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Brahman genetics influence muscle fiber properties, protein degradation, and tenderness in an Angus-Brahman multibreed herd

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ABSTRACT

The objective of this study was to determine the influence of Brahman genetics on muscle contractile and metabolic phenotype and postmortem proteolysis. Cattle used in this study represent a continuous spectrum of Angus-Brahman genetic variation. Steers were harvested and *Longissimus* samples were collected at 1.5 h, 24 h, and 14 d postmortem. Proteolysis during the 14d aging period was evaluated, along with Warner-Bratzler shear force (WBSF) and trained sensory panel tenderness. Myosin heavy chain composition and enzymatic activity were used to evaluate fiber type characteristics. As Brahman influence increased, WBSF increased and sensory tenderness decreased. Calpain-1 autolysis decreased as Brahman percentage increased, and corresponded with reduced degradation of troponin-T, desmin, and titin. Increasing Brahman percentage was associated with greater citrate synthase activity and greater cross-sectional area of type IIx fibers. Brahman-influenced cattle produced tougher steaks and exhibited decreased protein degradation. Thus, Brahman genetics impacted not only the calpain-calpastatin system, but also muscle fiber size and metabolic properties.

1. Introduction

Brahman and Brahman-influenced cattle are widely utilized in the Southeastern and Gulf Coast regions of the United States. Their heat tolerance and parasite resistance, among other adaptations, make Brahman an integral part of cattle herds in sub-tropical climates (Turner, 1980). While Brahman provide beneficial influence to the herd, they exhibit less desirable carcass and palatability traits. When compared to Angus and Angus × Brahman crossbred cattle, Brahman produce carcasses with smaller ribeye areas and their ribeye steaks are tougher than Angus ribeye steaks as measured by Warner-Bratzler shear force (WBSF) values and sensory panelists (Elzo, Johnson, Wasdin, & Driver, 2012; Gonzalez et al., 2014; Huffman, Williams, Hargrove, Johnson, & Marshall, 1990; Whipple et al., 1990).

While several factors influence meat tenderness, a primary determinant is the calpain system (Boehm, Kendall, Thompson, & Goll, 1998; Koohmaraie, 1992; Koohmaraie & Geesink, 2006). Calpains are calcium-activated cysteine proteases that initiate proteolysis but do not completely degrade their targets. Of the fifteen members within the calpain family, calpain-1 is the most related to meat tenderness; calpain-1 generates myofibrillar degradation products that closely follow the pattern observed during aging of meat (Huff-Lonergan et al., 1996) and calpain-1 knockout mice show limited postmortem proteolysis (Geesink, Kuchay, Chishti, & Koohmaraie, 2006). In the presence of Ca^{2+}, the 80 kDa subunit of calpain-1 autolyzes to a 76 kDa subunit which indicates that the calpain has become proteolytically activated. In postmortem muscle, calpain-1 targets several myofibrillar proteins, including titin, nebulin, desmin, and troponin-T (Huff-Lonergan et al., 1996; Huff-Lonergan, Parrish, & Robson, 1995; Koohmaraie, 1992). Calpastatin, the endogenous inhibitor specific to calpain, is composed of four domains (I, II, III, and IV) that can each inhibit the proteolytic activity of calpain (Goll, Thompson, Li, Wei, & Cong, 2003; Wendt, Thompson, & Goll, 2004). *Bos indicus* cattle have a higher calpastatin activity when compared to *Bos taurus* cattle, and as Brahman influence increases, the calpastatin: calpain-1 ratio increases. Augmented calpastatin activity in *Bos indicus* cattle decreases postmortem proteolysis, which negatively impacts tenderness (Pringle, Harrelson, West, Williams, & Johnson, 1999; Pringle, Williams, Lamb, Johnson, & West, 1997; Wheeler, Savell, Cross, Lunt, & Smith, 1990; Whipple et al., 1990).

During the conversion of muscle to meat, the changing cellular environment also influences proteolysis and tenderization. As homeostatic mechanisms are lost, muscle undergoes energetic, biochemical, and physical changes that dictate meat quality development. Energy

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metabolism shifts to anaerobic glycolysis, which generates lactate and H^+, and results in a decline in pH. Temperature and pH decline are well-known to affect protein functionality, including calpain-1 activity (Maddock Carlin, Huff-Lonergan, Rowe, & Lonergan, 2009; Mohrhauser, Lonergan, Huff-Lonergan, Underwood, & Weaver, 2014). Postmortem muscle also loses the ability to maintain reducing conditions. Oxidative modifications to proteins increase during meat aging, and even low levels of oxidation reduce tenderness (Harris, Huff-Lonergan, Lonergan, Jones, & Rankins, 2001). The rate of oxidation differs between muscles and may be affected by processing procedures, diet, and the inherent antioxidant systems within muscle (Martinaud et al., 1997; Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004). The intrinsic characteristics of muscle fibers play an important role in shaping these cellular changes during the conversion of muscle to meat.

Several factors, including genetics, breed, and body location, influence muscle fiber characteristics; and fibers are also highly adaptable to external cues, such as environment and exercise (Klont, Brocks, & Eikelenboom, 1998; Ozawa et al., 2000; Rosser, Norris, & Nemeth, 1992). Muscle fibers are classified based on contractile speed (slow or fast) and predominant type of energy metabolism (oxidative or glycolytic). Myosin heavy chain (MHC) isoform composition is the primary determinant of contractile speed. Cattle express three myosin heavy chain (MHC) isoforms: type I, type IIa, and type IIx (Lefaucheur, 2010). Type I fibers are slow-twitch and rely on oxidative metabolism. In contrast, type II fibers are fast-contracting, but differ in predominant type of metabolism; type IIa fibers are relatively more oxidative while type IIx fibers are more glycolytic.

The influence of Brahman genetics on muscle fiber properties is not clear. Fiber area has been shown to differ between *Bos indicus* and *Bos taurus* despite no differences in contractile fiber type (Coles et al., 2014; Seideman, 1985; Waritthitham, Lambertz, Langholz, Wicke, & Gauly, 2010; Whipple et al., 1990). The impact of breed on metabolic characteristics is also uncertain, though it seems logical that Brahman muscle may acquire adaptations that relate to heat tolerance. Muscle fiber properties affect cellular changes in postmortem muscle and thus may be important to understanding meat tenderization in Brahman influenced cattle. Therefore, the objectives of this study are to establish if contractile and metabolic phenotype of muscle may be related to the proteolysis and tenderness differences observed in cattle varying in Brahman composition.

2. Materials and methods

2.1. Animal breeding and management

Cattle used in this study were part of a long-term genetic study involving Angus, Brahman, and Angus-Brahman crossbreeding. Standards for animal care and use were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC number 201003744). Although these cattle represent a continuous spectrum of Angus-Brahman genetic variation (Table 1), they were divided into six breed groups for analysis: 1 = Angus; 2 = ¾ Angus, ¼ Brahman; 3 = Brangus; 4 = ½ Angus, ½ Brahman; 5 = ¼ Angus, ¾ Brahman; and 6 = Brahman. A diallel mating system was used in this multibreed herd, where sires from the six breed groups were mated across to dams of the same six breed groups (Elzo & Wakeman, 1998). Calves were born from late November 2013 to early February 2014, castrated at birth, and weaned in August 2014.

Table 1
Specific composition of Angus and Brahman breed within each breed group.

	Breed group	Angus	Brahman
1	Angus	0.80–1.00	0.00–0.20
2	¾ Angus, ¼ Brahman	0.60–0.79	0.21–0.40
3	Brangus	0.625	0.375
4	½ Angus, ½ Brahman	0.40–0.59	0.41–0.60
5	¼ Angus, ¾ Brahman	0.20–0.39	0.61–0.80
6	Brahman	0.00–0.19	0.81–1.00

Preweaning calves were kept with their dams on bahiagrass pastures (*Paspalum notatum*) at the Beef Research Unit of the University of Florida (UFBRU), with free access to a complete mineral supplement (Lakeland Animal Nutrition, Lakeland, FL). Postweaning, calves continued to be kept on bahiagrass pastures at the UFBRU where they received a supplement of bahiagrass hay, concentrate (1.6 to 3.6 kg/d; 14.0% CP, 488 Pellet Medicated Weaning Ration, Lakeland Animal Nutrition, Lakeleand, FL; soy hull pellets), and free access to mineral supplement. Yearling steers were transported to a contract feeder (Suwannee Farms, O Brien, Florida) where they were provided a standard commercial corn-protein diet with vitamins and minerals. Subsequently, steers were selected and sorted into one of three slaughter dates in 2015 based on physiological endpoint; steers were in the finishing phase for 181, 201, and 236 d. The goal was to have cattle finished with 1.27 cm of subcutaneous fat. Finished steers were transported 100 km to the University of Florida Meat Laboratory (Gainesville, FL) on the day prior to harvest.

2.2. Sample collection

Steers (n = 12 per day; 2 per breed group) were harvested under USDA-FSIS inspection at the University of Florida Meat Laboratory (Gainesville, FL) on one of three days. Samples from the *Longissimus lumborum* (LL) muscle were collected from the left side of the carcass at 1.5 h, 24 h, and 14 d postmortem. At 1.5 h, approximately 50 g muscle was removed 15 to 20 cm caudal to the last costa. A portion of the 1.5 h muscle sample was mounted on a cork and frozen in liquid nitrogen cooled isopentane for histology, and another portion was frozen in liquid nitrogen. At 24 h, roughly 15 g sample was removed from the LL at least 5 cm anterior from the previous location; for the 14 d sample, a 1.0 cm slice was collected and packaged identical to the steaks used for analysis of shear force and sensory evaluation. Muscle samples from all three time points were frozen in liquid nitrogen and stored at − 80 °C for subsequent analysis. The pH of the LL was determined at 1.5, 3, 6, 9, and 24 h postmortem with a Hanna HI 99163 meat pH meter (Hanna Instruments, Woonsocket, RI).

After initial 1.5 h sampling, hot carcass weight (HCW, kg) was determined, then carcasses received a final wash and were placed in a 0 °C cooler. At 48 h postmortem, carcasses were ribbed between the 12th and 13th rib and carcass data was recorded. Dressing percentage (DP, %) was calculated as a percentage of the carcass weight over the live weight. Carcass traits evaluated at the 12th rib were fat over the ribeye (FOE, cm), ribeye area (REA, cm^2), and marbling score (MAB; 100 to 199 = practically devoid, 200 to 299 = traces, 300 to 399 = slight, 400 to 499 = small, 500 to 599 = modest, 600 to 699 = moderate, 700 to 799 = slightly abundant, 800 to 899 = moderately abundant, 900 to 999 = abundant). Kidney, pelvic, and heart fat (KPH, %) was evaluated as a percentage of the carcass weight. Two 2.54 cm thick steaks were removed from the left side of the carcass posterior to the 12th/13th rib interface for subsequent analysis of Warner-Bratzler shear force (WBSF, N) and sensory evaluation. Steaks were individually bagged and then placed in heat shrink vacuum bags (B2570; Cryovac, Duncan, SC) and vacuumed sealed with a Multivac C500 (Multivac Inc., Kansas City, MO). Steaks were aged at 2 ± 3 °C for 14 d postmortem, then frozen at − 40 °C until further analysis.

2.3. Warner-Bratzler shear force

Steaks used for WBSF measurements and sensory evaluation were allowed to thaw at 2–5 °C for approximately 24 h prior to cooking. Steaks were prepared according to the American Meat Science Association Sensory Guidelines (Belk et al., 2015). Copper-constantan thermocouples (Omega Engineering Inc., Stanford, CT) were placed in

the geometric center of each steak to continuously measure internal temperature. Temperatures were monitored using 1100 Labtech Notebook Pro Software version 12.1 (Computer Boards Inc., Middleboro, MA). Steaks were cooked on open-hearth grills (Hamilton Beach Brand, Washington, NC) to an internal temperature of 35 °C, flipped once and removed when they reached the final internal temperature of 71 °C.

Cooked steaks used for WBSF were placed on a tray, overwrapped, and chilled at 4 ± 2 °C for 24 h. After chilling, steaks were trimmed to expose muscle fiber alignment. Six cores (1.27 cm diameter) were removed parallel to the longitudinal axis of the muscle fibers. An Instron Universal Testing Machine (Instron Corporation, Canton, MA) with a Warner-Bratzler shear head (crosshead speed of 200 mm/min) attached to a 490 N load cell was used to measure the force required to shear through the core. Each core was placed so that the sample was sheared through the center of the core, perpendicular to the longitudinal axis of the muscle fibers. Maximum shear force values for each core were recorded and values from all six cores were used to generate a single average shear force value for each steak.

2.4. Sensory evaluation

Steaks used for sensory analysis were removed from the grill and trimmed of any fat and connective tissue. Remaining muscle was sliced on a grid into 1 cm squares that were approximately 2.54 cm thick. Each panelist received two samples per steak and evaluated six steaks in a session. Sessions were held in a positive pressure ventilated room with lighting and cubicles designed for objective sensory analysis. The panel consisted of eight to eleven members trained in accordance with the American Meat Science Association Sensory Guidelines for Sensory Analysis (Belk et al., 2015). The panelists evaluated each sample for 5 attributes: juiciness (1 = extremely dry, 2 = very dry, 3 = moderately dry, 4 = slightly dry, 5 = slightly juicy, 6 = moderately juicy, 7 = very juicy, 8 = extremely juicy), beef flavor intensity (1 = extremely bland, 2 = very bland, 3 = moderately bland, 4 = slightly bland, 5 = slightly intense, 6 = moderately intense, 7 = very intense, 8 = extremely intense), overall tenderness (1 = extremely tough, 2 = very tough, 3 = moderately tough, 4 = slightly tough, 5 = slightly tender, 6 = moderately tender, 7 = very tender, 8 = extremely tender), connective tissue (1 = abundant, 2 = moderately abundant, 3 = slightly abundant, 4 = moderate amount, 5 = slight amount, 6 = traces amount, 7 = practically devoid, 8 = none detected), off-flavor (1 = extreme off-flavor, 2 = strong off-flavor, 3 = moderate off-flavor, 4 = slight off-flavor, 5 = barely detected, 6 = none detected).

2.5. Enzyme activity

Citrate synthase (CS) and lactate dehydrogenase (LDH) activities were determined on 1.5 h muscle samples. Powdered muscle was diluted 1:20 (w/v) in buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.4) and homogenized at 5000 rpm for 10s. Muscle homogenates were sonicated and diluted for determining CS and LDH activity. Citrate synthase activity was determined by measuring the reduction of DTNB (5,5′-dithiobis-[2-nitrobenzoic acid]) at 412 nm using a microplate spectrophotometer (Biotek; Winooski, VT) according to Scheffler et al. (2014). Lactate dehydrogenase activity was determined in homogenates by the decrease in NADH measured at 340 nm. Diluted muscle homogenate (10 μl) was added to 170 μl reaction media (90 mM sodium phosphate, 4.5 mM EDTA, and 0.6 mM NADH, pH 7.5). After a 2 min background reading, the reaction was initiated by the addition of 30 μl of 8.4 mM sodium pyruvate. Absorbance was measured at 37 °C every 20s for 7 min. Enzyme activities were calculated and reported as nmol/min/mg tissue for CS and μmol/min/mg tissue for LDH.

2.6. SDS-PAGE and western blotting

Muscle samples from 1.5 h, 24 h and 14 d were used for SDS-PAGE and western blotting to assess proteolysis during the 14 d aging period. Assessment included the degree of autolysis of the proteolytic enzyme, calpain-1; content of the calpain inhibitor, calpastatin; and the extent of degradation of troponin-T and desmin. Whole muscle samples used for the calpain, troponin-T and desmin analyses were finely powdered with liquid nitrogen and then 100 mg of powdered muscle was diluted with 10 volumes of buffer containing 10 mM sodium phosphate, pH 7.0 and 10% (w/v) SDS. For calpastatin content, powdered muscle was diluted 1:50 (w/v) in buffer (0.05 M Tris-HCl, pH 6.8) containing 8 M urea, 2 M thiourea, 3% (w/v) SDS, and 75 mM dithiothreitol (DTT). All samples were homogenized at 5000 rpm for 3 x 10s using a bead-beating homogenizer (Precellys, Bertin Corp.; Rockville, MD), and then centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was transferred to a new tube and protein concentration was determined for all samples using the RC DC protein assay (Bio-Rad 500-0119, Bio-Rad Laboratories, Hercules, CA). Protein samples for calpain, troponin-T and desmin were diluted in Laemmli buffer (final concentration: 40 mM Tris, pH 6.8; 100 mM dithiothreitol, 2% SDS, 0.01% bromophenol blue, 10% glycerol) to yield equal protein concentrations and heated to 95 °C for 5 min. Calpastatin protein samples were diluted with extraction buffer plus 0.03% bromophenol blue to yield equal protein concentrations and heated to 60 °C for 10 min. A 7% polyacrylamide separating gel (23% [v/v] 30% acrylamide/0.8% bisacrylamide, 0.37 M Tris, pH 8.8, 0.1% [w/v] SDS, 0.13% [w/v] ammonium persulfate, and 0.07% [v/v] TEMED) was used to determine calpain-1 autolysis and calpastatin content, a 15% polyacrylamide separating gel was used to detect the degradation of troponin-T, and a 10% polyacrylamide separating gel was used to determine the degradation of desmin. A 5% polyacrylamide gel (17% [v/v] 30% acrylamide/0.8% bisacrylamide, 0.125 M Tris, pH 6.8, 0.1% [w/v] SDS, 0.15% [w/v] ammonium persulfate, and 0.1% [v/v] TEMED) was used for the stacking gel. Gels were loaded with 15 μg protein for calpain-1, 30 μg protein for calpastatin, 5 μg protein for troponin-T, and 10 μg protein for desmin. For each target protein, four gels were needed to accommodate all samples. Within each gel, all breed groups were represented (1–2 steers per breed group on a gel). Reference samples for each time point (1.5 h, 24 h, 14 d) were included on each gel to verify consistency across gels, transfer, and blotting. The reference sample for each time point consisted of pooled protein samples from at least two steers from each breed group. Gels (20 cm wide × 8.5 cm tall × 0.75 mm thick) were run at 60 V for 20 min and then 125 V for 2 h for calpain and desmin and 3 h for calpastatin and troponin-T in a MGV-202-20 electrophoresis unit (C.B.S. Scientific, San Diego, CA).

Proteins were transferred from gels to nitrocellulose membranes at 4 °C for 60 min at 500 mA in EBU-402 transfer tanks (C.B.S. Scientific, San Diego, CA) using transfer buffer (50 mM Tris, 0.38 M glycine, 0.01% [w/v] SDS) with 10% methanol. Membranes were dried and then blocked with Tris-buffered saline, pH 7.5 (TBS) blocking buffer (StartingBlock 37542, Thermo Scientific, Rockford, IL) for 1 h at room temperature. Membranes were incubated with primary antibodies diluted in blocking buffer with 0.2% Tween 20 overnight at 4 °C. Primary antibodies were diluted as follows: Calpain-1, 1:10,000 (MA3-940, Thermo Scientific, Rockford, IL); calpastatin, 1:5000 (MA3-944, Thermo Scientific, Rockford, IL); troponin-T, 1:20,000 (T6277, Sigma, St. Louis, MO); desmin, 1:10,000 (D1033, Sigma, St. Louis, MO). Membranes were washed 3 × 5 min in TBS with 0.1% Tween and then incubated with secondary antibodies diluted in blocking buffer with 0.2% Tween 20 for 1 h at 4 °C. Goat anti-mouse secondary antibodies were diluted as follows: Calpain-1, 1:10,000 (IRDye 680RD, LI-COR, Lincoln, NE); calpastatin, 1:10,000 (IRDye 800CW, LI-COR, Lincoln, NE); troponin-T, 1:20,000 (IRDye 800CW, LI-COR, Lincoln, NE); desmin, 1:15,000 (IRDye 800CW, LI-COR, Lincoln, NE). Membranes were washed an additional 3 × 5 min in TBS with 0.1% Tween and

then rinsed once with TBS. Blots were imaged with an Odyssey CLx (LI-COR, Lincoln, NE) and bands were quantified using Image Studio version 5.2.

Total calpain-1 was calculated as a ratio of the total signal at 1.5 h (80 kDa + 78 kDa + 76 kDa) to the total signal of a reference sample. The degree of calpain-1 autolysis at 24 h was calculated by determining the ratio of the 76 kDa signal over the total signal (80 kDa + 78 kDa + 76 kDa) at 24 h. Calpastatin content at 24 h was calculated as a ratio of the 135 kDa signal at 24 h over the 135 kDa signal at 1.5 h. Within each time point (24 h or 14 d), degradation of troponin-T was calculated as a ratio of the degradation products (total signal minus intact troponin-T) over the total signal. Desmin was determined similarly, with 55 kDa considered the 'intact' band.

2.7. Agarose gels

Muscle samples from 1.5 h, 24 h and 14 d were used for determining the extent of degradation of titin in agarose gels according to Warren, Krzesinski, and Greaser (2003) with some modifications. Whole muscle samples were finely powdered with liquid nitrogen and diluted in extraction buffer (8 M urea, 2 M thiourea, 3% SDS, 75 mM DTT and 0.05 M Tris-Cl, pH 6.8) using a 1:100 sample: buffer ratio (w/v). Samples were homogenized at 5000 rpm for 3 × 10 s (Precellys, Bertin Corp.; Rockville, MD), and centrifuged at 13,200 × *g* for 12 min at 4 °C; the supernatant was transferred to a new tube. Sample protein concentration was determined using the RC DC protein assay (Bio-Rad 500-0119, Bio-Rad Laboratories, Hercules, CA). Samples were diluted in extraction buffer with 0.03% bromophenol blue to yield equal final concentrations, and 5 μg of protein was loaded into each well. Gels (20 cm wide × 16 cm tall × 1.5 mm thick) were run on a Protean II xi Cell electrophoresis unit (Bio-Rad Laboratories, Hercules, CA) at a constant 30 mA for 5 h. Gels were rinsed with double distilled water for two min prior to staining with coomassie (Imperial Protein Stain, Thermo Scientific, Rockford, IL). Gels were scanned with an Odyssey CLx (LI-COR, Lincoln, NE) and bands were quantified using Image Studio version 5.2. Titin degradation was calculated for each time point as the ratio of the degradation product (T2) to the total signal (T1 + T2).

2.8. Immunohistochemistry

Immunohistochemistry was used to detect myosin heavy chain (MHC) types I, IIa and IIx, and determine fiber cross-sectional area (CSA). Cross sections (10 μm) of LL were cut on a cryostat (Microm HM 525, Tritech Inc., Edgewater, MD), placed on silanized slides (Angerer & Angerer, 1991), and stored at −80 °C until subsequent analysis. Sections were blocked in 1 × PBS containing 5% (v/v) goat serum for 1 h at room temperature. Primary antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA) of unique isotypes were used to detect MHC β/slow (BA-F8; IgG2b), and MHC slow and 2a (BF-32; IgM) fibers. Sections were incubated with primary antibodies (2 μg/ml in blocking solution) for 1 h at 37 °C. Then, sections were washed 3 × 5 min with PBS and incubated for 1 h at room temperature with goat anti-mouse secondary antibodies conjugated to Alexa Fluor fluorescent dyes (IgG 350 [emission 450, blue]; and IgM 594 [emission 620, red]; Thermo Scientific, Rockford, IL) diluted 1:500 in blocking solution. Wheat germ agglutinin tagged with Alexa Fluor 488 (W11261, Thermo Scientific, Rockford, IL) was included (1:200) with the secondary antibodies to label the muscle fiber membrane. After incubation, sections were washed, 3 × 5 min in PBS, and coverslips were mounted (ProLong Gold Antifade, Life Technologies, Eugene, OR) and sealed. Sections were imaged with an EVOS FL fluorescence microscope (Advance Microscopy Group, Bothell, WA). Fibers staining purple (positive for BA-F8 and BF-32) were considered type I, and fibers staining red (positive for BF-32 only) were considered type IIa. Unstained fibers (black) were assumed to be type IIx. Approximately 900 fibers were counted per animal, and MHC fiber type composition was calculated from a ratio of the number of each fiber type over the total number of fibers analyzed. Muscle fiber CSA was obtained using ImageJ 1.46r, and the average CSA of each fiber type was determined for every animal.

2.9. Statistical analysis

Data were analyzed using linear mixed models with SAS-JMP Pro 11. The model for pH decline included postmortem time (1.5 h, 3 h, 6 h, 9 h, and 24 h) and breed group (1 to 6) as subclass fixed effects and kill day, age at slaughter, and residual as random effects. The model for fiber type composition contained the same effects except for time. The model for all other traits (proteolysis at 24 h and 14 d, autolysis at 24 h, calpastatin content at 24 h, fiber area, enzyme activity, sensory, WBSF, and carcass traits) comprised the fixed effect of Brahman percentage (linear covariate), and the random effects of kill day, age at slaughter, and residual. Postmortem time was considered a repeated measure when analyzing pH decline. Breed group least squares means (LSM) and their standard errors were computed for all traits, and compared using Bonferroni's t-tests. A value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Carcass and palatability traits

Based on previous data from this multibreed herd, crossbred cattle exhibit heavier HCW, larger REA, and a higher KPH when compared to high percentage Angus and Brahman cattle (Elzo et al., 2012). Of the carcass traits measured (Table 2), there was only a difference between breed compositions when comparing FOE. The FOE decreased as the percentage of Brahman increased ($R^2 = 0.14$, $P = 0.0228$). Cattle were chosen based on finishing at the same compositional endpoint, approximately 1.27 cm of FOE. It is challenging to select animals with the same amount of subcutaneous fat from each breed group to be represented on each slaughter day because these breeds grow at different rates (Turner, 1980). Despite the difference in fat, there was no difference in the age of the animals at slaughter. There were no differences in HCW, REA, and KPH between breeds. Dressing percentage tended to increase as the percentage of Brahman increased ($R^2 = 0.09$, $P = 0.0713$). This trend is consistent with the data presented by Elzo et al. (2012) and Pringle et al. (1997). Marbling score decreased as Brahman percentage increased ($R^2 = 0.47$, $P < 0.0001$; Fig. 1), which is also consistent with previous work (Elzo et al., 2012; Pringle et al., 1997; Wheeler et al., 1990; Whipple et al., 1990).

The impact of Brahman composition on palatability attributes was determined using objective tenderness and a trained sensory panel (Fig. 2). As Brahman percentage increased, WBSF increased ($R^2 = 0.28$, $P = 0.0009$). According to the USDA Tenderness Program, steaks are considered tender if they have a WBSF value less than or equal to 4.4 kg

Table 2
Carcass traits and pH decline across all animals.

Traits	Mean ± SD
HCW, kg	318 ± 36
DP, %	57.22 ± 2.42
FOE, cm	1.22 ± 0.34
REA, cm^2	73.30 ± 8.74
REA/100 kg HCW	23.18 ± 2.57
KPH, %	2.4 ± 0.5
pH	
1.5 h	6.47 ± 0.07
3 h	6.21 ± 0.09
6 h	5.89 ± 0.07
9 h	5.72 ± 0.06
24 h	5.47 ± 0.03

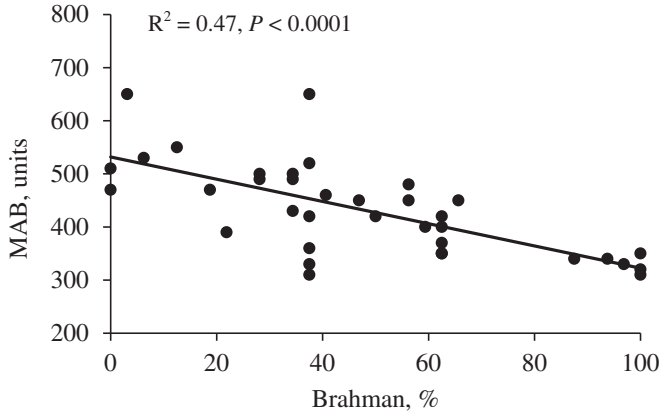


Fig. 1. Marbling (MAB) scores in the in the ribeye (longissimus) of steer carcasses ranging in breed composition from 0% Brahman (100% Angus) to 100% Brahman. (MAB: 100 to 199 = practically devoid, 200 to 299 = traces, 300 to 399 = slight, 400 to 499 = small, 500 to 599 = modest, 600 to 699 = moderate, 700 to 799 = slightly abundant, 800 to 899 = moderately abundant, 900 to 999 = abundant).

or 43 N (USDA, 2011). While 4 out of 6 Brahman cattle produced "tough" steaks, two Brahman steaks were classified as "tender." The opposite was true for Angus, with only 33% (2 out of 6) of steaks being considered "tough." Several researchers have reported increased WBSF values in Brahman or *Bos indicus* cattle compared to Angus or *Bos taurus* cattle (Elzo et al., 2012; Gonzalez et al., 2014; Huffman et al., 1990; Pringle et al., 1997; Whipple et al., 1990). Sensory panelists also detected reduced tenderness as Brahman influence increased (Fig. 3; $R^2 = 0.53$, $P < 0.0001$). On average, Brahman and 3/4 Brahman (breed group 5) were considered "slightly tough," and the remaining groups were rated "slightly tender." While two Brahman were classified as tender according to WBSF, only one Brahman steak attained "slightly tender" by panelists, and all Brahman steaks scored below Angus steaks for sensory tenderness. Sensory scores for juiciness and connective tissue also decreased as percentage Brahman influence increased, indicating a less juicy steak with more connective tissue ($R^2 = 0.40$, $P < 0.0001$ and $R^2 = 0.52$, $P < 0.0001$, respectively). Juiciness scores may be partly related to MAB ($R^2 = 0.22$, $P = 0.0041$). The same trends for tenderness, connective tissue, and juiciness were reported by Elzo et al. (2012). According to Gonzalez et al. (2014), Huffman et al. (1990), and Whipple et al. (1990) panelists assigned lower scores for tenderness and connective tissue in Brahman, but found no differences in juiciness. Although panelists perceive differences in connective tissue, there is little evidence that breed composition affects collagen content or solubility (Gonzalez et al., 2014). There were no differences detected in beef flavor intensity or off-flavor between breed compositions.

3.2. Calpain autolysis and calpastatin content

Calpain-1 activity is a major determinant of proteolysis and tenderness. In postmortem muscle, calpain activity is regulated by calcium concentration, calpain autolysis, and calpastatin. In the presence of Ca^{2+}, the 80 kDa catalytic subunit of calpain-1 progressively autolyzes to a 78 kDa intermediate, and then an active 76 kDa subunit. The degree of autolysis is used to provide evidence for calpain activity (Cruzen, Paulino, Lonergan, & Huff-Lonergan, 2014; Goll et al., 2003; Lomiwes, Farouk, Wu, & Young, 2014). Breed composition did not affect total calpain content. However, the degree of calpain-1 autolysis at 24 h decreased as Brahman percentage increased ($R^2 = 0.24$, $P = 0.0025$; Fig. 3). This is evidenced by a greater percentage of the 78 kDa intermediate signal and 80 kDa subunit signal remaining in Brahman at 24 h. Pringle et al. (1997) reported a decrease in calpain-1 activity with an increasing percentage of Brahman. Wheeler et al.

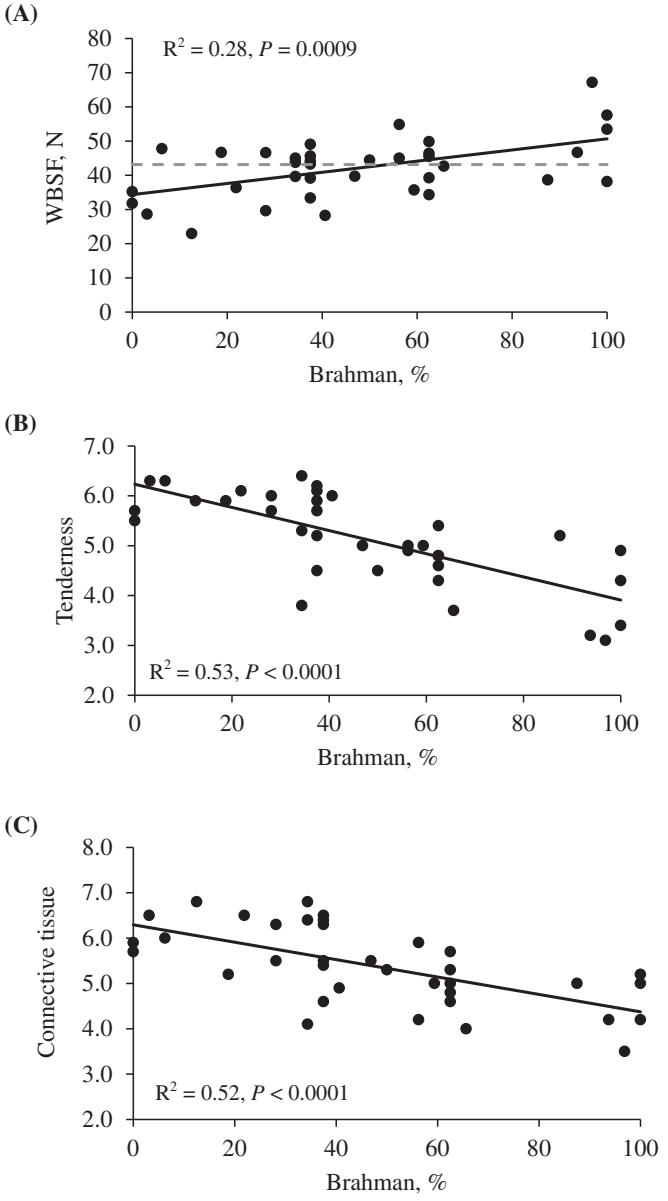


Fig. 2. Palatability attributes of strip loin steaks from steers ranging in breed composition from 0% Brahman (100% Angus) to 100% Brahman. (A) Objective tenderness assessed by Warner Bratzler shear force (WBSF). Dashed line is at 43.1 N (4.4 kg), the boundary between tough and tender as described by USDA (2011). Subjective evaluations by a trained sensory panel for (B) tenderness and (C) connective tissue. (Tenderness: 1 = extremely tough, 2 = very tough, 3 = moderately tough, 4 = slightly tough, 5 = slightly tender, 6 = moderately tender, 7 = very tender, 8 = extremely tender; Connective tissue: 1 = abundant, 2 = moderately abundant, 3 = slightly abundant, 4 = moderate amount, 5 = slight amount, 6 = traces amount, 7 = practically devoid, 8 = none detected).

(1990) also observed decreased calpain-1 activity at 10 min postmortem in Brahman compared to Hereford, but activity was similar by 24 h. Others have reported no difference in calpain-1 activity when comparing *Bos indicus* and *Bos taurus* (Johnson, Calkins, Huffman, Johnson, & Hargrove, 1990; Pringle et al., 1999; Whipple et al., 1990). Similar calpain-1 activities across breed types within the first hour postmortem is consistent with calpain activity being regulated primarily by cellular conditions (such as pH) and calpastatin, rather than calpain content. However, cellular environment is subject to breed and slaughter conditions, which could impact calpain-1 activity early postmortem. Moreover, conflicting results regarding genetic influence on calpain-1 activity may relate to proportion of *Bos indicus* genetics

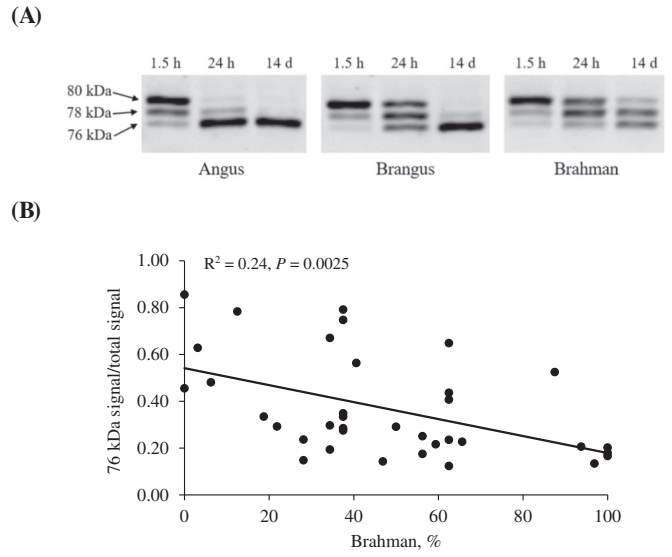


Fig. 3. Effect of breed composition on calpain-1 autolysis in the longissimus. (A) Western blot of Calpain-1 autolysis at 1.5 h, 24 h, and 14 d postmortem in Angus, Brangus, and Brahman breed groups. (B) Degree of calpain-1 autolysis at 24 h ranging in breed composition from 0% Brahman (100% Angus) to 100% Brahman. Autolysis at 24 h was calculated as the ratio of 76 kDa signal to total signal (80, 78, and 76 kDa).

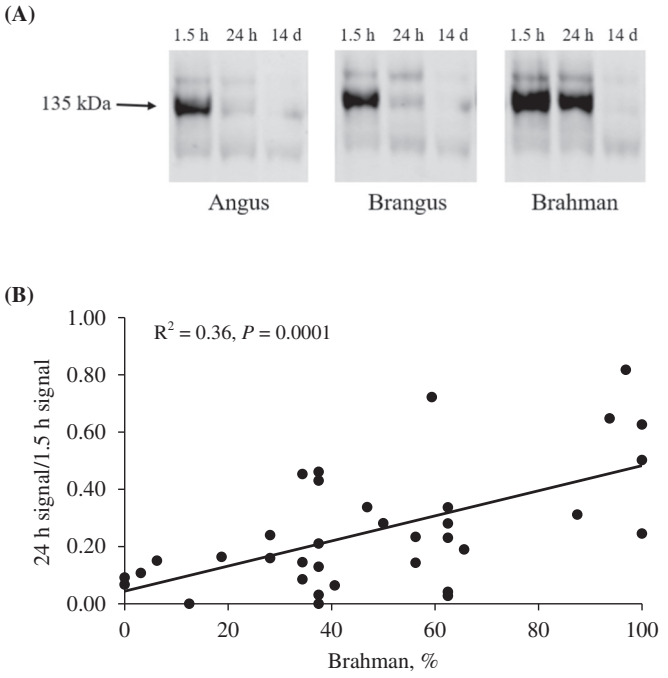


Fig. 4. Effect of breed composition on calpastatin content (135 kDa) in the longissimus. (A) Western blot of calpastatin at 1.5 h, 24 h, and 14 d postmortem in Angus, Brangus, and Brahman breed groups. (B) Calpastatin content at 24 h in longissimus from steer carcasses ranging in breed composition from 0% Brahman (100% Angus) to 100% Brahman. Calpastatin content at 24 h was calculated as the ratio of the 135 kDa signal at 24 h relative to the signal at 1.5 h.

and specific breeds utilized, as Whipple et al. (1990) and Johnson et al. (1990) used Sahiwal and Brahman influenced animals, respectively, but did not include purebred *Bos indicus*. Markers in the CAPN1 gene are associated with beef tenderness, but specific mechanisms relating to calpain content or activity have not been established.

Calpastatin, the inhibitor of calpain-1, consists of four domains that can each inhibit calpain activity. This inhibitor is labile to proteolytic degradation by calpain and other proteases. However, as calpastatin is degraded postmortem, fragments can still retain inhibitory activity (Boehm et al., 1998). Even so, calpastatin loses activity early postmortem and during subsequent aging (Boehm et al., 1998; Koohmaraie, Seideman, Schollmeyer, Dutson, & Crouse, 1987). When calpastatin is present in mammalian skeletal muscle and contains all four domains, it migrates at approximately 135 kDa in SDS-PAGE (Goll et al., 2003). Content of intact calpastatin at 24 h increased as the percentage of Brahman increased ($R^2 = 0.36$, $P = 0.0001$; Fig. 4). There was no difference in the calpastatin content at 14 d between breed compositions. Increased calpastatin content at 24 h would be expected to reduce calpain-1 autolysis and activity. Consistent with this, greater calpastatin content at 24 h coincided with decreased calpain-1 autolysis ($R^2 = 0.33$, $P = 0.0003$). Elevated calpastatin activity is well-known to contribute to reduced tenderness in indicine compared to taurine breeds. As Brahman influence increases, the calpastatin: calpain-1 activity ratio increases (Pringle et al., 1997, 1999; Wheeler et al., 1990; Whipple et al., 1990). Greater calpastatin activity typically persists from pre-rigor (< 1 h postmortem) to post-rigor (24 h to several days) (Pringle et al., 1999; Wheeler et al., 1990). However, some have reported increased calpastatin activity in postrigor but not prerigor muscle (Whipple et al., 1990), indicating other factors such as Ca^{2+} concentration, may also limit calpain activation in Brahman muscle early postmortem.

3.3. Protein degradation

In postmortem muscle, calpain-1 degrades key myofibrillar and cytoskeletal proteins, which disrupts the structure and integrity of the sarcomere, thereby contributing to tenderness (Huff-Lonergan et al., 1995, 1996; Koohmaraie, 1992; Lomiwes et al., 2014; Whipple et al., 1990). Postmortem degradation of several calpain targets, including troponin-T, desmin, and titin, was evaluated. Troponin-T is part of the troponin complex and interacts with the thin filament during muscle contraction. Because of its location, there is debate whether troponin-T degradation directly contributes to development of tenderness; regardless, troponin-T is a reliable indicator of postmortem proteolysis (Huff-Lonergan, Zhang, & Lonergan, 2010). In comparison, desmin and titin play important roles in maintaining muscle structure and organization. Desmin is an intermediate filament that links adjacent myofibrils at their Z-lines, and titin preserves alignment of the sarcomere. Titin spans half the sarcomere from the Z-line to the M-line and is the largest protein (approximately 3000 kDa) found in mammalian tissues.

As Brahman influence increased, troponin-T degradation at 24 h and 14 d decreased (Fig. 5; $R^2 = 0.16$, $P = 0.0141$ and $R^2 = 0.67$, $P < 0.0001$, respectively). Similarly, desmin degradation at 24 h and 14 d decreased with increasing percentage Brahman (Fig. 6; $R^2 = 0.19$, $P = 0.0077$ and $R^2 = 0.42$, $P < 0.0001$, respectively). Whipple et al. (1990) found that desmin degradation was greater at 0, 1, and 14 d postmortem when comparing *Bos taurus* to *Bos indicus* cattle. Titin degradation paralleled the results of troponin-T and desmin; titin degradation at 24 h and 14 d decreased with greater Brahman influence (Fig. 7; $R^2 = 0.20$, $P = 0.0059$ and $R^2 = 0.19$, $P = 0.0331$, respectively). For all three proteins, the degree of calpain-1 autolysis largely explained degradation at 24 h (troponin-T: $R^2 = 0.84$, $P < 0.0001$; desmin: $R^2 = 0.78$, $P < 0.0001$; titin, $R^2 = 0.66$, $P < 0.0001$).

Reduced proteolysis contributed to meat toughness, evidenced by increased WBSF with decreased troponin-T degradation at 14 d ($R^2 = 0.32$, $P = 0.0003$). There also was a tendency for titin degradation at 14 d to decrease as WBSF increased ($R^2 = 0.09$, $P = 0.0735$). However, when two samples that exhibited the lowest troponin-T degradation at 24 h were removed, relationships between 14 d protein degradation and WBSF essentially disappeared (troponin T, $R^2 = 0.08$, $P = 0.09$; titin, $R^2 = 0.0003$, $P = 0.92$). This suggests that troponin-T may be more reliable at predicting shear force only when proteolysis is dramatically decreased. In contrast, troponin-T degradation was highly

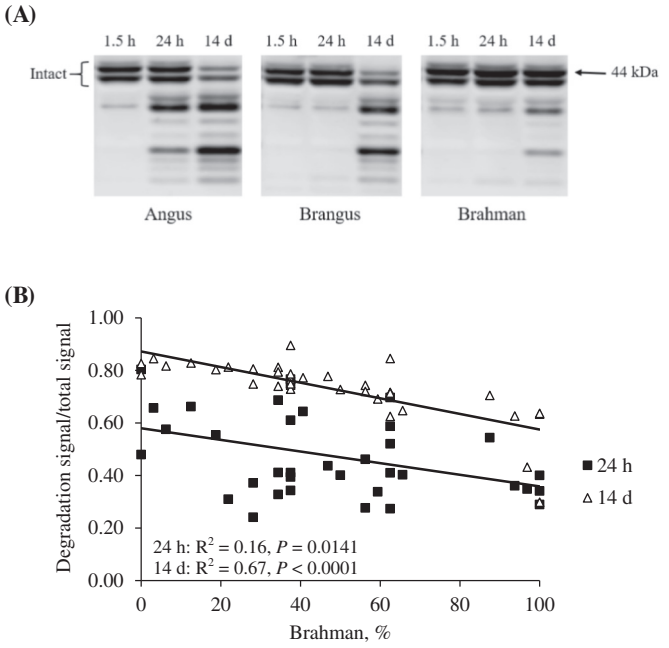


Fig. 5. Effect of breed composition on troponin-T degradation in the longissimus. (A) Western blot of troponin-T degradation at 1.5 h, 24 h, and 14 d postmortem in Angus, Brangus, and Brahman breed groups. (B) Degradation of troponin-T at 24 h and 14 d postmortem ranging in breed composition from 0% Brahman (100% Angus) to 100% Brahman. Troponin-T degradation was determined within each time point, and calculated as a ratio of the degradation products (total signal minus intact signal) over the total signal.

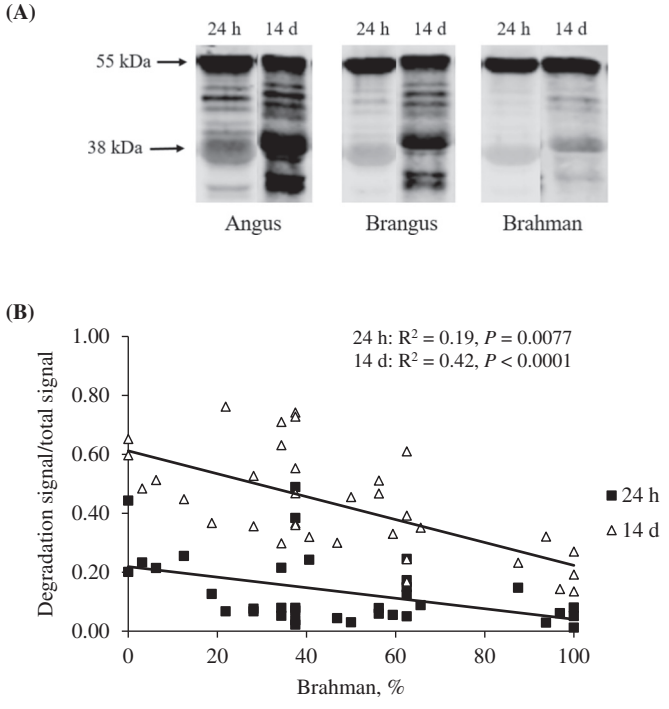


Fig. 6. Effect of breed composition on desmin degradation in the longissimus. (A) Western blot of desmin degradation at 1.5 h, 24 h, and 14 d postmortem in Angus, Brangus, and Brahman breed groups. (B) Degradation of desmin at 24 h and 14 d postmortem ranging in breed composition from 0% Brahman (100% Angus) to 100% Brahman. Desmin degradation was determined within each time point, and calculated as a ratio of the degradation products (total signal minus 55 kDa signal) over the total signal.

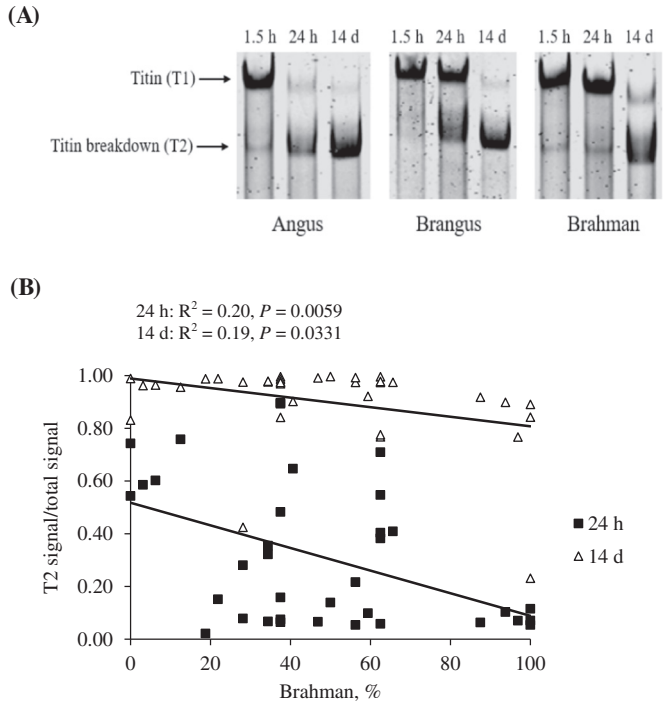
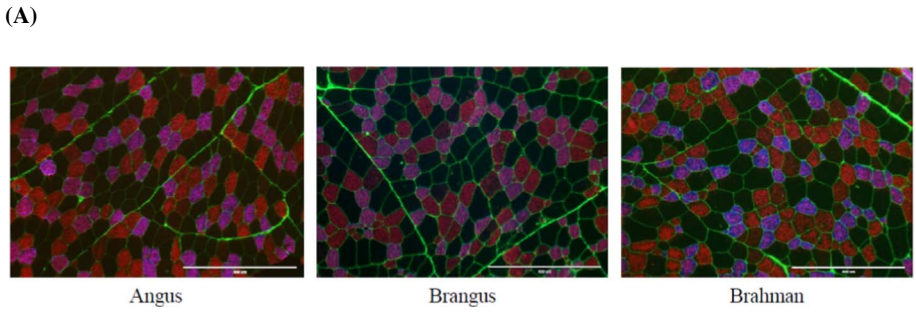


Fig. 7. Effect of breed composition on titin degradation in the longissimus. (A) Agarose gel of titin degradation, stained with coomassie, at 1.5 h, 24 h, and 14 d postmortem in Angus, Brangus, and Brahman breed groups; B) Degradation of titin at 24 h and 14 d postmortem ranging in breed composition from 0% Brahman (100% Angus) to 100% Brahman. Titin degradation was determined within each time point and calculated as a ratio of degradation product (T2) signal to total signal (T1 + T2).

related to sensory tenderness ($R^2 = 0.53$, $P < 0.0001$), even when the two aforementioned samples were removed ($R^2 = 0.50$, $P = 0.0002$). It is not clear why troponin-T degradation is more limited in predicting objective tenderness by WBSF compared to sensory analysis. Objective tenderness varied from ~20 to 55 N, a range that includes steaks that are very tender to tough; and shear force is appropriate for evaluating variation in tenderness of a muscle, but not for comparing different muscles (Belk et al., 2015). For example, longissimus and biceps femoris have similar WBSF, but differ greatly in sensory tenderness (Rhee, Wheeler, Shackelford, & Koohmaraie, 2004; Shackelford, Wheeler, & Koohmaraie, 1995). Therefore, it is possible other factors besides proteolysis are more important to explaining variation in WBSF in Brahman-influenced steaks.

3.4. Muscle fiber characteristics

Muscle fiber type properties represent an important source of variation in meat quality. The cells within a muscle vary in their contractile and metabolic properties, and during the postmortem period, these properties influence pH decline, rigor formation, and the development of meat quality characteristics. Muscle fibers are classified based on contractile speed (slow or fast) and predominant type of energy metabolism (oxidative or glycolytic). Muscle fiber contractile type was determined based on immunofluorescent detection of MHC proteins (Fig. 8). There were no differences in the percentage of type I fibers between breed groups. The percentage of type IIa fibers was higher in breed group 2 (¾ Angus, ¼ Brahman) when compared to Angus ($P = 0.0434$), and correspondingly, the percentage of type IIx muscle fibers was higher in breed group 1 (Angus) when compared to breed groups 2 (¾ Angus, ¼ Brahman, $P = 0.0067$) and 3 (Brangus, $P = 0.0218$). Others have shown that breed type does not affect fiber type composition when comparing *Bos taurus* to *Bos indicus* cattle (Seideman, 1985;Waritthitham et al., 2010; Whipple et al., 1990).



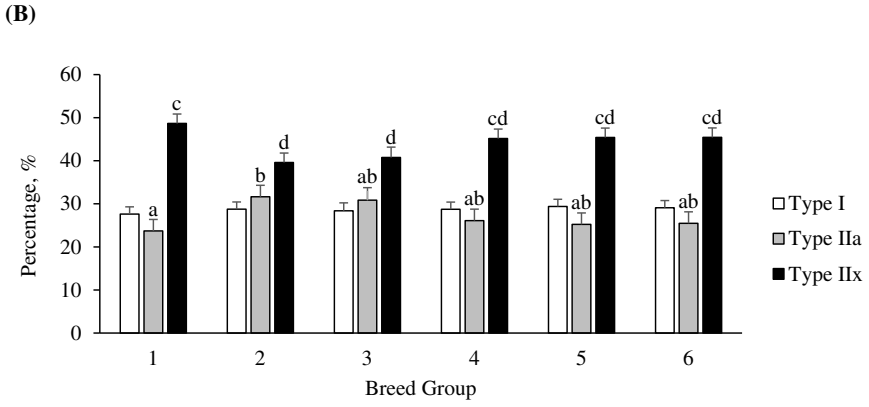


Fig. 8. Myosin heavy chain (MHC) isoform composition of longissimus from steers ranging in breed composition from 0% Brahman (100% Angus) to 100% Brahman. (A) Immunohistochemical staining of MHC isoforms in Angus, Brangus, and Brahman breed groups (type I = purple; type IIa = red; type IIx = black; scale bar = 400 μm). (B) MHC composition (percentage of fibers) for each breed group (LSM ± SE). Within a fiber type, means with different superscripts are significantly different ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Within the population used in this study, the extreme breed compositions of Angus and Brahman were not different. Therefore, it is unlikely that contractile fiber type directly explains differences in meat aging and tenderness.

Mean muscle fiber CSA was also determined for each fiber type for all animals. Breed composition did not affect the mean CSA of types I and IIa fibers. However, the CSA of type IIx muscle fibers increased as the percentage of Brahman increased (Fig. 9; $R^2 = 0.32$, $P = 0.0004$), resulting in a shift in CSA distribution in Brahman compared to Angus. The effect of breed composition on fiber CSA is conflicting; some reported that breed type does not influence fiber CSA (Waritthitham et al., 2010; Whipple et al., 1990), while others showed mean CSA of IIx or all fiber types is increased *Bos indicus* compared to *Bos taurus* cattle (Coles et al., 2014; Seideman, 1985). Increasing CSA corresponds with greater toughness, assessed by both sensory panelists and WBSF (Chriki et al., 2012; Crouse, Koohmaraie, & Seideman, 1990). Crouse et al. (1990) reported that CSA is associated with WBSF within the first 3 d postmortem, but not at 14 d; therefore, as proteolysis increases, CSA of fibers may become less important in modulating tenderness. In contrast, Chriki et al. (2012) found that CSA was related to WBSF of longissimus even at 14 d aging, and CSA was also positively related to total and insoluble collagen content. Considering the reduced proteolysis observed in Brahman, the greater CSA of Brahman IIx fibers could also contribute to reduced tenderness.

To assess metabolic characteristics of the LL, activity of key enzymes involved in glycolytic and oxidative metabolism were quantified. Lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate in anaerobic glycolysis, whereas citrate synthase is a marker of mitochondrial content in muscle. Breed composition did not affect LDH activity (not shown). However, CS activity (Fig. 10) increased as the percentage of Brahman increased ($R^2 = 0.20$, $P = 0.0056$). While Brahman exhibited a greater mitochondrial content as evidenced by CS activity, this was not related to MHC composition. Even though a muscle expresses specific contractile proteins, it is not necessarily mirrored in the metabolic phenotype. Shifting metabolic characteristics and expression of protein isoforms besides MHC allows muscle greater flexibility to adapt to specific conditions. Metabolic differences between

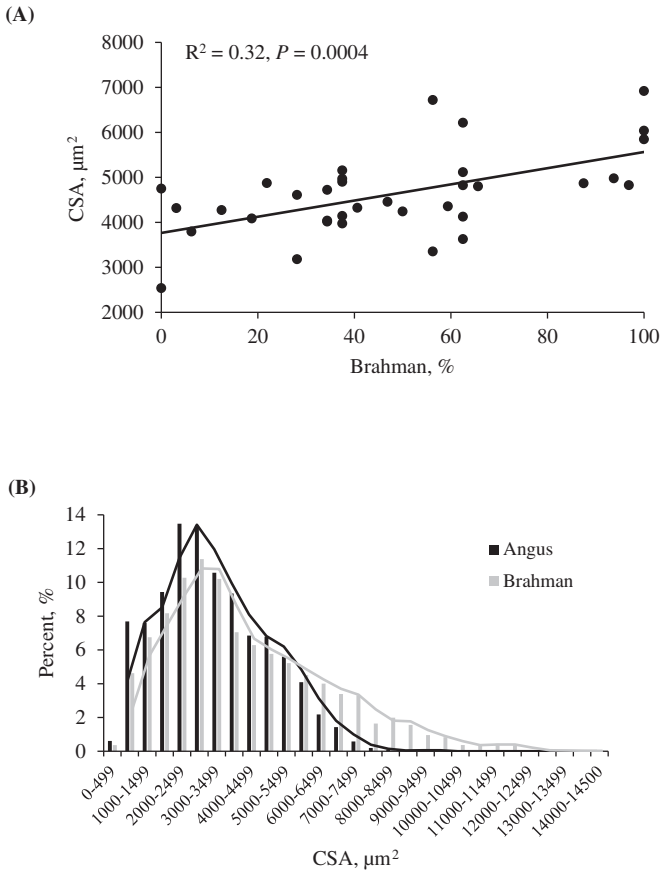


Fig. 9. Cross-sectional area (CSA) of type IIx fibers in longissimus. (A) CSA of IIx fibers from steers ranging in breed composition from 0% Brahman (100% Angus) to 100% Brahman. (B) Distribution of CSA across all muscle fiber types in Angus (80–100%) and Brahman (80–100%).

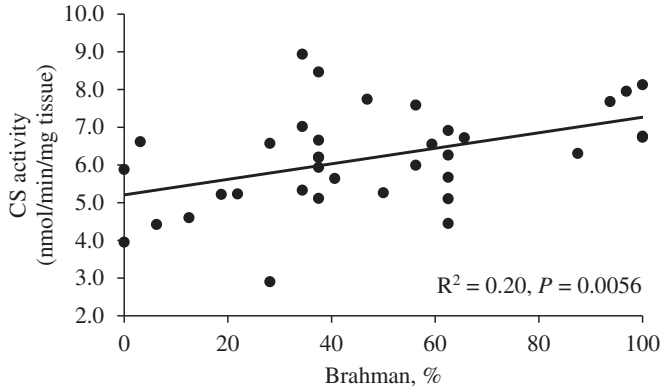


Fig. 10. Citrate synthase activity of longissimus from steers ranging in breed composition from 0% Brahman (100% Angus) to 100% Brahman.

taurine and indicine cattle are not well understood, but increased mitochondrial content may be an adaptation that allows Brahman muscle to function efficiently in its environment.

The energetic properties of muscle may be critical to postmortem energy metabolism and meat aging. The rate of glycolysis dictates pH decline, which may directly impact activity of calpain-1; more rapid postmortem metabolism may also indirectly augment calpain-1 by producing favorable calcium conditions, resulting from reduced ATP available to sequester Ca^{2+}. Postmortem pH decline was monitored (Table 2), but breed composition did not influence pH decline. However, a contrast of Brahman versus Angus at 3 h showed that Brahman exhibited a higher pH compared to Angus (6.30 vs. 6.04, $P = 0.02$). If content of glycolytic enzymes does not differ, post-translational mechanisms may be important for regulating enzymatic activity and glycolytic rate. For instance, phosphorylation sites on the glycolytic enzyme phosphoglucomutase-1 are expected to hasten glycolysis, and these same sites have been identified as potential biomarkers for predicting tenderness in longissimus steaks from British crossbred or Angus and Nellore cattle (Anderson, Lonergan, & Huff-Lonergan, 2014; Rodrigues et al., 2017). In addition, mitochondria may serve key roles in meat aging. Mitochondria contribute to sequestration, transfer, and release of Ca^{2+}, which could delay or accelerate meat aging through regulation of sarcoplasmic Ca^{2+}. In Brahman muscle, greater mitochondria content may increase Ca^{2+} sequestering capacity and prevent increases in free sarcoplasmic Ca^{2+}, thereby delaying activation of calpain-1. Moreover, accumulation of high levels of Ca^{2+} in mitochondria triggers induction of apoptosis and caspase-mediated cell death. Although caspase-mediated proteolysis is controversial, several researchers have provided evidence that greater susceptibility to apoptosis is associated with accelerated rate of beef tenderization (Gagaoua, Claudia Terlouw, Boudjellal, & Picard, 2015; Laville et al., 2009; Rodrigues et al., 2017). Brahman composition influences muscle metabolic properties, but further work is necessary to understand the mechanisms by which glycolytic rate and mitochondria affect tenderization.

4. Conclusions

Overall, tenderness is a complex attribute resulting from inherent muscle properties and the cellular and processing milieu during the conversion of muscle to meat. Elevated calpastatin activity is well-known to contribute to reduced tenderness in indicine compared to taurine breeds. Consistent with this, Brahman cattle produced tougher meat based on WBSF and sensory panel scores. Troponin-T degradation, an indicator of proteolysis, explained considerable variation in sensory tenderness, but was more limited in predicting WBSF. Factors other than proteolysis, such as type IIx fiber CSA or connective tissue, may explain variation in objective tenderness. Importantly, despite similar contractile phenotype, Brahman composition affects metabolic phenotype of longissimus. Greater Brahman influence was associated with increased CS activity, a marker of mitochondria content. Shifting muscle metabolic properties may impact events that modulate proteolytic activity, including pH decline, sarcoplasmic Ca^{2+}, and apoptosis. Thus, further work is needed to evaluate impact of muscle metabolic phenotype on postmortem energy metabolism and tenderness development in *Bos taurus* and *Bos indicus* breeds.

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