory panel

Descriptive texture and flavor attributes were evaluated by expert panelists with more than 200 h of training and experience. Training for tenderness and juiciness assessment on an 8-point scale (1 = extremely tough/extremely dry to 8 = extremely tender/extremely juicy) was conducted as described by the American Meat Science Association (AMSA, 2016), and training for flavor assessment on a 16-point universal scale (0 = none to 15 = extremely intense) followed the methods of Adhikari et al. (2011). Descriptors for attributes assessed by the panel are presented in Table 1. Prior to the present study, panelists underwent a 2-wk refresher training. The panel was initially trained by the research team that developed the beef flavor lexicon to validate the usefulness of the lexicon (Philip, 2011). In that study, beef flavor intensity scores were lower than the other panels, although treatment differences were consistent across the panels. We have maintained the panel training for beef flavor intensity to keep scores of the same magnitude so that data can be compared across studies (King et al., 2021). Pairs of 2.54-cm-thick steaks were cut from each frozen section designated for sensory analysis and were held at 5°C for 24 h to ensure complete thawing before being cooked on an electric belt grill to a final temperature of 71°C as previously described (Wheeler et al., 1998). External fat and connective tissue were removed before the steaks were cubed (1-cm × 1-cm × steak thickness). Three random cubes from each sample were presented to panelists immediately after cooking was accomplished.

pH and myoglobin concentration

The iodoacetate method of Bendall (1973) was used to measure muscle pH. Powdered muscle tissue (2.5-g) was homogenized in 10 vol of a 5 mM iodoacetate, 150 mM KCl solution (pH = 7.0). Homogenates were held for 1 h at room temperature and vortexed, and pH was measured with a Reed SD-230 handheld pH meter with a pH probe (Omega PHE 2385 pH probe, Omega Engineering Inc., Stamford, CT). The pH meter was calibrated with standardized pH solutions (pH = 4 and pH = 7), and calibration was conducted approximately every 10 samples.

Myoglobin was quantified from duplicate 2.5g (±0.05 g) samples according to the method reported by Hunt and Hedrick (1977) with modification. Samples were homogenized with 10 volumes of 800 m*M* sodium acetate (pH = 4.5) and shaken for 1 h at 4°C before centrifugation (38,000 × *g*) for 35 min at 4°C. Pellets were washed two additional times in 10 mL sodium acetate buffer with 30 min of shaking and centrifugation. Supernatant was filtered (Nalgene 0.45 µm, surfactant-free cellulose acetate membrane; Thermo Fisher Scientific, Rochester, NY), and triplicate aliquots (200 µL) were pipetted into a 96-well plate and blanked against sodium acetate solution. Absorbance at 525 nm and 700 nm was collected using a Spectramax plus 96-well plate reader (Molecular Devices, Sunnydale, CA). Extracted myoglobin

pigment concentration (mg/g meat) was calculated taking the difference between the absorbance at 525 nm and 700 nm and the equation defined by King et al. (2023)

Oxygen consumption and nitric oxide metmyoglobin reducing ability

A 2.54 cm × 2.54 cm × steak thickness cube was removed from each steak avoiding large pieces of marbling and connective tissue. A horizontal cut was made through the center of the cube exposing the interior of the steak. The top portion (previous steak surface) was used for nitric oxide metmyoglobin reducing ability. The remaining portion (previous steak interior) was used for oxygen consumption measurement.

Oxygen consumption measurement was conducted as described by King et al. (2011). The sample was covered with polyvinyl chloride film (stretchable meat film 55003815; Prime Source, St. Louis, MO), which had an oxygen transmission rate of 1.4 mL/(cm2 · 24 h) at 23°C to prevent dehydration and was exposed to air at 4°C for 2 h. Samples were scanned with the previously described colorimeter, which was set to collect spectral data, immediately after vacuum packaging and again immediately after 30 min of deoxygenation. Initially, oxygen consumption was calculated as the percentage change in oxymyoglobin between the two measurements. However, these values were not comparable across dark cutter classes, so oxygen consumption (Bloom) was reported as the percentage of surface myoglobin in the oxymyoglobin form after 2 h of oxygenation.

Initially, nitric oxymyoglobin reducing ability was measured as described by King et al. (2023). Initial levels of metmyoglobin formed after incubation with 0.03% sodium nitrite for 30 min at 20°C were determined with a colorimeter as previously described. The sample then was incubated under vacuum at 30°C for 2 h before surface metmyoglobin was again determined. Ultimately, initial metmyoglobin formed after incubation with sodium nitrite was used to represent reducing ability because percentage reduction of nitric oxide metmyoglobin could not be compared across dark cutter classes.

Glycolytic potential

Glycolytic potential was assessed with the protocol of Miller et al. (2000) and Souza et al. (2011) with further modification. Briefly, samples were assayed for glucose using a Glucose (HK) Assay Kit (Sigma-Aldrich, St. Louis, MO) with and without amyloglycosidase digestion. Residual glycogen was calculated as the difference between these assays. Aliquots (20 µl) of the Amyloglucosidase digested sample were combined with 180 µl of assay buffer (0.2 M glycine, 0.003 M NAD, and 3 units of lactate dehydrogenase). Glycolytic potential was reported as the total of lactate equivalents present in the muscle (Monin and Sellier, 1985), where glycolytic potential = 2[(glycogen) + (glucose) + (glucose-6-phosphate)] + [lactate].

Mitochondrial DNA abundance

Relative mitochondrial abundance was assessed as the total number of copies of mitochondrial DNA present in the muscle relative to the genomic DNA present in the muscle.

Extraction of DNA was accomplished using the Promega Wizard SV 96 Genomic DNA Purification System (Promega Corp, Madison, WI) following the manufacturer's instructions. Previously reported primers for mitochondrial (Iwata et al., 2011) and genomic (Marson et al., 2008) genes in cattle were used for real-time polymerase chain reaction. Copy numbers of mitochondrial amplicons were calculated as described by Schmittgen and Livak (2008).

Measurement of protein solubility and carbonyl determination

Longissimus muscle tissue (5 ± 0.1 g) was homogenized in 15 mL of Post Rigor Extraction buffer (100 m*M* Tris, 10 m*M* EDTA, 0.05% 2-mercaptoethanol [MCE]; pH = 8.3) before being centrifuged (4°C) at 27,000 x *g* for 30 min. Supernatants were filtered through cheesecloth. Concentration of protein was determined with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA). The amount of soluble protein (mg) in 1 g wet tissue was reported as protein solubility. Carbonyls on sarcoplasmic proteins were quantified as described by Keller et al. (1993) and Reznick and Packer (1994).

Proximate analysis

Total lipid was determined on duplicate samples via diethyl ether extraction (AOAC, 1985). Briefly, duplicate 100-g samples of ground *longissimus* tissue were wrapped in cheesecloth. Moisture content was determined by oven drying at 100°C for 24 h. Total lipids then were obtained by diethyl ether extraction.

Statistical analysis

Data from carcasses with no missing values ($n = 309$) were utilized in these analyses. Variables

with many missing values were removed from the analysis, and sensory variables revealed to have little contribution to variation in these samples by preliminary analyses were also omitted from the final analyses. Pearson correlation coefficients were generated using the rcorr function of the Hmisc package (Harrel et al., 2020) of R software (R Core Team, 2019). Euclidian distance matrices were generated for scaled and centered flavor variables as well as for biochemical and physical traits using the get_dist() function of the factoextra package (Kassambara and Mundt, 2020). Clustering of variables and generation of dendrograms were accomplished using hclust(), with Wards method clustering, and as.dendrogram() functions of base R, respectively. Correlation coefficients and dendrograms were combined to generate a heatmap depicting relationships between flavor and chemical variables using the Heatmap() function of the ComplexHeatmap package (Gu et al., 2016).

Hierarchical agglomerative clustering was used to group carcasses using muscle pH, glycolytic potential, *L*, a*, b**, myoglobin concentration, mitochondrial DNA copy number, carbonyls in the sarcoplasmic fraction, sarcomere length, desmin degradation, slice shear force, overall tenderness rating, juiciness rating, intramuscular lipid content, intramuscular moisture content, initial metmyoglobin formation, and bloom. Clustering was accomplished using the Agglomerative Hierarchical Clustering (AHC) function in XLSTAT (v. 2020.3.1, Addinsoft Inc., Paris, France) to determine level of dark cutting categories. The clustering function utilized dissimilarity and the Euclidean distance parameters and the Ward's method was selected. The analysis evaluated 3 to 8 classes, and based on the within-class variance, 4 clusters were selected. The clusters were defined as Severe ($n = 75$), Moderate ($n = 35$), Shady ($n = 111$), and Control ($n = 88$) by the analysis. In the original data analysis, dark cutter carcasses were classified within day into 4 levels of dark cutter. This cluster analysis did not consider day effects, only the independent variables defined earlier. The experimental units were assigned an AHC category, which were compared by ANOVA with the PROC GLM function of SAS (v. 9.4, SAS Institute, Inc., Cary, NC). The model included cluster as a fixed effect and selection day as a random effect. The LSMEANS function with the pdiff option was used to generate least-squares means. Differences were considered statistically significant at $P < 0.05$.

Variables were arbitrarily partitioned into classes (Table 1) for multiple factor analysis (MFA). Variable groups included cluster grouping, carcass characteristics, pH characteristics, mitochondrial function characteristics, proximate composition characteristics, tenderness characteristics, and flavor characteristics. All data except for flavor attributes (which were collected on a common scale) were centered and scaled prior to MFA using the MFA() function of the FactoMineR package (Le et al., 2008). Multiple functions from the factoextra package (Kassambara and Mundt, 2020) enabled visualization of MFA results.

Results and Discussion

Univariate analyses of these data were previously reported in Grayson et al. (2016) and McKeith et al. (2016). Those reports were informative but did not fully elucidate relationships among metabolic factors and flavor attributes. Pearson correlations between flavor attributes and chemical traits as well as clustering of both sets of variables are presented in Figure 1. The dendrogram of chemical variables indicated 4 main branches. The largest cluster (C1) indicated relationships among color traits, tenderness component traits, and mitochondrial abundance and function. Oxygenation of *longissimus* muscle upon exposure to atmospheric oxygen (Bloom) and the accumulation of metmyoglobin during incubation with sodium nitrite (Initial_mMb) were closely related to glycolytic potential. The extent of oxygenation is largely determined by the competition between mitochondria and myoglobin for oxygen, and greater values for Bloom indicate reduced oxygen consumption by the muscle. Increased metmyoglobin formation during incubation in oxidative conditions is an indicator of decreased overall reducing capacity in the muscle. Mechanisms for reduction include direct reduction of metmyoglobin by mitochondrial activity and indirect mechanisms via production of reducing equivalents by mitochondrial activity (Ramanathan and Mancini, 2018; Mitacek et al., 2019). The link between these measures of mitochondrial activity and glycolytic potential is interesting and may be due to the maintenance of mitochondrial function in postmortem muscle with low glycolytic potential, which does not undergo normal pH decline.

The next cluster (C2) indicated close relationships between adjusted fat thickness and vision yield grade, which is not surprising given that adjusted fat thickness is a large contributor to the yield grade calculation. Marbling score and intramuscular lipid were also highly related, which again is not surprising, given that marbling score is a visual appraisal of intramuscular fat. Cluster C3 indicated close relationships in the

Table 1. Description of variables and variable partitioning included in multiple factor analysis

Variable	Description
Clustered groups (supplementary)	
Cluster	Clusters determined on agglomerative hierarchical clustering of biochemical variables
Carcass characteristics (supplementary)	
AFT	Adjusted fat thickness
LMA	*Longissimus* muscle area
YG	Vision yield grade
Marbling	Marbling score
pH traits	
pH	Muscle pH determined at 14 d postmortem
Glycolytic potential	
Color	
*L**	Muscle lightness
*a**	Muscle redness
*b**	Muscle yellowness
Myoglobin concentration	Concentration of myoglobin in *longissimus* muscle
Tenderness characteristics	
Overall tenderness	Sensory panel overall tenderness ratings
Juiciness	Sensory panel juiciness ratings
SSF	Slice shear force
Sarcomere length	Extent of muscle contraction
Desmin degraded	Percentage of desmin degraded at 14 d postmortem
Mitochondrial characteristics	
Mitochondrial copy number	Copies of mitochondrial genes relative to nuclear gene
Carbonyl	Carbonyls on proteins in sarcoplasmic fraction
Bloom	Amount of oxymyoglobin present after incubation of freshly exposed muscle tissue to atmospheric oxygen at 4°C at 14 d postmortem. Measure of oxygen consumption.
Initial metmyoglobin formation	Amount of metmyoglobin formed on 14 d postmortem muscle surface incubated with 0.3% $NaNO_3$ at 20°C. Measure of overall reducing capacity.
Proximate characteristics	
Lipid	Percentage of denuded muscle sample comprised of extractable lipid
Moisture	Percentage of muscle tissue comprised of water
Flavor attributes	
Beef flavor identity	Amount of beef flavor identity in the sample
Brown/roasted	Round, full aromatic generally associated with beef suet that has been broiled
Bloody/serumy	Aromatic associated with blood on cooked meat products. Closely related to metallic aromatic.
Fat-like	Aromatic associated with cooked animal fat
Metallic	Impression of slightly oxidized metal
Liver-like	Aromatic associated with cooked organ meat/liver
Overall sweet	Combination of sweet taste and sweet aromatics
Musty/earthy/humus	Musty, sweet, decaying vegetation
Bitter	Fundamental taste factor associated with a caffeine solution
Salty	Fundamental taste factor of which sodium chloride is typical
Sweet	Fundamental taste factor associated with a sucrose solution
Sour	Fundamental taste factor associated with a citric acid solution
Umami	Flat, salty, somewhat brothy. Taste of glutamate, salts of amino acids and other nucleotides.

perception of overall tenderness and juiciness. These traits were clustered closely to ultimate muscle pH and carbonylation of sarcoplasmic proteins. The final branch of the dendrogram (C4) grouped *longissimus* muscle area, intramuscular moisture content, myoglobin concentration, and slice shear force values.

Flavor attributes clustered into 2 large clusters and 1 cluster consisting of only beef flavor identity (F2). One cluster, F1, indicated relationships between metallic, sour, and fat-like flavor attributes. These attributes also were closely related to brown/roasted, umami, and bloody/serumy. The second cluster (F3) indicated that

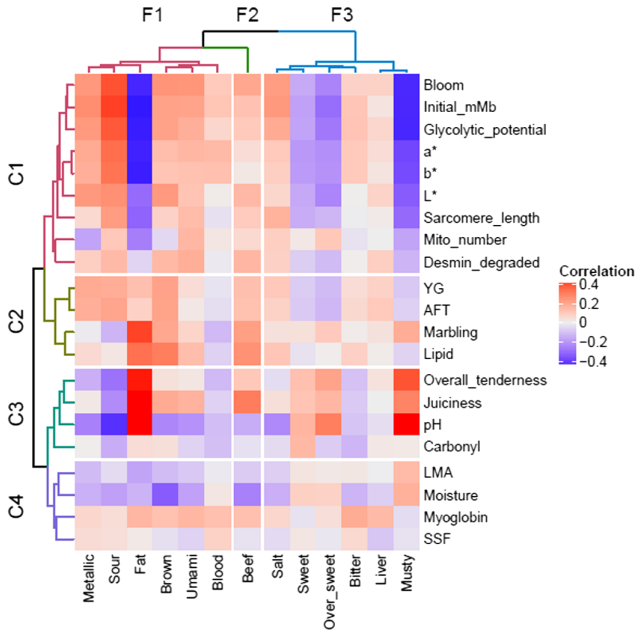

Figure 1. Heatmap of Pearson correlation coefficients among biochemical traits and flavor attributes categorized by agglomerative hierarchical clustering.

salty and sweet notes were closely related, as were overall sweet and bitter flavor notes. Perceptions of liver-like and musty/earthy/humus notes were very closely related and were more distant from the other attributes in this branch.

Correlation coefficients indicated that the variables in cluster C1, comprising color, mitochondrial function, and tenderness component traits, were generally positively correlated to the variables in cluster F1. The most positive relationship was between the C1 variables and the sour attribute. In contrast to the other flavor attributes in cluster F1, fat-like rating was negatively related to mitochondrial function and color traits. Beef flavor identity (cluster F2) had lowly positive correlations to variables in cluster C1. Those variables were negatively correlated to most of the variables in the cluster F3. Thus, increased ratings for musty/earthy/humus, sweet and overall sweet notes were related to increased mitochondrial activity and lesser values for CIE color space values. Alternatively, this cluster of chemical variables was weakly positively related to salty, bitter, and liver-like flavor notes.

Cluster C2 were traits related to carcass fatness and lipid content of the muscle. These variables were positively related to beef flavor identity ratings. Relationships between C2 variables and F1 variables were generally positive. This was particularly true for the relationship between fat-like ratings and both marbling score and intramuscular lipid content.

Cluster C3 consisted of overall tenderness, juiciness, ultimate pH, and carbonyls in sarcoplasmic proteins. The strongest relationships between these variables and those in cluster F1 were positive correlations to fat-like flavor and negative correlations to sour ratings. Beef flavor identity was positively related to juiciness ratings and negatively related to muscle pH. Variables in cluster C3 had positive relationships to musty/earthy/humus and less strong positive relationships to sweet and overall sweet notes. Cluster C4 included *longissimus* muscle area, longissimus muscle moisture content, myoglobin concentration, and slice shear force values. Moisture content of the *longissimus* muscle had negative correlations to most of the flavor attributes in the final analysis, except sweet, overall sweet, and musty/earthy humus. This was particularly true with brown/roasted flavor and beef flavor identity. Myoglobin content had a lowly positive relationship to attributes in cluster F1 and beef flavor identity.

Cluster analysis of metabolic traits

Carcasses originally were included in the present study based on visual appraisal of lean color, which was confirmed with muscle pH. Segregation into dark cutter classes was done via visual appraisal within a selection day for previous analyses (Grayson et al., 2016; McKeith et al., 2016). For those reports, this method of classification was selected after evaluating several other sorting criteria such as muscle pH and CIE color space values. The statistical analyses in our previous reports accounted for selection day effects, and provided a detailed characterization of metabolic, tenderness, and flavor differences across levels of the dark cutting condition.

Comparisons made across these classes provided insight into the contribution of variation in metabolic status to flavor but did not directly test these relationships. Thus, carcasses in this experiment were classified using agglomerative hierarchical cluster analysis of biochemical and physical traits characterizing pH, lean color, mitochondrial function, tenderness traits, and carcass characteristics into 4 groups that were similar within the group, which have been named for dark cutter classes by the clustering software. The within class variation was 86.99%, and the between class variation was 13.01% of total variation indicating that the 4 clusters mainly explained within-class variation. These 4 clusters were defined as Severe, Moderate, Shady and Control nodes by the clustering software (Table 2, Figure 2).

Table 2. Frequency table of carcasses classified by *longissimus lumborum* pH in Grayson et al. (2016) or by aglommerative hierarchical clustering

	Classification based on muscle pH[1]					
Cluster classification[2]	Control	Shady	Mild	Moderate	Severe	Cluster totals
Control	88					88
Shady	62	28	17	3	1	111
Moderate	6	6	9	5	9	35
Severe	1	6	11	28	29	75
Original class totals	157	40	37	36	39	309

[1]Classification based on muscle pH and visual lean color reported by Grayson et al. (2016).

[2]Classification by agglomerative hierarchical clustering using metabolic data. Cluster names were assigned by the statistical software.

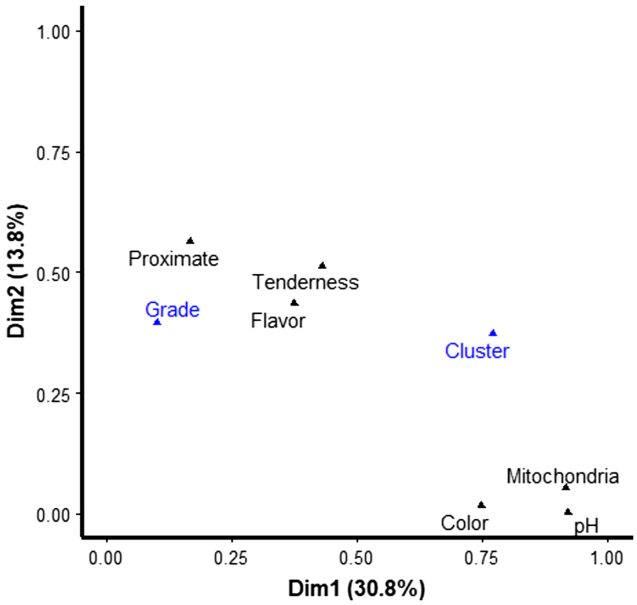

Figure 2. Loadings of variable groupings from multiple factor analysis (MFA) for dimensions 1 and 2. Variables were assigned to groupings for MFA as described in Table 1. Variable groupings labelled in black were used in the calculation of the MFA. Variable groupings labelled in blue were not used in the calculation of the MFA but are mapped to show relationships to other variable groupings.

The frequency distribution of the samples included in this study by the original muscle pH and lean color and by the clustering method are presented in Table 2. Clearly, ranking the carcasses strictly by muscle pH did not completely correspond to the clustering based on biochemical traits related to metabolism. All carcasses classified as Controls by the clustering used in the present analysis were included as controls in the original study. However, at least one carcass included in the original study as a control was clustered in each of the other three classifications, although most discrepancies were assigned to the Shady classification. It should be noted that, in the previous analysis, samples were assigned to pH classes within selection days; thus, some overlap existed in muscle pH across the classifications. However, all the carcasses included in Control category in the original analysis were selected to have normal lean color and muscle pH. Moreover, the dispersion of samples across the clusters derived in the present analysis was not completely explained by that overlap. The general trends in these distributions indicate that the clustering was largely consistent with, but not entirely in agreement with muscle pH. Hence, it is apparent that muscle pH does not completely reflect the differences in capacity for glycolytic and mitochondrial metabolism existing within these muscles. Further investigation of the relationships between variation in muscle metabolism and resulting variation in muscle pH is warranted. Moreover, we suggest that groupings accounting for several metabolic factors are more appropriate than muscle pH alone for investigating the contributions of muscle metabolism to animal variation in beef flavor attributes.

To understand whether the four clustering nodes differed in biochemical, color, carcass characteristics and tenderness measures, data are presented in Table 3. It is apparent from differences reported in Table 3, that the clusters were mainly segmented on differences in pH, glycolytic potential, and color attributes.

Muscle pH increased ($P < 0.0001$) as dark cutter severity class differentiated by cluster analysis increased. Carcasses classified as Control had a mean pH value which is well within the range considered to be "normal" for beef. Muscle pH of carcasses classified in the Shady cluster was still in the upper portion of the "normal" range, which is also associated with less tender beef products. Muscle pH of the remaining 2 classes were well within the range to be considered dark cutters. Differences in glycolytic potential mirrored those in muscle pH, indicating large differences in the amount of glycogen present in the *longissimus lumborum* at harvest. Differences in muscle pH across dark cutter classes were also reflected in color attributes. As dark cutter class increased, progressive

Table 3. Biochemical, color, carcass characteristics, and slice shear force value least-squares means for *longissimus lumborum* steaks from four dark cutting beef categories based on four nodes from Agglomerative Hierarchical Clustering

	Dark Cutting Beef Category From Agglomerative Hierarchical Clustering Nodes					
Attribute	Severe	Moderate	Shady	Control	*P* value[a]	Root Mean Square Error
Biochemical Measures						
Glycolytic potential	49.7[a]	82.6[b]	113.5[c]	157.0[d]	<0.0001	31.0
pH	6.40[d]	6.10[c]	5.75[b]	5.53[a]	<0.0001	0.230
Carbonyl	2.44	2.45	2.04	2.28	0.08	1.158
Color Measures						
*L** color space value	34.63[a]	36.95[b]	40.37[c]	44.51[d]	<0.0001	3.082
*a** color space value	22.74[a]	24.68[b]	28.31[c]	31.82[d]	<0.0001	2.328
*b** color space value	14.53[a]	16.72[b]	20.25[c]	23.90[d]	<0.0001	2.381
Myoglobin, mg/g	4.63	4.59	4.36	4.40	0.06	0.727
Bloom[1], %	63.64[a]	75.70[b]	83.74[c]	92.54[d]	<0.0001	7.717
Initial metmyoglobin formation[2], %	50.29[a]	57.59[b]	64.38[c]	71.24[d]	<0.0001	5.756
Mitochondrial copy number[3]	240.00[a]	411.88[b]	571.14[c]	415.71[b]	<0.0001	289.3
Carcass Characteristics						
Yield grade	1.94[a]	3.40[c]	2.18[b]	3.17[c]	<0.0001	0.601
Preliminary yield grade	2.82[a]	3.81[b]	3.91[c]	3.62[b]	<0.0001	0.453
Adjusted preliminary yield grade	2.81[a]	3.68[c]	2.99[b]	3.57[c]	<0.0001	0.367
Ribeye area, inches[2]	15.28[b]	13.78[a]	15.09[b]	14.23[a]	<0.0001	1.613
Marbling	424[b]	562[d]	398[a]	459[c]	<0.0001	75.56
Tenderness Measures						
Sarcomere length, *u*m	1.688[a]	1.716[a]	1.790[b]	1.874[c]	<0.0001	0.1195
Desmin degraded, %	40.77[a]	47.47[ab]	49.43[b]	67.66[c]	<0.0001	23.989
Slice shear force, kg	19.05[b]	19.76[b]	23.42[c]	14.82[a]	<0.0001	5.696
Lipid, %	3.07[a]	5.88[c]	3.23[a]	5.16[b]	<0.0001	1.256
Moisture, %	74.59[c]	71.97[a]	74.06[b]	71.34[a]	<0.0001	1.094

[1]Bloom = the percentage of surface myoglobin in the oxymyoglobin form after incubation in atmospheric oxygen.

[2]Initial memyoglobin formation = the percentage of surface metmyoglobin in the metmyoglobin form after incubation with 0.3% sodium nitrite.

[3]The number of copies of mitochondrial DNA present after being corrected for the number of copies of genomic DNA.

[a]*P* value from Analysis of Variance Table.

[b–d]Mean values within a row and main effect followed by the same letter are not significantly different ($P < 0.05$).

decreases were detected ($P < 0.0001$) in muscle lightness (L^*), redness (a^*), and yellowness (b^*). Myoglobin concentration was not affected ($P = 0.06$) by dark cutter clustering groups.

Copy number of mitochondrial genes indicated that *longissimus* muscles from carcasses classified in the Shady cluster had the greatest ($P < 0.05$) number of mitochondria, and muscles from carcasses classified in the Severe cluster had the lowest number of mitochondria. This differs somewhat from the results of McKeith et al. (2016). However, it must be noted that, in addition to differences in classification, the calculation of mitochondrial copy number used in the present report was quite different from the ratio of polymerase chain reaction-cycle threshold values used in McKeith et al. (2016). Measures of oxygen consumption and nitric oxide metmyoglobin reducing ability commonly used in lean color stability research (King et al., 2023) reflect mitochondrial function, both directly and indirectly. As the severity of dark cutting increased, the oxygenation of myoglobin in freshly cut meat surfaces after incubation in atmospheric oxygen decreased ($P < 0.05$). Similarly, the amount of metmyoglobin formed on meat surfaces after incubation in 0.03% sodium nitrite decreased ($P < 0.05$) as severity of dark cutting increased. Thus, it is apparent that mitochondrial function was greater in *longissimus* muscles in carcasses classified as dark cutters by data clustering. It is not clear whether this was strictly due to higher muscle pH facilitating the retention of mitochondrial function, or whether muscles from dark cutting carcasses possess greater mitochondrial function *in vivo*.

In our previous report, we attributed increased mitochondrial abundance and decreased mitochondrial efficiency as causative factors in dark cutting beef (McKeith et al., 2016). We suggested that depletion of muscle glycogen was due to inefficient mitochondria attempting to generate ATP during a stress response. Mahmood et al. (2018) reported a decreased capacity for glycolytic metabolism in dark cutting beef as evidenced by decreased abundance of several glycolytic proteins relative to normal-colored beef. In that case, the muscle may possess less glycogen under baseline conditions. Either of these conditions could contribute to decreased pH decline in postmortem muscle but would not necessarily cause the dark cutter condition in all cases. Thus, categorizing muscles based on several metabolic factors rather than just muscle pH may be more conducive to increasing understanding of the contribution of muscle metabolism to beef flavor.

Overall tenderness ratings and slice shear force values both differed ($P < 0.0001$) across dark cutter classes. Overall tenderness ratings were greatest ($P < 0.05$) for *longissimus* steaks in the Severe and Moderate clusters and least ($P < 0.05$) for *longissimus* steak from carcasses identified as Shady. Slice shear force values were lowest ($P < 0.05$) for steaks from carcasses classified as Controls and greatest ($P < 0.05$) for *longissimus* steaks from carcasses classified as Shady. Both measures of tenderness indicated that *longissimus* steaks from carcasses identified as Shady were substantially less tender than carcasses from other classes. Previous investigators have reported identified beef *longissimus* steaks with pH values similar to those in the Shady classification as being less tender than *longissimus* steaks with either higher or lower ultimate pH values (Jeremiah et al., 1991; Wulf and Page, 2000). Differences in juiciness ratings across dark cutting classes mirrored ($P < 0.05$) those observed for overall tenderness. Desmin degradation was greatest ($P < 0.05$) in *longissimus* steaks classified as Controls. *Longissimus* steaks from carcasses classified as Shady exhibited greater ($P < 0.05$) desmin degradation than steaks from carcasses classified as Severe dark cutters. Sarcomere length was longer ($P < 0.05$) in *longissimus* muscles from Control carcasses than those from carcasses classified as Shady, which were longer ($P < 0.05$) than steaks from carcasses classified as either Moderate or Severe dark cutters. *Longissimus* muscle lipid content was least ($P < 0.05$) in carcasses classified as Severe or Shady. Lipid content of *longissimus* steaks from Control carcasses was lower ($P < 0.05$) than from carcasses classified as Moderate. Differences in *longissimus* muscle moisture content were inversely related to those detected for lipid content.

The dark cutter classes determined by cluster analysis differed regarding carcass traits. Vision yield grade was greatest ($P < 0.05$) for carcasses classified as Moderate or as Controls and lowest ($P < 0.05$) in carcasses classified as Severe dark cutters, with carcasses Classified as Shady having intermediate vision yield grades. These differences across dark cutter classes were the same as those detected for adjusted fat thickness. *Longissimus* muscle area was greater ($P < 0.05$) in carcasses classifies as Severe and Shady than in carcasses classified as Control or Moderate. Marbling scores differed ($P < 0.0001$) across dark cutter classes. Ranking of the dark cutter classes for marbling score were Moderate >> Control > Severe > Shady.

Least-squares means of flavor attributes stratified across the clusters of carcasses differing in dark cutter severity are presented in Table 4. Beef flavor identity, liver-like, and bitter attributes were not ($P = 0.18$, 0.59, and 0.17, respectively) affected by dark cutter class. Brown/roasted flavor was higher ($P < 0.05$) in steaks from carcasses classified as Control and Moderate than in steaks from carcasses classified as Severe. Brown/roasted flavor did not differ ($P > 0.05$) between steaks from carcasses classified as Moderate and those from carcasses classified as Shady. Generally, as dark cutter severity class increased, the flavor attributes of fat-like, overall sweet, musty/earthy/humus, and sweet increased. Conversely, as dark cutter severity class increased, the flavor attributes of bloody/serumy, metallic, salty, sour, and umami generally decreased. These results are in agreement with Denzer et al. (2020), which reported that *longissimus lumborum* steaks with pH greater than 6.0 had increased sour flavor and tenderness ratings than normal *longissimus lumborum* steaks. `

Multiple factor analysis

Multiple factor analysis summarizes and enables visualization of complex relationships when individuals are characterized by groupings of variables, which can be either qualitative or quantitative. These results indicated that use of agglomerative hierarchical clustering analysis segmented these carcasses into categories that expressed differences in dark cutting. In addition, these data had sufficient variability to understand relationships between biochemical, color, carcass characteristics, tenderness measures, and flavor and texture descriptive attributes. The overall loadings for groupings of variables to dimensions (factors) 1 and 2, which accounted for 30.8 and 13.8% of the variation, are

Table 4. Flavor aromatics, basic tastes, and texture descriptive attributes least-squares means for *longissimus lumborum* steaks from four dark cutting beef categories based on four nodes from Agglomerative Hierarchical Clustering (0 = none; 15 = extremely intense)

	Dark Cutting Beef Category from Agglomerative Hierarchical Clustering Nodes					
Attribute	Severe	Moderate	Shady	Control	*P* value[a]	Root Mean Square Error
Flavor Aromatics						
Beef identity	4.18	4.33	4.24	4.30	<0.18	0.39
Brown/roasted	2.08[a]	2.28[bc]	2.15[ab]	2.36[c]	0.0003	0.42
Bloody/serumy	2.75[ab]	2.66[a]	2.83[bc]	2.88[c]	0.03	0.40
Fat-like	1.95[b]	2.05[b]	1.47[a]	1.46[a]	<0.0001	0.45
Metallic	1.79[a]	1.82[ab]	1.96[b]	1.96[b]	0.002	0.35
Liver-like	0.08	0.05	0.07	0.08	0.59	0.13
Overall sweet	0.75[b]	0.77[b]	0.62[a]	0.62[a]	0.0001	0.25
Musty/earthy/humus	0.21[b]	0.17[b]	0.06[a]	0.04[a]	<0.0001	0.17
Basic Tastes						
Bitter	0.61	0.67	0.62	0.73	0.17	0.39
Salty	0.98[a]	1.09[ab]	1.05[ab]	1.12[b]	0.009	0.25
Sweet	1.05[b]	1.05[b]	0.92[a]	0.93[a]	0.001	0.25
Sour	1.66[a]	1.72[a]	2.07[b]	2.22[c]	<0.0001	0.46
Umami	2.21[a]	2.33[ab]	2.35[b]	2.43[b]	0.006	0.38
Texture						
Juiciness	5.68[c]	5.75[c]	5.13[a]	5.39[b]	<0.0001	0.367
Overall tenderness	6.03[c]	5.77[c]	4.67[a]	5.29[b]	<0.0001	0.70

[a]*P*-value from Analysis of Variance table.

[b–d]Mean values within a row and main effect followed by the same letter are not significantly different ($P < 0.05$).

shown in Figure 2. Carcass grade characteristics (Grade) and the dark cutter clustering (Cluster) were included as supplementary variables and did not contribute to the calculation of the dimensions. However, loadings for these variables allow visualization of how they relate to the other variables in relation to the dimensions.

Variables in the pH, mitochondrial function, and color attribute groupings were the strongest contributors to dimension 1. Dimension 2 was the result of strong contributions of proximate composition, tenderness, and flavor variable groups. Loadings for the Cluster variable indicated strong relationships to both dimension 1 and 2 (Figure 2). Loadings for the Grade variable group indicate strong relationships to proximate composition, tenderness, and flavor. Thus, it appears that loadings for dimensions 1 and 2 will provide insight into the relationships between metabolic factors associated with the dark cutting condition and flavor attributes.

The loadings for individual variables and individual samples for dimensions 1 and 2 are presented in Figure 3A and 3B, respectively. Positive values for dimension 1 indicate a carcass with lower *longissimus* pH, higher glycolytic potential, higher mitochondrial function, and lower ratings for overall tenderness and juiciness. Positive loadings for dimension 1 also were associated with increased sour flavor. Positive loadings for dimension 2 were associated with greater overall tenderness and juiciness ratings, as well as greater lipid content. Flavor attributes associated with positive loadings for dimension 2 were beef identity and brown/roasted. Fat-like flavor had loadings that were negative for dimension 1 and positive for dimension 2, which were in the same direction as overall tenderness and juiciness ratings.

Previous results from our laboratory identified animal-to-animal variation in beef *longissimus lumborum*, *gluteus medius*, and *biceps femoris* (sirloin cap) flavor that was not associated with quality grade or aging time but was, to some extent, related to production lot (King et al., 2021). That report identified 2 groupings of beef flavor attributes. One grouping consisted of beef flavor identity, brown/roasted, fat-like, and salt flavors. The second grouping consisted of bloody-serumy, metallic, bitter, and sour flavor attributes. These relationships among flavor attributes are similar to those observed by Foraker et al. (2020) across beef

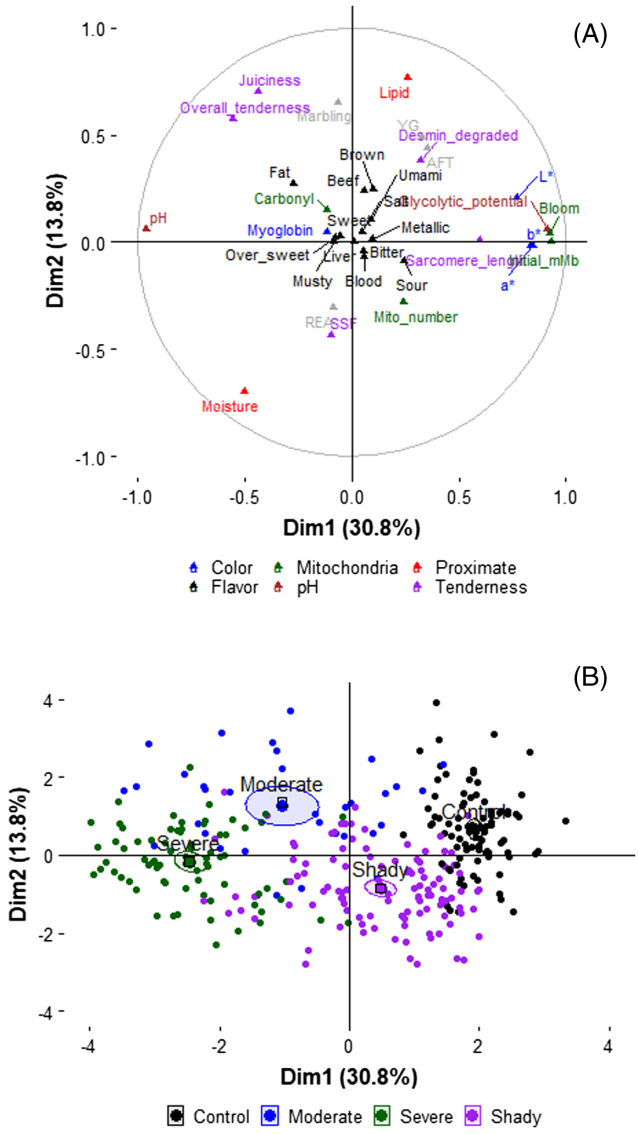

Figure 3. Loadings of individual variables (panel A) and individual samples and cluster grouping (panel B) for dimensions 1 and 2 from multiple factor analysis.

longissimus muscles treated with varying aging protocols. Both of those studies were limited to samples within the normal muscle pH range. In the present experiment, clustering of beef flavor attributes resulted in a grouping (F1) that included most of the flavor attributes from those two groupings, which, except for fat-like, were generally positively correlated to metabolic factors, indicating increased glycolytic metabolism and lesser mitochondrial function, and negatively correlated to ultimate muscle pH. The loadings of these flavor attributes in relation to the dark cutter cluster groupings indicate that muscles from carcasses in the Control or Shady clusters tended to have greater sour, bitter, bloody, and umami ratings, while carcasses from the Moderate and Severe clusters tended to have higher ratings for fat-like and musty, which is in general agreement with the results of our previous analysis (Grayson et al., 2016).

In the present study, positive loadings for dimension 2 indicate positive relationships between overall tenderness ratings and desmin degradation to flavor attributes such as beef flavor identity, brown-roasted, and fat-like. This is consistent with previous findings that postmortem proteolysis is related to the production of volatile compounds that are precursors for favorable beef flavor development (Yang et al., 2018; Yang et al., 2019). This would also be consistent with flavor development associated with increased postmortem aging (Lepper-Blilie et al., 2016; Foraker et al., 2020; Tuell et al., 2022).

The clusters for dark cutting categories are clearly segregated along dimension 1, with the Control cluster grouped on the right (positive) end of the axis and the Severe cluster on the left (negative) end of the axis. The Shady and Moderate clusters were intermediate and with more overlap along dimension 1. This arrangement along dimension 1 clearly confirms that the clusters were associated with variation in lean color and muscle pH indicative of the dark cutting condition. Whereas carcasses classified in the Moderate and Control clusters generally had positive values for dimension 2, carcasses classified as Shady and Severe tended to have negative loadings indicating more favorable flavor attributes in the Moderate and Control carcasses.

Conclusions

While ultimate postmortem muscle pH does reflect differences in glycolytic and mitochondrial metabolism, pH alone does not entirely characterize the metabolic differences affecting meat quality parameters such as flavor. Results from the present analysis indicate that variation in the capacity and extent of muscle metabolism impacts the flavor profile of beef *longissimus lumborum* steaks. Moreover, increased tenderness attributes were associated with increased ratings for favorable beef flavor attributes. These results suggest that there are direct effects of both the metabolic machinery and ultimate muscle pH that are independent but confounded with one another. More work is necessary to parse out those effects. The use of multivariate statistical approaches enhanced the understanding of the relationships described by previous univariate approaches.

Acknowledgments

Mention of trade names or commercial products in this publication is solely for the purpose of providing

specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture (USDA). The authors are grateful to Patty Beska, Kristen Ostdiek, Peg Ekeren, and Casey Trambly of the U.S. Meat Animal Research Center for their assistance in the execution of this experiment and to Jody Gallagher of the U.S. Meat Animal Research Center for her secretarial assistance. The USDA is an equal opportunity provider and employer.

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