



## Effects of Marbling Texture on Muscle Fiber and Collagen Characteristics

Kelly R. Vierck, Travis G. O'Quinn, Jere A. Noel, Terry A. Houser, Elizabeth A. E. Boyle, and John M. Gonzalez\*

Department of Animal Sciences and Industry, Kansas State University, Manhattan, KS 66502, USA

\*Corresponding author. Email: johngonz@ksu.edu (J. M. Gonzalez)

**Abstract:** The objective of this study was to evaluate the effects of marbling texture on muscle fiber morphometrics, adipocyte cross-sectional area, perimysial thickness, collagen solubility, and thermal transition temperature of perimysial collagen of beef strip loin steaks from 3 marbling scores. Beef strip loins ( $n = 117$ ) were selected from three marbling scores [Top Choice (Modest<sup>00</sup> – Moderate<sup>100</sup> marbling), Low Choice (Small marbling), and Select (Slight marbling)] to equally represent 3 different marbling texture groups: fine, medium, and coarse, via visual appraisal. There were no marbling texture  $\times$  marbling score interactions for all traits evaluated. Marbling texture impacted ( $P < 0.05$ ) adipocyte cross-sectional area, where coarse steaks displayed larger adipocytes in comparison to fine marbled steaks, but medium marbled steaks were similar ( $P > 0.05$ ) to both coarse and fine marbled steaks for the same trait. However, marbling texture did not impact ( $P > 0.05$ ) perimysial thickness. Marbling texture did not affect collagen traits, as no differences ( $P > 0.05$ ) were found among marbling texture treatments for soluble collagen, insoluble collagen, and total collagen concentrations. Furthermore, all marbling texture groups (fine, medium, and coarse) were similar ( $P > 0.05$ ) for the peak thermal transition phase of the perimysial fraction of collagen. Marbling score affected adipocyte size, as Top Choice and Low Choice possessed larger ( $P < 0.05$ ) adipocytes than Select steaks. There were no differences ( $P > 0.05$ ) among marbling scores for fiber type; nor were differences found among marbling textures or marbling scores for fiber cross-sectional area. These results indicate that marbling texture does not contribute to differences in collagen characteristics or fiber cross-sectional area that may impact eating quality of beef strip loin steaks.

**Keywords:** adipocyte, beef, collagen, marbling texture, muscle fiber

*Meat and Muscle Biology* 2:75–82 (2018) doi:10.22175/mmb2017.10.0054

Submitted 31 Oct. 2017

Accepted 21 Jan. 2018

## Introduction

Marbling texture is often considered by USDA graders when applying quality grades, though it is not formally included in the beef grading standards (USDA, 1997; Smith et al., 2001). Coarse marbled beef is often discriminated against, preventing these carcasses from receiving premiums associated with most branded beef programs. Of the branded beef programs supervised by the USDA-Agricultural Marketing Service, approximately 75% require beef

carcasses to have medium or fine textured marbling in the ribeye at the time of grading to qualify (USDA, 2017). This prejudice against coarse marbled beef is based upon a study by Moody et al. (1970). Earlier work had demonstrated marbling texture and distribution were weakly associated with Warner-Bratzler shear force (WBSF) values, but had not evaluated this association further (Goll et al., 1965). Following up on this work, Moody et al. (1970) evaluated the impact of marbling texture on the tenderness and palatability traits of beef rib roasts. They found that fine marbled samples had lower WBSF values than samples with coarse marbling and speculated that the observed difference was related to perimysial thickness differences, though they reported no measure of this trait (Moody

Contribution no. 18-007-J of the Kansas Agricultural Experiment Station, Manhattan, KS 66506

et al., 1970). In the time since this first study was published, no other authors have evaluated the impact of marbling texture on perimysial thickness or tenderness.

Additionally, other biological factors impact beef tenderness. Increased fiber cross-sectional area has been linked to increased shear force and reduced sensory tenderness ratings (Seideman et al., 1987; Crouse et al., 1991; Ebarb et al., 2016). Furthermore, intramuscular collagen content has also been implicated in increasing shear force values (Cross et al., 1973; Ebarb et al., 2016). Currently, no research has addressed the effects of marbling texture on muscle histology or collagen characteristics. Therefore, the objective of the current study was to determine the effects of marbling texture on muscle histology, adipocyte cross-sectional area, and collagen traits of beef strip loin steaks.

## Materials and Methods

### *Sample collection and preparation*

Beef strip loins [IMPS #180;  $n = 117$ ; 39/marbling score group (Top Choice, Low Choice, and Select)] of 3 marbling texture treatments (fine, medium, and coarse) were selected through visual appraisal using the USDA-AMS-LS-SB-02 marbling texture reference card. This reference card provides a visual reference for fine, medium, and coarse marbling based on marbling spec size. All strip loins were graded and selected by trained Kansas State University (KSU) research personnel to fit each of the various marbling score and texture treatments. To be selected for the study, greater than 75% of the marbling in the ribeye was required to meet the USDA visual standard for the designated marbling texture treatment. After selection, strip loins were transported under refrigeration (2°C) to the KSU Meat Laboratory for steak cutting and processing. Strip loins were fabricated into 2.54-cm thick steaks from anterior to posterior using a manual electric slicer (Model KAMS-14; Pro-Cut, Houston, TX) 72 h postmortem. The anterior most “face steak” was removed and the next steak was used for histology analysis.

### *Histochemistry and immunohistochemistry*

Within each histology steak, 3 marbling flecks that best represented the marbling texture treatment were selected for extraction from the medial, central, and lateral portion of the steak to provide the best representation of the entire steak in the analyses. A 1.27 cm × 1.27 cm × 2.54 cm area around the fleck was extract-

ed and embedded in optimum cutting temperature tissue freezing medium (Tissue Tek OCT; VWR; Radnor, PA), frozen in liquid nitrogen cooled isopentane, and stored at -80°C until subsequent analysis. After sample embedding, the remaining portions of each steak were vacuum packaged in 3 mil standard barrier vacuum bags with oxygen transmission rates of  $4.5 \text{ cm}^3 \times 100 \text{ cm}^{-2} \times 24 \text{ h}^{-1}$  at 23°C and 65% humidity (Prime Source Vacuum Pouches; Bunzl Processor Division, Koch Supplies, Kansas City, MO) using a Multivac C500 (Cryovac Inc., Elmwood, NJ) and aged for 21-d postmortem at 2 to 4°C.

The methods of Phelps et al. (2014) were used for cryosection collection and immunohistochemical analysis. Seven-micrometer cryosections were collected on positively charged glass microscope slides. For each marbling fleck, 1 cryosection was taken for immunofluorescent fiber type staining and a serial cryosection was taken for Masson’s trichrome staining for perimysial collagen and adipocyte staining. Samples were trimmed 2 mm from the location of the first cryosection and 2 serial cryosections were collected.

Immunohistochemical cryosections were incubated in a blocking solution of 10% horse serum and 0.2% Triton X-100 in phosphate buffer solution (PBS) for 30 min at room temperature. Slides were then incubated in the primary antibody solution of 1:500 rabbit  $\alpha$ -dystrophin (Thermo-Fisher Scientific, Waltham, MA), 1:10 supernatant mouse anti-MHC, slow IgG2b (BA-D5; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), and 1:10 mouse anti-MHC all but type IIX, IgG1 (BF-35; Developmental Studies Hybridoma Bank). After the rinsing step of incubation of slides with PBS for 5 min, a solution of secondary antibodies was applied for 30 min. The secondary antibody solution included 1:100 Alexa-Fluor 488 goat anti-mouse IgG1 (Invitrogen, Grand Island, NY); 1:1000 Alexa Fluor 633 goat anti-mouse IgG2b (Invitrogen); 1:1000 Alexa-Fluor 594 goat anti-rabbit H&L (Invitrogen); and 1:1000 DAPI (Thermo-Fisher Scientific). After another washing step, slides were coverslipped for imaging.

A Masson’s trichrome staining kit was used (Sigma-Aldrich; St. Louis, MO) for staining histology cryosections. Slides were incubated in deionized water for 2 min, followed by a Bouin’s solution (Sigma-Aldrich; St. Louis, MO) incubation for 15 min at 56°C. Samples were then successively incubated in tap water, Weigert’s Hematoxylin solution, tap water again, deionized water, Beibrich Scarlet-Acid Fuchsin stain, a phosphotungstic and phosphomolybdic acid solution, and aniline blue solution for 5 min each. Following these solutions, slides were then incubated in a 1% acetic acid

solution for 2 min, and then successively incubated in deionized water, 100% ethanol, and xylenes for 1 min each. Slides were coverslipped for imaging.

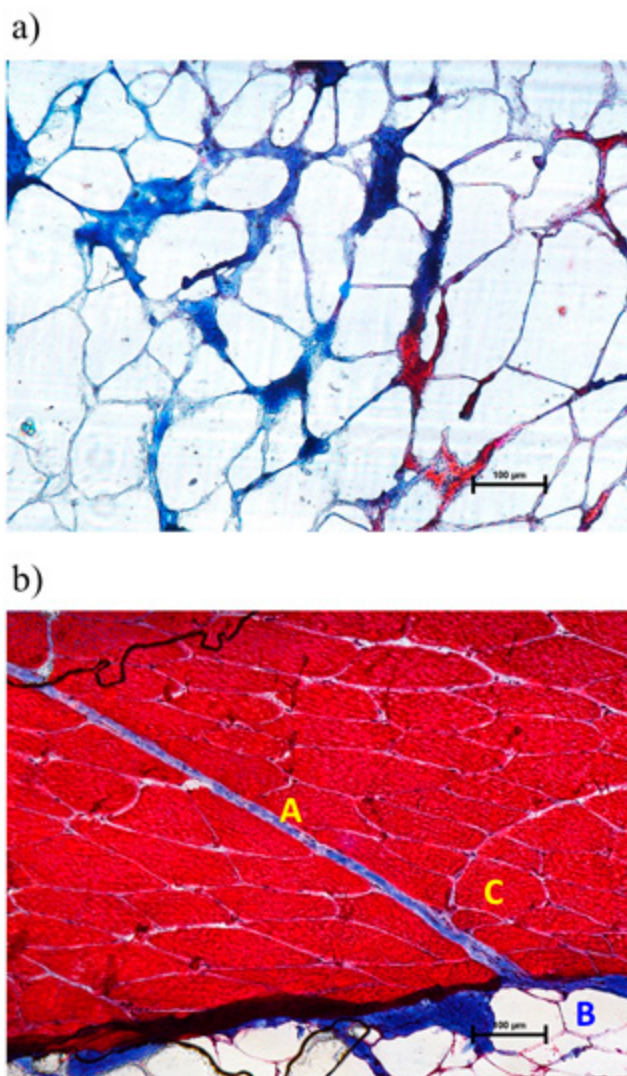
Cryosections were imaged at 100-fold magnification with a Nikon TI-U inverted microscope (Nikon; Lewisville, TX) fitted with an X-Cite 120XL epifluorescence illumination system (EXFO; Mississauga, Ontario, Canada), a DS-QiMC digital camera (for immunohistochemical imaging), and a DS-Fi1 digital camera (for histochemical imaging). Images were taken specifically near the marbling fleck in order to evaluate the impact of marbling texture on the specified traits.

Immunohistochemistry photomicrographs were analyzed for muscle fiber cross-sectional area and myosin heavy chain (MHC) type distribution using NIS-Elements Imaging Software (Basic Research 3.3; Nikon Instruments, Inc., Melville, NY). For each marbling fleck sample, a minimum of 3 photomicrographs and 300 fibers were analyzed per section. Fibers that stained positive for BA-D5 antibody were considered as MHC Type I fibers. Fibers that stained positive for the BF-35 antibody and negative for BA-D5 antibody were considered as MHC type IIA fibers. Any fibers that did not stain positive for both BF-35 and BA-D5 were considered as MHC Type IIX fibers. The  $\alpha$ -dystrophin ring around each fiber identified the fiber cross-sectional area (Ebarb et al., 2016).

Histochemistry photomicrographs were analyzed for adipocyte cross-sectional area and perimysial thickness using NIS-Elements Imaging Software (Basic Research 3.3; Nikon Instruments, Inc.). For each marbling fleck, a minimum of 200 adipocytes per steak were measured and perimysium thickness was measured every 10 micrometers with a minimum of 10 measurements per image (Figure 1).

### Collagen solubility

A modified method described by Gonzalez et al. (2014) was used to determine hydroxyproline content of each sample. Briefly, following the 21-d aging period, steaks were diced and frozen in liquid nitrogen, then homogenized using a Waring blender (Waring Products Division; Hartford, CT) and stored at  $-80^{\circ}\text{C}$  until further analysis. Three grams of ground sample in duplicate were mixed with 0.25 strength Ringer's Solution. The tubes were then placed in a hot water bath at  $77^{\circ}\text{C}$  for 70 m and stirred every 10 m. Samples were then centrifuged at  $20^{\circ}\text{C}$  for 15 m at  $5,200 \times g$ . Soluble samples were then decanted into autoclave milk bottles through Fisher 09-795 filter paper (Thermo-Fisher Scientific). Soluble samples were re-centrifuged with 0.25 strength Ringer's solution under the same conditions, and then



**Figure 1.** Representative photomicrographs of Masson's trichrome staining of bovine longissimus lumborum intramuscular adipose and muscle tissue; a) Photomicrograph utilized for intramuscular adipocyte cross-sectional area analysis, b) Photomicrograph utilized for perimysium thickness measures (A = perimysium; B = marbling adipocyte; C = muscle cell). Scale bars = 100  $\mu\text{m}$ .

re-decanted. After the sample was filtered, 25 mL of 12 N hydrochloric acid was added to the bottles and they were sealed. The remaining pellet was transferred into autoclave bottles with the filter paper used for decantation and labeled as the insoluble fraction. The centrifuge tubes were rinsed with 25 mL of 6 N hydrochloric acid and decanted into the bottles.

Following decantation, all samples were autoclaved for 18 h at  $121^{\circ}\text{C}$  at 18 to 21 psi. One gram of decolorizing charcoal was added to each sample bottle and mixed thoroughly. Following the addition of charcoal, all samples were filtered through Whatman #2 filter paper (Thermo-Fisher Scientific). Sample bottles were rinsed with deionized water to bring the soluble

samples to 175 mL and insoluble samples to 300 mL. Each sample's pH was adjusted to 6 using 12 N sodium hydroxide and various concentrations of hydrochloric acid. Soluble samples were diluted to 250 mL and insoluble samples were diluted to 500 mL in volumetric flasks. All samples were thoroughly mixed and filtered through Fisher 09-795 filter paper into 15 mL centrifuge tubes and frozen at  $-40^{\circ}\text{C}$  until reading. Hydroxyproline determination was accomplished using procedures of Bergman and Loxley (1963). A BioTek Eon spectrophotometer (Biotek Instruments Inc., Winooski, VT) was used to read absorbance at 558 nm and oriented using a blank of distilled water and a standard curve created for each group of analyses. To determine total and fractional collagen contents, hydroxyproline content was multiplied by 7.52 for the soluble fraction and 7.25 for the insoluble fraction (Cross et al., 1973).

### Transition temperature of collagen

The protocol of Light and Champion (1984) was used to extract the perimysial fraction of muscle. In short, 50 g of tissue was diced, homogenized in cold 0.05 M calcium chloride in a glass Waring blender (Waring Products Division; Hartford, CT), and filtered through a 1-mm<sup>2</sup> sieve. This process was repeated 3 times with tissue remaining on top of the screen. Following separation, samples were freeze dried for 24 h and 20 mg of the dried tissue were weighed into aluminum pans (Shimadzu Scientific Instruments, Kyoto, Japan). Peak thermal transition temperature was analyzed using a differential scanning calorimeter (Shimadzu Scientific Instruments), and analyzed using TA 60WS software (Shimadzu Scientific Instruments).

### Statistical analysis

Statistical analysis was performed using the PROC GLIMMIX procedure of SAS (Version 9.4; SAS Inst. Inc., Cary, NC). Data were analyzed with strip loin as the experimental unit as a completely randomized design with a 3 × 3 factorial arrangement, with marbling score group, marbling texture and the marbling score group × texture interaction serving as fixed effects. The Kenward-Roger adjustment was used for all analyses. Additionally, effects were considered significant with an  $\alpha$  of 0.05.

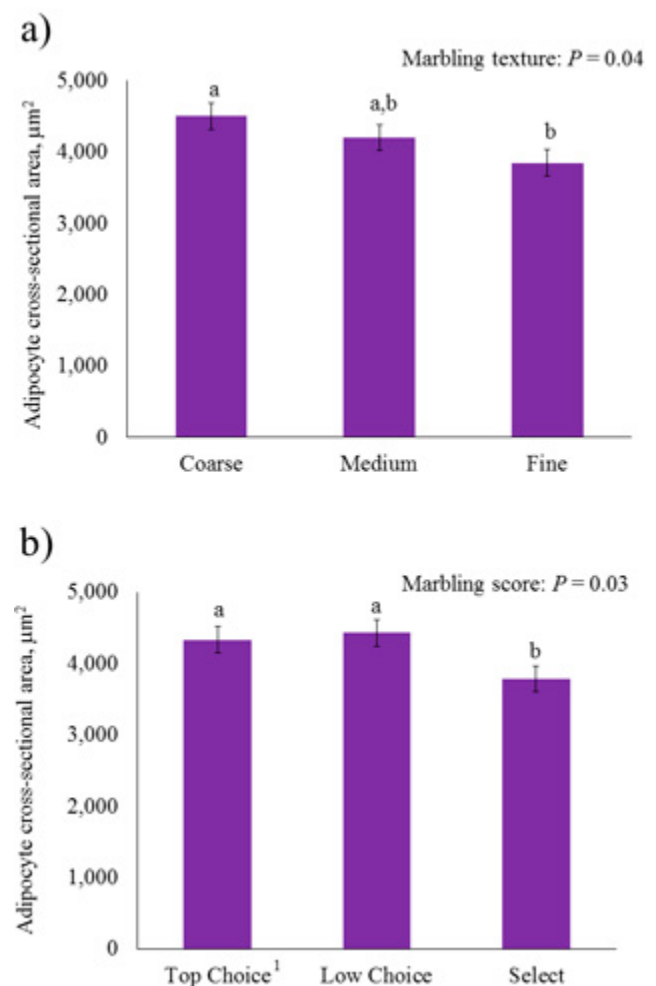
## Results

There were no marbling texture × marbling score group interactions ( $P > 0.05$ ) for all traits evaluated.

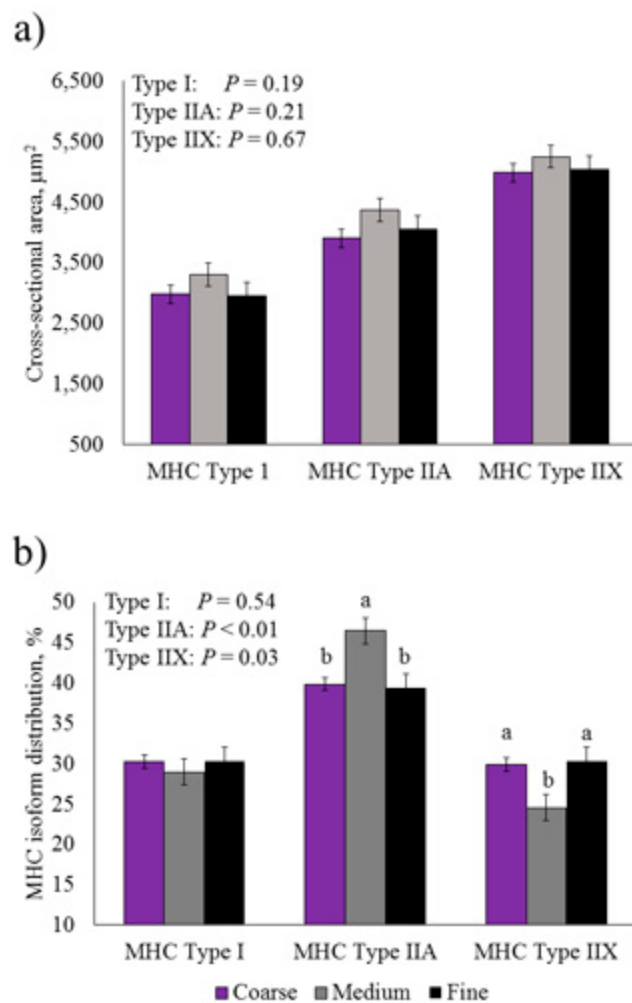
## Histology

Marbling texture and marbling score impacted ( $P < 0.05$ ) adipocyte cross-sectional area (Figure 2). Coarse marbled steaks possessed larger ( $P < 0.05$ ) adipocytes than fine marbled steaks. Adipocytes of medium marbled steaks were similar ( $P > 0.05$ ) in size to adipocytes of both fine and coarse marbled steaks. Adipocytes of Top Choice and Low Choice steaks were larger ( $P < 0.05$ ) than adipocytes of Select steaks. Adipocytes of Top Choice and Low Choice steaks were similar ( $P > 0.05$ ) in size.

Marbling texture did not impact ( $P > 0.05$ ) fiber cross-sectional area for any of the 3 MHC (Type I, Type IIA, and Type IIX) isoforms, however, marbling texture affected ( $P < 0.05$ ) the distribution of the MHC isoforms (Figure 3). Medium marbled steaks possessed a greater ( $P < 0.05$ ) percentage of MHC Type IIA fibers than both fine and coarse marbled steaks, which were similar ( $P > 0.05$ ). A contrasting



**Figure 2.** Least squares means of adipocyte cross-sectional area of beef strip loin steaks of varying a) marbling texture and b) marbling scores. <sup>1</sup>Top Choice = marbling score of Modest<sup>00</sup> to Moderate<sup>100</sup>. <sup>ab</sup>Means within a panel without a common superscript differ ( $P < 0.05$ ).

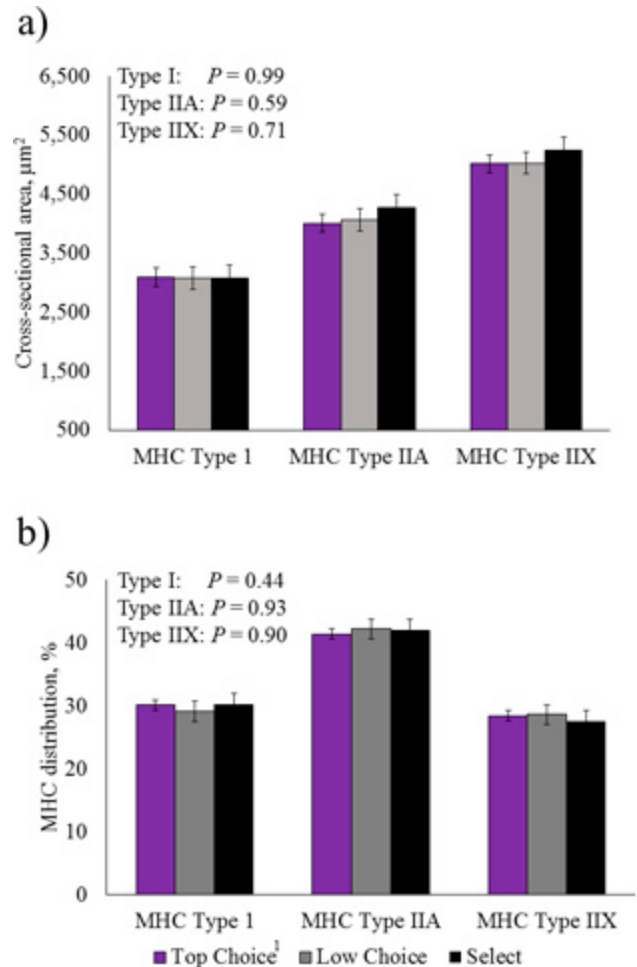


**Figure 3.** Least squares means of myosin heavy chain (MHC) a) cross-sectional area and b) fiber type distribution of beef strip loin steaks of varying marbling texture treatments. <sup>ab</sup>Means within a panel and same MHC isoform without a common superscript differ ( $P < 0.05$ ).

effect occurred in MHC Type IIX fibers, where fine and coarse marbled steaks possessed a greater ( $P < 0.05$ ) percentage of MHC Type IIX fibers compared to medium marbled steaks. Fine and medium marbled steaks were similar ( $P > 0.05$ ) for the percentage of MHC Type IIX fibers. Marbling score did not impact ( $P > 0.05$ ) fiber cross-sectional area or distribution for MHC Type I, Type IIa, and Type IIX (Figure 4).

### Collagen traits

All texture groups (fine, medium, and coarse) possessed a similar ( $P > 0.05$ ) amount of both soluble and insoluble collagen (Table 1). In addition, each marbling texture group exhibited a similar ( $P > 0.05$ ) amount of total collagen. All marbling score groups had a similar ( $P > 0.05$ ) amount of soluble, insoluble, and total collagen. Similarly, marbling texture did not influence perimysial



**Figure 4.** Least squares means of myosin heavy chain (MHC) a) cross-sectional area and b) fiber type distribution of beef strip loin steaks of varying marbling scores. <sup>1</sup>Top Choice = marbling score of Modest<sup>00</sup> to Moderate<sup>100</sup>.

thickness, as coarse, medium, and fine marbled steaks were all similar ( $P > 0.05$ ) for this trait. Additionally, marbling score did not impact ( $P > 0.05$ ) perimysial thickness, as Top Choice, Low Choice, and Select steaks were similar ( $P > 0.05$ ) for perimysial thickness. Moreover, marbling texture did not affect ( $P > 0.05$ ) peak thermal transition temperature of the perimysial fraction. Marbling score also had no effect ( $P > 0.05$ ) on peak transition temperature of the perimysial fraction, as Top Choice, Low Choice, and Select steaks possessed a similar ( $P > 0.05$ ) perimysial thermal transition temperature.

### Discussion

Marbling texture is believed to have an influence on beef tenderness (Moody et al., 1970). However, it is important to assess whether this factor truly plays a role in beef palatability. Moody et al. (1970) proposed the observed marbling texture difference in tenderness

**Table 1.** Least squares means of collagen characteristics of beef strip loin steaks of varying marbling texture and marbling score treatments

Treatment	Soluble collagen, mg/g	Insoluble collagen, mg/g	Total collagen, mg/g	Perimysial peak transitional temperature, °C	Perimysial thickness, µm
<b>Marbling texture</b>					
Coarse	1.48	9.49	10.98	48.87	43.23
Medium	1.74	9.81	11.56	54.07	46.26
Fine	1.72	9.92	11.64	47.10	41.44
SEM <sup>1</sup>	0.12	0.34	0.38	2.72	2.20
<i>P</i> -value	0.27	0.65	0.41	0.17	0.31
<b>Marbling score</b>					
Top Choice <sup>2</sup>	1.50	9.86	11.36	50.29	44.56
Low Choice	1.77	9.61	11.37	47.84	42.54
Select	1.67	9.77	11.44	51.91	43.84
SEM <sup>1</sup>	0.12	0.34	0.38	2.72	2.20
<i>P</i> -value	0.31	0.87	0.99	0.57	0.81
<b>Texture × Marbling score</b>					
<i>P</i> -value	0.19	0.28	0.12	0.47	0.36

<sup>1</sup>SEM (largest) of the least squares means in the same main effect (marbling texture or marbling score).

<sup>2</sup>USDA marbling score of Modest<sup>00</sup>-Moderate<sup>100</sup>.

was related to potential differences in perimysial thickness between the texture treatments, but the authors did not measure perimysial thickness within that study. Perimysial thickness has been linked to reduced tenderness ratings and is associated with the background tenderness of beef products (Smith and Carpenter, 1974; Purslow, 2005; Purslow, 2014), and thus any potential differences due to marbling texture could negatively impact tenderness measures. In the more than 45 years since this hypothesis was proposed, no other published study has evaluated the impact of marbling texture on collagen characteristics, including perimysial thickness.

Our results do not support the theory proposed by Moody et al. (1970), as no differences were found among marbling texture categories for not only perimysial thickness, but for all of the collagen characteristics evaluated in the current study. This is further supported by similar tenderness ratings among the marbling texture groups as measured by both instrumental (WBSF) and sensory panelists (Vierck, 2017). Other authors have demonstrated the impact of collagen on beef tenderness (Cross et al., 1973; Seideman et al., 1987; Ebarb et al., 2016), but have often limited these comparisons between breeds, muscles, diet, and different maturity groups. Additionally, in the current study, no differences were found among the marbling score groups evaluated for any of the collagen traits evaluated. These findings for total collagen

are similar to previous studies comparing marbling levels and collagen content, where other authors have reported no differences in total collagen among beef from various quality grades (Oler et al., 2015). Similarly, in a study comparing cattle breeds, Martins et al. (2015) reported no differences in collagen content between Angus and Nellore cattle, despite a large marbling difference observed between those breeds. In contrast, Oler et al. (2015) reported as marbling levels increased in Polish Lowland × Limousin crossbred heifers, the percentage of soluble collagen was reduced. Our results indicate marbling texture has no impact on collagen characteristics nor the collagen-related aspects of tenderness.

In addition to collagen solubility measures, thermal transition temperature data further supports the similarity in tenderness observed between marbling texture groups, as there were also no differences among marbling texture groups. Peak transition temperature of collagen, particularly the perimysial and endomysial fractions, have been investigated as an indication of tenderness, as it indicates the temperature at which collagen present would denature (Tamilmani and Pandey, 2016). There has been very limited research to investigate differences within perimysial peak transition temperature, with most published work primarily focusing on differences within LM and semitendinosus muscles (Li et al., 2006; Li et al., 2010). However, when making comparisons within the same muscle, similar to the current study, most authors have reported no differences (Li et al., 2006; Li et al., 2010). Our results show that the perimysial fraction of muscle, as well as other connective tissue factors, are not impacted by marbling texture.

Previously published literature does not include research that has evaluated the effect of marbling texture on adipocyte size. The marbling score effects observed in the current study for adipocyte size are in agreement with Moody and Cassens (1968), who also observed both Small and Moderate marbled steaks possessed larger adipocytes in comparison to steaks with Traces marbling scores. Larger depositions of marbling, accompanied with larger adipocytes, could have contributed to possible splintering and thinning of the perimysium, as reported by Nishimura et al. (1999) in Japanese Black cattle, a breed known for extreme marbling. Because the coarse marbled steaks possessed larger adipocytes than fine marbled steaks, this could have thinned and splintered the perimysium, resulting in the lack of difference observed between these 2 marbling texture treatments for perimysial thickness.

To further identify any possible histological effects on eating quality as a result of marbling texture, muscle fiber type and cross-sectional area were evaluated.

Increased fiber cross-sectional area has previously been linked to increased WBSF and sensory tenderness scores (Seideman et al., 1987; Crouse et al., 1991; Chriki et al., 2013; Ebarb et al., 2016). However, in the current study, there were no differences found among marbling texture groups nor among marbling score groups in fiber cross-sectional area. Nevertheless, marbling texture impacted fiber type distribution, with medium marbled steaks possessing a greater percentage of MHC Type IIA type fibers and a corresponding reduction in MHC Type IIX fibers. The fiber distribution in the current work differs from previous investigations of the longissimus muscle, where MHC Type IIX have been reported to be in greater amounts than both MHC Type I and IIA (Hunt and Hedrick, 1977; Kirchofer, 2002). However, the results found in the current study are similar to a more recent study by Ebarb et al. (2016), which reported increased amounts of MHC Type IIA fibers in strip loin steaks in comparison to MHC Type IIX. This difference could be attributed to the use of immunofluorescence staining in the current study and the study by Ebarb et al. (2016) in comparison to ATP-ase activity and succinate dehydrogenase techniques used in previous research. Studies have found the percentage of slow and fast twitch fibers affect postmortem proteolysis (Xiong et al., 2007) and tenderness (Maltin et al., 1998), but the exact role of fiber type on palatability is not adequately defined in the literature (for review see Lee et al., 2010). Because the fiber type differences noted in the current study are within the fast fiber isoforms, this could be a reason the lack of palatability differences seen by Vierck (2017).

Results from the current study indicate that marbling texture influences adipocyte cross-sectional area, but has no impact on collagen characteristics or muscle fiber cross-sectional area, 2 main biological influencers of beef tenderness and eating quality. Therefore, the lack of palatability differences seen by Vierck (2017) were due to the lack of a texture effect on collagen and muscle fiber characteristics.

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