RESEARCH REPORT
PORK QUALITY

Title: Predicting Pork Quality: Discovering protein biomarkers for fresh pork loin tenderness. **#18-199 IPPA**

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Industry Summary:
The production of high-quality pork is important to the success and sustainability of food and agriculture industries. This is true for competition in domestic and international markets. Even more important is a quality differentiation with trendy plant-based products that are on the market today. Tenderness and water binding capacity of fresh meat are among the most critical attributes determining consumer satisfaction of fresh pork. While there is no question that the quality of fresh pork impacts the profitably of value-added pork industries, most of the successful innovations to measure pork value determine the quantity of pork, rather than quality. This is primarily because it is far easier to measure and control carcass weight and lean percentage/yield than it is to measure quality. Controlling and predicting fresh pork quality is extremely challenging because despite years of research, we still do not fully understand the biology of early postmortem muscle that governs major quality features, including tenderness and water-holding capacity The only way the research community and the food industry can develop reliable pork quality indicators is to understand factors that really determine quality. Our experience is that the primary factors that influence the fresh pork loin quality are protein factors and these are influenced by postmortem metabolism (pH decline) and handling (postmortem aging). In order to learn more about these processes and to apply them in a meaningful way, we proposed the following objectives:

Objective 1: Determine the contribution of key proteins in the postmortem aging process to the development of fresh pork tenderness.

Objective 2: Define the protein profile of early postmortem pork loin chops that is associated with a beneficial postmortem aging process and improved pork loin tenderness.

To address the objectives, we used fresh pork loin selected from a commercial processing facility one day post-harvest. Fresh pork quality (tenderness, color, water holding capacity) was determined on pork loin aged 1, 8, 14, and 21 days. Importantly, we evaluated product fresh at those days of aging and frozen at the end of each of those aging periods. We also measured the protein features (degradation of meat proteins troponin-T and desmin, length of protein structure in muscle called the sarcomere).

The most extreme examples of tough and tender pork loin in the experiment were chosen to investigate the protein profile (at day 1 postmortem) of the most tough and tender pork loin chops (observed after aging 14 days).The method used (2D -DIGE) provides a direct comparison of proteins in samples that represent the tough (High star probe (HSP)) and tender groups (Low star probe (LSP)).

Aging fresh pork loin for 8 d showed a 22% and 32% improvement in SP and WBS values, respectively. The current results suggest that aging 14 or 21 d did not result in improved SP or WBS values compared 8 d aging. Degradation of troponin-T and Desmin both showed that as degradation progressed with aging, so did improvement in star probe. Sarcomere length did not change during the postmortem aging process. Protein degradation data were numerically more highly correlated with instrumental tenderness measurements later postmortem when compared with sarcomere length, suggesting protein degradation may have a greater impact on tenderness values later postmortem. Importantly, freezing pork at 1 d postmortem will not allow products to improve in SP or WBS values so a recommended best practice is aging pork prior to freezing.

The immediate application of this research is that fresh pork loin should be allowed to age before freezing in order to improve fresh pork quality. This might have immediate considerations for smaller processors. In addition, it is clear that pork aging must be considered as a source of variation in fresh pork loin quality. This must be clarified, especially in local markets or "farmers' markets" where product might be frozen very early postmortem. Further, these results may have implications for merchandizing pork in home delivered meal kits that might have a frozen fresh pork product.

Variations in star probe values were attributed to differences in pH, marbling, water holding capacity, proteolysis, and sarcoplasmic protein profile at 1 d aging. The HSP (less tender) had a lower pH, less marbling, and poorer water holding capacity. In addition, the HSP group showed less degradation of key proteins desmin and troponin. In the protein profile experiment, (comparing protein profile at day 1 postmortem to fresh pork tenderness at day 14), the HSP group had greater abundance of metabolic, regulatory, and mitochondrial associated proteins whereas the LSP group had greater abundance of stress response proteins. The sarcoplasmic proteome analysis results confirm a difference in glycolytic metabolism capabilities between star probe groups, thus demonstrating the need to investigate more deeply the role of metabolic and regulatory proteins in the development of pork tenderness. Identification of many mitochondrial proteins in the sarcoplasmic proteome may suggest solubilization of by-products of the mitochondrial electron transport chain due to rupture of mitochondria. The rupture of mitochondria in muscle may increase the need for stress related proteins or need for antioxidant proteins. Once the status of these proteins is defined, robust protein biomarkers can identify products of differing tenderness.

Immediate researchable questions surround the topic of the heretofore un-reported observation that mitochondrial disruption in post mortem or post rigor pork can be an indication of the progression of proteolysis and improvement in tenderness. The rupture of mitochondria in muscle may increase the need for stress related proteins or need for antioxidant proteins. Once the status of these proteins is defined, robust protein biomarkers can identify products of differing tenderness.

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Key Findings:

- Protein degradation was confirmed to be a key component of fresh pork loin quality
- Abundance of antioxidant proteins (peroxiredoxin-2) in aged pork was linked to pork tenderness
- Abundance of mitochondrial proteins in sarcoplasmic fraction from tough pork loins suggests that fiber type and mitochondrial disruption may be linked to processes that interrupt normal aging of pork.
- Fresh pork tenderness was affected by pH, marbling score, protein degradation and sarcomere length

Keywords: glycolytic proteins, pork quality, proteolysis, sarcomere length, mitochondrial proteins

Scientific Abstract:

The objectives were to determine 1) the interaction between aging duration and post-aging freezing on pork loin quality attributes and 2) the relationship between pork loin star probe (SP) and Warner-Bratzler shear force (WBS). Loins from 20 carcasses were collected one d postmortem. Chops (n=9; 2.54 cm thick) were fabricated from each loin and vacuum packaged. Four chops from each loin were aged at 4°C for 1, 8, 14, and 21 d and immediately evaluated (Fresh). Four adjacent chops were aged (1, 8, 14, and 21 d), frozen for 14 d, and thawed for evaluation (Frozen). An additional chop was used for evaluation of sarcomere length, intact desmin and troponin-T degradation. Purge, objective color, pH, subjective color and marbling score, cook loss, SP, and WBS were evaluated at each aging period. Desmin and troponin-T degradation, and sarcomere length were measured on fresh samples at each day of aging. Post-aging freezing had no significant impact on SP, WBS, pH, and subjective color or marbling score at any aging period. Fresh chop purge increased at each day of aging ($P<0.01$). Post-aging freezing resulted in greater purge at 1, 8, and 14 d aging ($P<0.01$). Fresh chop cook loss was greater than post-aging freezing chop cook loss at 14 and 21 d aging ($P<0.05$). Across all aging periods and treatments, SP was correlated ($r=0.85$; $P<0.01$) with WBS. Fresh chop SP and WBS decreased from 1 to 8 d aging but was not different after 8 d aging. The abundance of intact desmin decreased ($P<0.01$) between 1, 8, and 14 d aging. Troponin-T degradation increased ($P<0.01$) with each aging period. Sarcomere length was not different across aging periods ($P>0.05$). Aging, without freezing, for 14 or 21 d did not improve SP or WBS observed at 8 d, corresponding with changes in desmin degradation.

A second experiment was conducted to determine the extent to which the sarcoplasmic proteome explains variations in aged pork loin star probe (SP) value. Pork loins (n=12) were categorized by differences in SP at 21 d postmortem from a larger set of loins. Loins were categorized into Low SP group (LSP; n=6; SP<5.80 kg) and High SP group (HSP; n=6; SP>7.00 kg) based on 21 d SP value with inclusion criteria of marbling score (1.0-3.0) and 24 h pH (5.69-5.98). Quality traits were measured at 1, 8, 14, and 21 d aging. Desmin and troponin-T degradation, peroxiredoxin-2 abundance, calpain-1 autolysis, and sarcomere length were determined. Two-dimensional difference in gel electrophoresis and mass spectrometry were used to identify proteins that differed in abundance due to category. Star probe values were lower ($P<0.01$) in LSP at each d of aging compared with HSP. Greater pH values were observed ($P<0.05$) in LSP compared with HSP at each d of aging. Marbling score was greater ($P<0.05$) in LSP compared with HSP at each d of aging. Greater ($P<0.05$) desmin and troponin-T degradation was detected in LSP chops at 14 and 21 d aging and 8, 14, and 21 d aging, respectively. Greater($P<0.05$) sarcomere length was determined in LSP compared with HSP at 1, 8, and 21 d aging. Sarcoplasmic proteins from HSP chops had greater abundance($P<0.10$) of metabolic and regulatory proteins whereas the LSP chops had greater abundance ($P<0.10$) of stress response proteins. Star probe values were affected by pH, marbling score, protein degradation, sarcomere length, and sarcoplasmic proteome.

Introduction: Fresh pork quality is a critical factor in the future success and sustainability of the industry. Low drip loss/high water holding capacity and tenderness of fresh meat are attributes that that exert a strong influence on consumer satisfaction of fresh pork (Font-i-Furnols and Guerrero 2014). Also, water-holding capacity has a major impact on the suitability of fresh pork as a raw material for further processed products. From the processers' standpoint, water holding capacity has an impact on the profitability of products because drip that is lost represents a significant loss in weight of the product, not to mention a loss of valuable water-soluble proteins that are lost along with the purge (Huff-Lonergan and Lonergan 2005). While there is no question that quality attributes have a major impact on the profitably of the fresh pork industry, most of the innovations in measuring pork value have been with respect to improvement in the efficiency of the *quantity* of pork, rather than quality. One of the primary reasons for this is the fact that it is far easier to measure and to control carcass weight and lean percentage/yield than it is to measure/control quality. Controlling and predicting fresh pork quality has proven to be highly challenging because we do not fully understand the biology of early postmortem muscle that controls major quality features, including tenderness and water-holding capacity (Huff Lonergan et al. 2010). The research community cannot develop reliable pork quality indicators that can be used to drive value until we understand what factors really control quality. This lack of knowledge must be overcome if the industry is to make any progress in improving fresh pork quality. Our experience suggests that the protein component of the postmortem metabolism and structure of meat contributes to observed variation in fresh pork loin quality. Therefore, we proposed the objectives below.

Objectives:

Objective 1: Determine the contribution of key proteins in the postmortem aging process to the development of fresh pork tenderness.
Objective 2: Define the protein profile of early postmortem pork loin chops that is associated with a beneficial postmortem aging process and improved pork loin tenderness.

Materials & Methods:

Objective 1: Determine the contribution of key proteins in the postmortem aging process to the development of fresh pork tenderness.

Pork Loin Collection and Fabrication

Paired sides of fresh pork loins of similar genetics (Duroc sired crossbreds), management, diets, harvest, and chilling methods were collected one d postmortem from 20 carcasses harvested at a commercial processing facility. Loins were vacuum packaged and transported to the Iowa State University Meat Laboratory for fabrication one d postmortem. Each pair of loins were fabricated into 9

chops after removal of the sirloin end (approximately 10 cm). Loin chops (2.54 cm; n=8), containing only the longissimus muscle, were trimmed of external fat and connective tissue. Chops were vacuum packaged prior to aging. Four chops from each pair of loins were aged for 1, 8, 14, and 21 d at 4° C and immediately evaluated at the conclusion of the prescribed aging period (Fresh). Four adjacent chops were aged (1, 8, 14, and 21 d at 4° C), individually placed on racks, frozen (-29° C) post-aging for 14 d and then thawed (2° C, 22 h) for quality evaluation (Frozen). Quality evaluations of frozen chops occurred at 15, 22, 28, and 35 d postmortem. The final chop was divided into four equal sections (~100 g) and vacuum packaged. These samples followed the aging period of that loin to mimic each aging period. Following aging, the sample was frozen (-80° C) for protein extraction and analysis. Loin side for each set of aging times (1 and 8, or 14 and 21) was randomly assigned.

Quality Data Analysis

At completion of aging or freezing, chop purge was collected by weighing the chop and the package with the purge in the package. Chop purge was calculated using the following formula: (weight of package with purge - weight of package without purge)/ chop weight) x100. Chop pH was measured using a Hanna HI9025 pH meter (Hanna Instruments, Woonsocket, RI). The pH meter was calibrated using pH 4 and 7 buffers at room temperature (20° C). Accuracy of calibration of pH was checked before each measurement using pH 7 buffer (6.95 to 7.05 pH range). Chops were removed from refrigeration and allotted 15 min to bloom at room temperature (~22° C). Subjective color and marbling scores were assessed using the National Pork Board 6-point and 10-point scale standard pictures, respectively (Color: 1= pale pinkish gray to white; 6= dark purplish red; Marbling: 1= 1.0% intramuscular fat; 10= 10.0% intramuscular fat) (National Pork Board, 2000). Hunter L, a, and b values were measured on each chop at the center of the chop surface using a Minolta Chroma Meter with a D65 light source, 50 mm aperture, and 2° observer angle (CR-410; Konica Minolta Sensing Americas Inc., Ramsey, NJ) (AMSA, 2012). Hue angle and chroma values were calculated using the following equations: hue angle= arctangent (b/a) and chroma= $(a^2+b^2)^{1/2}$(AMSA, 2012). Each chop was cooked to an internal temperature of 68° C on clamshell grills (Cuisinart, East Windsor, NJ). Cook loss was calculated by using the following formula: [(raw chop weight-cooked chop weight)/raw chop weight] x100. Purge, pH, visual color and marbling scores, Hunter L, a, and b values, and cook loss measurements were averaged for both chops of each treatment at each aging period.

Instrumental Tenderness Analysis

Star probe punctures and compresses the chop to 20% of its original height. Value from this analysis are similar to the nature of chewing (Huff-Lonergan et al. 2002). An Instron (Instron, Norwood, MA) fitted with a five-point star probe attachment was used to measure instrumental tenderness on one cooked chop per treatment per day of aging (Arkfeld et al. 2015; Carlson et al. 2017a,b). Three replicate compressions were made on each chop and averaged for a final instrumental tenderness value. Adjacent chops were cooked in the same manner and analyzed with a WBS force attachment to an Instron (Instron, Norwood, MA). Three 1.27-centimeter diameter cores were removed from each chop for analysis. Cores were removed parallel to the muscle fibers.

Sample Preparation and Western Blot analysis

Frozen meat (*longissimus dorsi;* 100 g) was homogenized in liquid nitrogen. Samples from each aging time (fresh) (0.5 g) were homogenized and whole muscle protein extracts were completed as described by Carlson et al. (2017a,b). Consistency of protein concentrations were assured using 15% SDS-PAGE gels and Colloidal Coomassie blue staining (1.7% ammonium sulfate, 30% methanol, 3% phosphoric acid, and 0.1% Coomassie G-250). Desmin and troponin-T degradation were determined using one-dimensional SDS-PAGE gel electrophoresis and western blot analysis as described by Carlson et al. (2017b). At completion of running the SDS-PAGE gels, the gels were transferred to polyvinylidene difluoride (PVDF) membranes with pore sizes of 0.2 µm as described by Carlson et al. (2017a,b).

Following transfer, the membrane was incubated for 60 min at 22° C in PBS-Tween mixed with 5% non-fat dry milk (NFDM). Desmin primary antibody was added to the blot at a dilution of 1:40,000 polyclonal rabbit anti-desmin antibody produced at Iowa State University (Huff-Lonergan et al. 1996; Carlson et al. 2017a,b) with PBS-Tween. Troponin-T primary antibody was added to separate blots at a dilution containing of 1:10,000 using monoclonal mouse anti-troponin-T primary antibody (T6277, JLT-12; Sigma-Aldrich, St. Louis, MO) with PBS-Tween. Blots were incubated in primary antibodies overnight at 4° C. After incubation with primary antibody, blots were washed with PBS-Tween 3 times for 10 min. Secondary antibodies were diluted with PBS-Tween and incubated with each blot for 1 hour at room temperature. Secondary antibody dilution for desmin and troponin-T blots contained 1:20,000 goat anti-rabbit-HRP antibody (for desmin blots) and 1:5,000 goat anti-mouse HRP antibody (32430; Pierce) for troponin-T blots. Following incubation with secondary antibodies, blots were washed with PBS-Tween 3 times for 10 min. A chemiluminescent detection kit (ECL Prime, GE Healthcare, Piscataway, NJ) was used to detect proteins. Blots were imaged and analyzed using a ChemiImager 5500 (Alpha Innotech, San Leandro, CA) and Alpha Ease FC software (v 3.03 Alpha Innotech). Using the internal reference on each blot, the intensity of the 55-kDa intact desmin band and 30-kDa troponin-T degradation product was quantified as a comparative ratio of the sample protein band to the internal reference protein band on each gel. All western blots were completed in at least duplicate with a coefficient of variance less than 20%.

Sarcomere Length

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

Statistical Analysis

Each individual chop was the experimental unit. All quality data (cook loss, pH, purge, subjective color and marbling, Hunter L, a, and b, hue angle, chroma, SP and WBS values (n=160 per measurement except for WBS (n=158)) measurements were analyzed using the MIXED procedure of SAS (v.9.4; SAS Inst., Cary, NC). Fixed effects included days aged and treatment (Fresh or Frozen) for all quality data measurements. Carcass was used as a random effect in all models.
Intact desmin (n=80) and troponin-T degradation (n=80) product were analyzed using the MIXED procedure of SAS (v9.4, SAS Inst., Cary, NC) with a fixed effect of days aged. Gel was used as a random effect in the model. Sarcomere length measurements (n=80) were analyzed using the MIXED procedure of SAS (v.9.4; SAS Inst., Cary, NC). Sarcomere length analysis fixed effects included days aged. Carcass was used as a random effect in all models. Least squares means, and standard errors were reported for all measured attributes. Least squares means were separated using pdiff procedure of SAS (v.9.4; SAS Inst., Cary, NC). Significance levels were denoted with a $P < 0.05$.
Pearson correlations were generated using PROC CORR. Correlations were considered lowly correlated at $r \leq 0.35$, moderately at $0.36 \leq r \leq 0.67$, and highly if $r \geq 0.68$ (Taylor, 1990). Correlations and correlation comparisons were considered significant when $P < 0.05$.

Objective 2: Define the protein profile of early postmortem pork loin chops that is associated with a beneficial postmortem aging process and improved pork loin tenderness.

Loins from the previous objective with high (star probe>7.0 kg) and low (star probe<5.8 kg) star probe values at 21 d postmortem were chosen to represent extreme differences in star probe value. Quality data (cook loss, pH, purge, subjective color and marbling, Hunter L, a, and b, and star probe value) were collected as previously described (Schulte et al., 2019). Marbling score and pH value parameters were set as inclusion criteria to further identify sample experimental groups. Loin marbling scores at 21 d aging ranged from 1.0 to 3.0. Loin pH at 21 d aging ranged from 5.69 to 5.93. This 21 d aging classification criteria narrowed the sample set to a balanced experiment of high (n=6) and low (n=6) star probe categories.

Sample preparation, western blot analysis, and sarcomere length

Sample preparation, western blot analysis (for desmin and troponin-T) nd sarcomere length were conducted as described for objective 1.

Sarcoplasmic Protein Extraction

Frozen meat containing only the *longissimus dorsi* (100 g) was homogenized in liquid nitrogen. Samples from each aging time (3 g) were homogenized and sarcoplasmic proteins were extracted (4°C; 50 mM Tris-HCl and 1 mM EDTA, pH 8.0) as described by Carlson et al. (2017b). Samples to be used for two-dimensional difference gel electrophoresis (2D-DIGE) were diluted to 10 mg/ml using cold sarcoplasmic extraction buffer (4°C; 50 mM Tris-HCl and 1 mM EDTA, pH 8.0) and frozen.

Western blot analysis: Peroxiredoxin-2 and Calpain-1

Sarcoplasmic protein extracts were used to determine peroxiredoxin-2 abundance (1, 8, 14, and 21 d aged) as well as calpain-1 autolysis at 1 d postmortem were determined using one-dimensional SDS-PAGE gel electrophoresis as described by Carlson et al. (2017a, b). Protein was extracted from porcine *longissimus dorsi* (aged 0, 1 and/or 7 d) with the identical protocol (Carlson et al. 2017a, b) to generate a reference sample (4 mg/ml of protein) that was included in one well on each gel. A reference (longissimus muscle, 0 d aging) was used for desmin analysis, a 0/7 d mixed reference sample was used for troponin-T analysis and a 1 d reference sample was used for peroxiredoxin-2 analysis. SE 260 Hoefer Mighty Small II electrophoresis units (Hoefer, Inc., Holliston MA) were used to run 15% gels for desmin, troponin-T, and peroxiredoxin-2 analysis, and 10% gels for calpain-1 autolysis.
Protein abundance was determined using antibodies for Peroxiredoxin-2 using monoclonal rabbit anti-peroxiredoxin-2 antibody (ab109367; ABCam, Cambridge, UK) and calpain-1 using monoclonal mouse anti-calpain-1 (MA3-940, Thermo Scientific, Rockford, IL). Blots were analyzed as described by Carlson et al. (2017a, b). Using the internal reference on each blot, the intensity of the 22-kDa intact peroxiredoxin-2 band was quantified as a comparative ratio of the sample protein band to the internal reference protein band on each gel. Calpain-1 autolysis was analyzed as a percentage of the 80-, 78-, or 76-kDa band within each sample. All western blots were completed in at least duplicate with a coefficient of variance less than 20%.

Two-Dimensional Difference in Gel Electrophoresis

Sarcoplasmic protein (50 µg) extracts from each sample of each experimental group were labeled alternatively with CyDye3 and CyDye5 (Carlson et al., 2017b) according to the manufacturer's directions (GE Healthcare, Piscataway, NJ). A pooled reference sample containing equal amounts of all samples (n= 12 total) were used for identification and pick gels. Three aliquots of the pooled reference sample (100 ug) were labeled with CyDye2. The final protein concentration of samples were 7.14 mg/ml. Samples were stored at -80° C until use to complete experiments at corresponding pH ranges.
Labeled samples were prepared for running on 11-centimeter pH 3-10 or pH 4-7 immobilized pH gradient (IPG) strips (GE Healthcare, Piscataway, NJ) as described previously (Cruzen et al., 2015) with minor adjustments.
Spots of interest between experimental groups were chosen for identification and the pooled reference sample was used for identification. For spot identification, gels were run as described above with the corresponding pH range to isolate spots of interest. Picked spots were excised from gels, sent to the Iowa State University Protein Facility, digested with trypsin using a Genomic Solutions Investigator ProGest automated digester (Genomic Solutions Inc., Ann Arbor, MI). After digestion, the solution was dried down and reconstituted in 25 µL water containing 0.1% formic acid. Spots were then separated through liquid chromatography (Thermo Scientific EASY nLC-1200 coupled to a Thermo Scientific Nanospray FlexIon source) using a pulled glass emitter 75 µm X 20 cm (Agilent capillary, part #16-2644-5), with the tip packed with Agilent SB-C18 Zorbax 5 µm packing material (part #820966-

922) and the remaining emitter packed with nanoLCMS Solutions UChrom C18 3 µm packing material (part #80002). Samples were analyzed by tandem mass spectrometry (MS/MS) using a Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA). Thermo Scientific's Proteome Discoverer Software (Waltham, MA) was used to analyze the raw data for identification of matched proteins and peptides against publicly available or user-provided databases. Peptide fragments were compared to a known database program using Mascot (London, United Kingdom) and Sequest HT against Sus Scrofa to identify proteins.

Statistical Analysis

All quality data (cook loss, pH, purge, subjective color and marbling, Hunter L, a, and b, and star probe value) were analyzed using the MIXED procedure of SAS (v.9.4, SAS Inst., Cary, NC). Fixed effects included days aged and category (HSP or LSP). Whole muscle intact desmin and troponin-T degradation product, sarcoplasmic peroxiredoxin-2 abundance and autolyzed sarcoplasmic calpain-1 data were analyzed using the MIXED procedure of SAS (v9.4, SAS Inst., Cary, NC) with fixed effects of days aged and category. Gel was used as a random effect in the model. Sarcomere length measurements were analyzed using the MIXED procedure of SAS (v.9.4; SAS Inst., Cary, NC) with fixed effects of days aged and category. Carcass was used as a random effect in all models. Least squares means and standard errors were reported for all measured attributes. Least squares means were separated using the pdiff procedure of SAS (v.9.4; SAS Inst., Cary, NC). Significance levels were denoted with a $P \leq 0.05$.

2D-DIGE gel images were analyzed using DeCyder 2D software v6.5 (GE Healthcare, Piscataway, NJ) using Student's paired t-test to determine differences in protein spot relative abundance between treatment groups. Significance was determined at $P \leq 0.10$ and trending at $0.10 < P \leq 0.15$.

Results:

Objective 1: Determine the contribution of key proteins in the postmortem aging process to the development of fresh pork tenderness.

The results of this objective are published in Meat and Muscle Biology (Schulte et al. 2019 *Meat and Muscle Biology* 3(1):313–323 (2019) doi:10.22175/mmb2019.05.0015). The results of the current experiment demonstrate the impact of post-aging freezing of pork loins at different aging periods. Table 1 summarizes the effects of aging and post-aging freezing on meat characteristics.

Instrumental Tenderness

Post-aging freezing had no significant impact on Star Probe (SP) or Warner-Bratzler Shear Force (WBS) values compared with fresh chops that were evaluated immediately after aging (Table 1; $P>0.05$). Chop SP and WBS values were greatest at 1 d aging than any other aging period regardless of treatment. These results align with previous data from beef, pork and ovine studies (Kim et al. 2011a, b, 2015, 2018) that demonstrate freezing meat prior to aging does not allow product to improve in tenderness value if cooked immediately after thawing frozen product. Star Probe and WBS values decreased significantly from 1 to 8 d aging ($P<0.01$). Star Probe value was not different at 8, 14, and 21 d aging ($P>0.05$) regardless of treatment whereas WBS values decreased in frozen chops from 14 to 21 d aging ($P<0.01$).

Post aging freezing tended ($P <0.08$) to result in lower WBS values in samples aged 21 d. Freezing induced decrease in WBS value may be caused by ice crystal formation between myofibrils (Leygonie et al. 2012b). From the literature and these results, it is understood that post-aging freezing does not significantly impact tenderness values if product is aged prior to freezing. Additionally, chops must be aged prior to freezing to allow for tenderization if chops will not be aged post-freezing.

Fresh SP value decreased by 23 % from 1 to 21 d aging across all fresh samples ($P<0.05$). Frozen SP value decreased by 27 % from 1 to 21 d aging across all frozen samples ($P<0.05$), indicating a similar response to its fresh counterparts. Fresh WBS value decreased by 41 % from 1 to 21 d aging across all fresh samples ($P<0.05$) whereas frozen WBS value decrease by 59 % from 1 to 21 d postmortem.

Color Analysis

Fresh chop L value increased from 1 to 8 d aging ($P<0.01$) but did not change after 8 d aging ($P>0.05$). Post-aging freezing chop L value also increased from 1 to 8 but also increased from 14 to 21 d aging ($P<0.01$). Chops demonstrated lesser redness values at 1 d aging ($P<0.01$) than any other aging period considered. Fresh chop b value increased from 1 to 8 d aging ($P<0.01$) but was not different after 8 d aging ($P>0.05$). Post-aging freezing chop b value increased from 1 to 8 and 8 to 14 d aging ($P<0.01$) but did not differ between 14 and 21 d aging ($P>0.05$). As expected, chops became lighter, more red, and more discolored over time indicating loss of color stability which could be caused by a large array of factors such as pH, muscle source, lipid oxidation, oxidation of myoglobin, and mitochondrial activity. Meat color stability is also impacted by storage time loss of water and freezing rate.

Fresh chops aged 1 d had lower a values ($P<0.01$) than their frozen counterparts that were aged 1 d ($P<0.01$). Post-aging freezing also resulted in greater b value compared with fresh chop b value at 1, 14, and 21 d ($P<0.01$) aging. Decreased redness and greater discoloration were expressed in fresh chops at 1 d aging compared to frozen chops aged 1 d. It is well known that freezing and thawing reduces blooming ability and color stability. It has been previously demonstrated that steaks from beef and lamb as well as chops from pork *longissimus* muscle subjected to post-aging freezing demonstrated greater discoloration compared with fresh chops This increase in discoloration in the post-aging freezing chops is most likely due to increased susceptibility of myoglobin to oxidation (MacDougall 1982; Kim et al. 2011). Contrary to previous results of other studies, the results of this study demonstrate decreased redness in the fresh chops compared with the post-aging freezing chops at 1 d aging. This could be due to the blooming ability (MacDougall 1982) and the mitochondrial reducing ability of the samples (Tang et al. 2005). Fresh samples may have had mitochondria that were continuing to respire, thus decreasing the blooming ability of myoglobin due to a lower partial pressure of

oxygen within chops aged 1 d. This can be compared to chops which experienced post-aging freezing at 1 d aging. These chops may have experienced damage to the mitochondria through the freezing and thawing process, thus impacting mitochondrial functionality and ultimately the blooming ability of myoglobin (Vestergaard et al. 2000).

The rate of freezing and confounding factors such as pH and myoglobin concentration can have a significant impact on color stability. In a study by Kim et al. (2018), fast-freezing pork loin sections after aging resulted in greater redness and greater discoloration compared with loin sections that were frozen slowly. The current results demonstrated differences in a and b values at 1 d aging between fresh and frozen samples ($P<0.01$) which may be impacted by the freezing method used. However, this difference in a and b values is also impacted by decreases in metmyoglobin reducing agent activity and alterations of mitochondrial function (Kim et al. 2011).

Post-aging freezing had no significant effect on pH, subjective color score and marbling score, or Hunter L value at any aging period (Table 1; $P>0.05$). Post-aging freezing was not expected to impact marbling due to no significant changes in the fat content of the chops throughout the freezing period. Previous research has demonstrated small, but significant impacts of post-aging freezing on quality attributes of pH and color scores (Kim et al. 2011, 2018; Leygonie et al. 2012b; Kim and Kim 2017). Kim et al. (2018) demonstrated that pork *longissimus* sections which were aged for 19 d, frozen, and then thawed maintained greater pH values than sections that were only frozen or frozen, thawed, and then aged (Kim et al. 2018). In a similar study examining the effects of aging and freezing/thawing sequence on beef *biceps femoris* and *gluteus medius*, freezing and thawing of muscles decreased the pH regardless of the sequence of freezing and thawing (Kim and Kim 2017). Kim et al. (2011) observed that freezing for 9 weeks reduced pH values in sheep *longissimus* muscle compared to post-aging freezing of steaks and wet aging of steaks (Kim et al. 2011). Differences in Hunter L values and visual color scores were not observed between fresh and post-aging freezing treatments. Since samples were stored in opaque boxes during storage, this result was expected.

Correlation of Star Probe and Warner-Bratzler Shear Force

Previous studies have shown a moderate correlation between SP and a trained sensory panel tenderness score. These results of the current experiment demonstrate that SP and WBS generate correlated measurements and they can be utilized to assess texture regardless of aging time or conditions. Across all aging periods and treatments (n=158), SP value was highly correlated ($r=0.85$; $P<0.01$) with WBS values. Fresh SP and WBS correlations across all days aging (n=80; $r=0.89$) were not significantly different ($P=0.13$) than post-aging freezing correlations (n=78; $r=0.83$) across all aging periods. Within aging periods, fresh SP and WBS values were trending ($P=0.06$) to be more highly correlated at 8 d aging (n=20; $r=0.93$) than 1 d aging (n=20; $r=0.78$) but did differ from 14 (n=20; $r=0.89$) or 21 d aging (n=20; $r=0.92$). Post-aging freezing SP and WBS values were significantly more highly correlated ($P<0.01$) at 14 d aging (n=20; $r=0.93$) than 8 (n=20; $r=0.76$) or 21 d aging (n=18; $r=0.65$). The results suggest that the relationship between SP and WBS is weaker at 21 d aging when pork chops are frozen after aging.

Sarcomere Length

During the conversion of muscle to meat, contraction of muscles and shortening of sarcomeres occurs impacting tenderness of whole muscle products. The extent and rate of shortening is impacted by many factors, one of those being temperature. This study demonstrated no significant changes in sarcomere length across days aging ($P>0.05$) in the fresh chops. This suggests that improvement of WBS or SP during aging is not due to changes in sarcomere length during aging. However, sarcomere length was correlated with WBS ($r=-0.46$) and SP ($r=-0.43$) across all d aging ($P<0.01$). At 1, 8, 14, and 21 d aging, sarcomere length was correlated with WBS ($r=-0.60$, -0.56, -0.71, and -0.51) and SP ($r=-0.53$, -0.60, -0.55, and -0.47), respectively. Across all days aging, correlation values were not significantly ($P>0.05$) different between sarcomere length and WBS or SP values. These results do propose a consistent sarcomere length contribution to textural measurements of fresh pork.

Postmortem Protein Degradation

A large array of biochemical changes occurs during the conversion of muscle to meat influencing meat quality attributes such as water holding capacity and tenderness. Postmortem protein degradation is one factor that has a significant impact on the development of meat tenderness and meat's ability to hold water (Taylor et al. 1995; Melody et al. 2004). Desmin is an intermediate filament protein that connects the myofibril with other myofibrils and integrates surrounding organelles. Troponin-T is part of the troponin complex which functions to modulate actin and myosin cross bridging (Clark et al. 2002). Greater degradation of these proteins has been linked to pork loin with lower star probe values (Carlson et al. 2017). Fresh samples used in this study were aged for 1, 8, 14, and 21 d to assess the development of pork tenderness throughout different aging periods to determine optimum time for tenderness development. Abundance of intact desmin and troponin-T degradation product is summarized in Table 1. The results show that abundance of intact desmin decreased from 1 to 8 and 8 to 14 d aging ($P<0.01$) but was not different at 21 d aging ($P>0.05$) compared with 14 d aging. Troponin-T degradation product was not detected at 1 d postmortem in any sample. Abundance of troponin-T degradation product significantly increased at each d postmortem ($P<0.01$). Additionally, intact desmin decreased by 56 % from 1 to 21 d aging across all fresh samples ($P<0.05$). Intact desmin and troponin-T were highly correlated ($P<0.01$) to SP ($r=0.61$ and -0.62, respectively) and WBS ($r=0.66$ and -0.67) across all d aging in the fresh samples. Within day correlations revealed that intact desmin was not significantly correlated with WBS ($r=0.26$; $P=0.26$) or SP ($r=0.70$; $P=0.09$;) at 1 d aging. Troponin-T degradation product was not detected in any sample aged 1 day, so no correlations were computed. In contrast, troponin-T degradation was moderately and highly correlated with WBS at 8, 14, and 21 d postmortem ($r=-0.73$, -0.64, and -0.77, respectively) and with SP at 8, 14, and 21 d postmortem ($r=-0.70$, -0.69, -0.79, respectively) but Fisher's Z transformation revealed that the correlations to troponin-T degradation were not different across days ($P>0.05$). Similarly, intact desmin was moderately and highly correlated at 8, 14, and 21 d postmortem

with WBS (r=0.45, 0.55, 0.82, respectively) and SP (r=0.46, 0.58, 0.73, respectively). Correlation values at 8, 14, and 21 d were not different from each other (P>0.05) but intact desmin correlations to SP and WBS at 8, 14, and 21 d were all significantly greater than what was determined at 1 d aging (P<0.01). However, intact desmin and troponin-T were weakly correlated to sarcomere length (r=-0.20; P=0.08 and r=0.24; P=0.04, respectively) across all d aging. This weak relationship demonstrates that in this experiment, sarcomere length did not contribute to variation in access of proteinases to substrates in different locations in the muscle cell and myofibril.

Degradation of desmin and troponin-T has consistently shown to account for differences seen in instrumental tenderness values of pork muscles (Wheeler et al. 2000; Melody et al. 2004; Carlson et al. 2017). Degradation of desmin protein can ultimately alter myofibril alignment and connection to conjoining structures and resulting in differences in tenderness. Degradation of troponin-T could demonstrate weakening of the complex formed between actin and myosin or skeletal muscle protein degradation overall. These results demonstrate the corresponding relationship between both instrumental tenderness measurements and protein degradation data. These results also demonstrate protein degradation, in general, is consistent with SP and WBS value decline across aging periods.

Objective 2: Define the protein profile of early postmortem pork loin chops that is associated with a beneficial postmortem aging process and improved pork loin tenderness.

The results from this objective are in a manuscript currently under review for publication in the Meat and Muscle Biology. Fresh pork characteristics of both experimental groups are summarized in Table 1. Star probe values were significantly greater in the HSP group compared with the LSP group at each day of aging *(*P<0.01). Pork loin star probe values at 21 d aging for LSP ranged from 4.76 to 5.77 kg and HSP ranged from 7.05 to 9.35 kg. Star probe groups had a 2.51 kg difference in average star probe value at 1 d aged and a 2.94 kg difference in average star probe at 21 d aging. The LSP probe group demonstrated a 25% decrease in star probe value from 1 to 21 d aging while the HSP group only had a 15% decrease in star probe value during the aging period.

The LSP group had less purge at 14 and 21 d aging (P<0.01) and less cook loss at 8 and 21 d aging (P<0.05) than the HSP group. This difference could be partially accounted for by significant differences in pH and desmin degradation. Postmortem proteolysis not only impacts meat tenderness, but also impacts the ability of meat to retain water. Bee et al. (2007) showed that drip loss at 1, 2, and 4 d of storage was positively correlated to intact desmin (0.41, 0.45, and 0.42, respectively) and talin (0.35, 0.60, and 0.51, respectively). In pork, early postmortem and ultimate pH is negatively correlated with drip and purge loss (Huff-Lonergan et al., 2002; Melody et al., 2004; Bee et al., 2007; Boler et al., 2010; Richardson et al., 2018; Watanabe et al., 2018).

High pH values will result in greater pork quality regardless of intramuscular fat content (Lonergan et al., 2007). However, intermediate pH ranges (5.50<pH>5.95) may be influenced by lipid content (Lonergan et al., 2007). However, when factors such as genetics, management techniques and harvest day were restricted along with a set range of ultimate pH (5.48 to 5.79), marbling did not influence eating quality (Rincker et al., 2008). Additionally, some research has suggested that consumers purchasing intent has shifted towards the desire to purchase pork with less intramuscular fat content (Brewer et al., 2001; Papanagiotou et al., 2013). This has influenced genetic decisions for leaner, more efficient growth in pigs and adversely, negatively impacting pork quality (Lonergan et al., 2001). A small, yet significant difference in pH was observed between star probe groups. The LSP group demonstrated greater pH values at 1, 14, and 21 d aging (P<0.05) when compared with the HSP group. Additionally, the LSP group had greater marbling scores at all aging timepoints (P<0.05) compared with the HSP group. The combination of pH and marbling score of these chops within these medium pH ranges may be influencing quality attributes and proteolysis, however, the differences were small. Chop visual color scores and Hunter L, a, and b values were not different between star probe groups at any aging time point (P>0.05).

Calpain-1 plays a significant role in the degradation of myofibrillar, cytoskeletal, and intermediate filament proteins (Wheeler et al., 2000; Lametsch et al., 2004; Geesink et al., 2006; Koohmaraie and Geesink 2006). The 76-kDa autolysis product of calpain-1 was negatively correlated to desmin (-0.57), vinculin (-0.18), and talin (-0.66) demonstrating the close association of calpain-1 degradation and autolysis (Bee et al., 2007). Calpain-1 activity and autolysis is impacted by pH decline (Melody et al., 2004; Bee et al., 2007), oxidative conditions (Maddock-Carlin et al., 2006), and nitric oxide (Li et al., 2014; Liu et al., 2016, 2019; Zhang et al., 2018); calpain-1 rate of activation and autolysis could impact the extent of proteolysis which occurs. Desmin, troponin-T, titin, tropomyosin, actin, and myosin light chain I are substrates of calpain-1 (Huff-Lonergan et al., 1996; Lametsch et al., 2004; Geesink et al., 2006; Anderson et al., 2012; Carlson et al., 2017a). Although some autolysis occurred in every sample at 1 d postmortem, no significant difference between experimental groups was identified (P>0.05) in the sarcoplasmic fraction. Melody et al. (2004) demonstrated that muscles with a faster rate of pH decline showed a quicker rate of calpain-1 autolysis in the sarcoplasmic protein fraction (Melody et al., 2004). The rate of pH decline during the early postmortem period was not collected with these samples. Differences in the rate of pH decline could explain differences in calpain-1 activity between samples. An extreme pH decline is not suspected due to no significant differences in Hunter L or b values between star probe groups, but this was not measured so cannot be concluded.

A representative Western blot of desmin degradation analysis and the corresponding star probe values for those specific samples is shown in Figure 3. Postmortem proteolysis has a significant impact on the development of meat tenderness (Taylor et al., 1995; Melody et al.,2004; Carlson et al., 2017a). Desmin functions to integrate the myofibril with surrounding organelles while also interlinking myofibrils at the Z-line region (Clark et al., 2002). Troponin-T is an integral part of the troponin complex which regulates the cross bridging of myosin and actin (Clark et al., 2002). Degradation of desmin and troponin-T has consistently shown to be related

to differences seen in instrumental tenderness values of pork muscles (Wheeler et al., 2000; Melody et al., 2004; Carlson et al.,2017a). Degradation of desmin can alter alignment of myofibrils ultimately increasing meat tenderness. The results demonstrate that abundance of intact desmin decreased from 1 to 8 d aging (Table 1; $P<0.01$) in both SP groups. The LSP group had significantly less intact desmin at 14 and 21 d aging ($P<0.01$) compared with the HSP group. Abundance of intact desmin in the LSP group decreased by 67% from 1 to 21 d aging whereas the HSP group only decreased by 30% during the entire aging period. No measurable 30-kDa troponin-T degradation product was detected at 1 d aging in either SP group. Troponin-T degradation product abundance increased from 8 to 14 and 14 to 21 d aging (Table 1; $P<0.01$) in the LSP group. Conversely, troponin-T degradation product increased from 14 to 21 d aging (Table 1; $P<0.01$) but was not different between 8 and 14 d aging in the HSP group. Within 8, 14 and 21 d aging, the LSP group had significantly greater abundance of 30 kDa troponin-T degradation product compared with the HSP group ($P<0.01$). While no differences were observed between LSP and HSP groups in calpain-1 at 1 d aging, the rate of pH decline and the activation of calpain-1 over d aging may explain the differences observed in intact desmin and degraded troponin-T after 1 d aging.

2D-DIGE Analysis

Both 2D-DIGE corresponding pH ranges identified a variety of proteins in the sarcoplasmic fraction related to glycolytic metabolism, other forms of energy metabolism, stress response, and regulatory proteins that differed in abundance between star probe groups. In the corresponding 3-10 pH range, of 444 spots, there was significance or a tendency for classification group to affect abundance of 23 spots. 7 of the most prevalent spots were chosen for identification (Figure 1 and Table 2). Spot 248 was also picked due to prevalence ($P=0.16$). In the refined pH range (4-7), of 462 spots, abundance of 32 spots were different or tended to be different due to classification group. The 13 most prevalent were chose for identification (Figure 2 and Table 2). Spots 272, 273, 371, and 409 were picked due to prevalence between experimental groups and previous identification in aged pork loins as potential biomarkers for star probe differences (Carlson et al. 2017b).

Glycolytic Metabolism

Several proteins spots were identified to be involved with glycolytic metabolism. These proteins included pyruvate kinase (Spot 248), triosephosphate isomerase (Spots 353 and 355), glyceraldehyde-3-phosphate dehydrogenase (Spots 305 and 314), and phosphoglycerate kinase 1 (Spot 332). Pyruvate kinase was numerically (84%) more abundant in the HSP samples, but not trending or significant (Figure 1; $P=0.16$). Two spots identified as triosephosphate isomerase (Spot 353 and 355) were trending to be more abundant (54% and 52%, respectively; Figure 1; $P=0.13$ for both spots) in the HSP group. This enzyme catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Alber et al. 1981). Triosephosphate isomerase has been identified as a potential biomarker for meat quality attributes including tenderness (Lametsch et al., 2003; Hwang et al., 2005; Carlson et al., 2017b) and drip loss in pork (Di Luca et al., 2013), as well as intramuscular fat deposition in beef (Kim et al. 2009). The results of the current experiments are consistent with Carlson et al. (2017b) demonstrating a greater abundance of triosephosphate isomerase in the less tender experimental group.

Two spots identified as glyceraldehyde-3-phosphate dehydrogenase (Spots 305 and 314) tended (Figure 1; $P= 0.11$ and 0.15, respectively) to be greater in abundance by 137% and 59% in the HSP group. Glyceraldehyde-3-phosphate dehydrogenase catalyzes the oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate (Seidler 2013). The only protein identified in the 4-7 pH range directly related to glycolytic energy production was phosphoglycerate kinase 1 (Spot 332; Figure 2). Phosphoglycerate kinase 1 was 36% more abundant in the HSP group ($P=0.04$). Phosphoglycerate kinase catalyzes the conversion of 1,3 bisphosphoglycerate to 3-phosphoglycerate through the transfer of a phosphoryl group (Ohlendieck 2010; Tymoczko et al., 2013). Combined, these proteins involved in glycolytic metabolism further identify the need to understand their role in postmortem metabolism, extent of impacting ultimate pH, and subsequent meat quality development. A greater understanding of the impact of different isoforms is also warranted for future research to enhance this understanding.

Other Energy Metabolism

Several other energy metabolism proteins were found to express differential abundance between experimental groups; creatine kinase M-type (Spot 272; Figure 1) as well as mitochondrial ATP synthase subunit beta (Spot 271; Figure 2) and mitochondrial isocitrate dehydrogenase subunit alpha (Spot 310; Figure 2). Creatine kinase M-type tended to be more abundant (64%; Figure 1; $P=0.12$) in the HSP group. Creatine kinase functions to reversibly catalyze the transfer of a phosphoryl group to ADP, through the phosphagen system, from phosphocreatine to produce ATP and creatine during postmortem anaerobic metabolism (Westerblad et al. 2010; Tymoczko et al. 2013). Mitochondrial isocitrate dehydrogenase subunit alpha, was 25% more abundant (Figure 2; $P=0.06$) in the HSP samples. Isocitrate dehydrogenase catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate in the citric acid cycle (Tymoczko et al., 2013). This is the rate-limiting step of the TCA cycle (Tymoczko et al., 2013).

In the refined pH range, 71% greater abundance (Figure 2; $P<0.01$) of mitochondrial ATP synthase subunit beta was observed in the HSP group. This protein is an enzyme functionally active in the production of ATP through the use of a proton gradient in the electron transport chain (Stock et al. 1999). Specifically, the beta subunit is the water soluble, catalytic domain. As demonstrated by Matarneh et al. (2018), this subunit in *in vitro* conditions is shown to extend ultimate pH decline when an inhibitor of ATP hydrolysis is present. Thus, a greater abundance of mitochondrial ATP synthase subunit beta in the sarcoplasmic fraction could help to explain why the HSP

samples may have had a lower ultimate pH. Combined, these proteins could identify different isoforms that could be impacting postmortem metabolism and the extent of postmortem glycolysis.

Other Proteins

Two proteins were identified in the corresponding 3-10 pH range, mitochondrial aldehyde dehydrogenase (Spot 246) and adenylate kinase isoenzyme 1 (Spot 244). Proteins found in experiment two included myc box-dependent-interacting protein 1 (Spot 214), mitochondrial dihydrolipoyl dehydrogenase (Spot 244), mitochondrial aldehyde dehydrogenase (Spot 264), Annexin A7 (Spot 272) and A5 (Spot 371), protein disulfide-isomerase (Spot 256), and mitochondrial isocitrate dehydrogenase subunit alpha (Spot 310). Spots of mitochondrial aldehyde dehydrogenase in both pH ranges demonstrated 114% and 32% greater abundance (Figure 1 & 2; P=0.14 and 0.02; respectively) in the HSP group. This enzyme functions to protect cells from oxidative stress by catalyzing the oxidation of acetaldehydes to acetate (Jelski and Szmitkowski 2008). The greater abundance of both protein spots could indicate cellular stress in the HSP group that may impact tenderness development.

Adenylate kinase is a phosphotransferase used to maintain homeostasis in the cell through the reversible reaction of ATP and AMP (adenosine monophosphate) to two molecules of ADP (Janssen et al., 2003; Dzeja and Terzic 2009). It was reported that the abundance of adenylate kinase increased over 24 h postmortem in beef longissimus thoracis muscle (Jia et al. 2007). However, the abundance of adenylate kinase was lesser in beef aged 7 d compared with beef aged 0 d (Oliveira et al., 2019). Laville et al. (2007) reported a greater abundance of adenylate kinase in the sarcoplasmic fraction of tough pork chops compared with tender pork chops (Laville et al., 2007). Comparable results in our study demonstrated a 108% greater abundance (P=0.08) of adenylate kinase (Spot 444) in the HSP group. This greater abundance of adenylate kinase could mean a greater need for ATP production in the HSP group. Many of these proteins originate in the mitochondria. Obtaining a greater understanding of what causes this location change and their impact on meat quality development is needed.

Stress Response Proteins

In the refined pH 4-7 range, 3 proteins were identified related to stress response; peroxiredoxin-2 (Fragment; Spot 409; Figure 2), Hsc 70 Interacting Protein (Spot 273), and heat shock cognate 71 kDa protein (Spot 161). Peroxiredoxin-2 was numerically (25%) more abundant (P=0.20) in the HSP group. To confirm the results from the 2D-DIGE experiment, peroxiredoxin-2 abundance was quantified using 1-dimensional western blots on the sarcoplasmic extracts at each d of aging. Peroxiredoxin is a family of peroxidases that protect against oxidative damage or are involved in signaling through regulation of hydrogen peroxide (Rhee, 2001). Specifically, peroxiredoxin-2 reduces the consequences of oxidative stress in cells by removing hydrogen peroxide produced from normal metabolism, influencing oxidative stress resistance (Rhee, 2001; Olahova, 2008). Carlson et. al (2017b) reported a greater abundance of peroxiredoxin-2 in HSP aged pork loins at 14 d aging compared with LSP pork loins, potentially impacted by oxidative stress early postmortem. In the current study, peroxiredoxin-2 abundance decreased between 1 and 8 d aging (Table 1; P<0.01) in LSP and did not change with further days aging. In HSP, peroxiredoxin-2 abundance decreased between 8 and 14 d aging (P<0.01). No differences between classification groups at 1 d aging were observed. The HSP group maintained a greater abundance of peroxiredoxin-2 at 8 and 21 d aging compared with the LSP group (P<0.01), potentially a response to oxidative stress. However, the role of peroxiredoxin-2 regarding meat tenderness remains unclear, warranting further investigation with oxidative stress and the impact on meat tenderness or proteolysis.

Heat shock cognate 71 kDa protein (Spot 161; P=0.09) and Hsc 70-interacting (Spot 273; Figure 2; P=0.20) were 14% more abundant in the sarcoplasmic fraction of the LSP group. The Hsc 70-interacting protein functions as a binder at the ATPase domains of at least two heat shock cognate 70 molecules for activation. Heat shock cognate 71 was more abundant in sarcoplasmic fraction of beef longissimus lumborum and triceps brachii after aging 7 d (Oliveira et al., 2019). Heat shock cognate 71 protein plays a crucial role in the initial folding of myosin as well as the assembly of myosin through its chaperoning function (Srikakulam 2004). Understanding the role of stress response proteins on postmortem meat is key to being able to predict tenderness variations.

Regulatory Proteins

The only regulatory protein identified in the sarcoplasmic protein fraction was myosin regulatory light chain 2, which was 46% more abundant (Spot 462; Figure 2; P=0.05) in the HSP group. Myosin light chains are important for regulation of muscle contraction (Weeds and Lowey 1971; Clark et al. 2002). The light chains may be phosphorylated, impacting the rate and extent of force being produced (Perrie et al., 1973; Sweeney et al., 1993). In beef bulls, the sarcoplasmic fraction of the LM was analyzed between tough and tender samples based on the 7 d shear force value (Bjarnadóttir et al., 2012). Three spots of myosin regulatory light chain 2 were found, two being more abundant in the tender group while the other was more abundant in the tough samples (Bjarnadóttir et al., 2012). A similar beef study analyzing the *longissimus* myofibrils of aged (36 h) samples identified 3 myosin light chain 2 proteins within bands as being negatively and 3 being positively associated with Warner-Bratzler shear force values at 36 h (Zapata et al., 2009). Myosin light chain 2 fragment abundance was found to be correlated to 1 and 4 d Warner-Bratzler shear force values (0.59 and 0.49, respectively) in pork *longissimus* muscle (Lametsch et al., 2003). The current results, in light of the published data, demonstrate that dynamic changes in myosin light chain 2 may be associated the development of tenderness early postmortem.

Table 1. Summary of effects of aging and post-aging freezing on pork loin chop (n=20) quality characteristics.

	Treatment									*P*-value		
	Fresh				Frozen							
Days Aged	1	8	14	21	1	8	14	21	SEM	Days Aged	Treatment	Days Aged* Treatment
SP (kg)[1]	8.44^{a}	6.58^{b}	6.33^{b}	6.54^{b}	7.92^{a}	6.22^{b}	6.30^{b}	5.82^{b}	0.37	<0.01	0.12	0.82
WBS (kg)[2]	5.62^{a}	3.83^{b}	3.62^{b}	3.31^{b}	6.19^{a}	3.36^{bc}	4.30^{b}	2.53^{c}	0.34	<0.01	0.94	0.06
Purge (%)[3]	0.15^{dx}	1.31^{cx}	2.23^{bx}	2.93^{a}	1.81^{cy}	2.56^{by}	3.83^{ay}	3.09^{b}	0.21	<0.01	<0.01	<0.01
pH[4]	5.88^{a}	5.82^{a}	5.89^{a}	5.86^{a}	5.82^{b}	5.90^{ab}	5.96^{a}	5.93^{a}	0.04	0.09	0.08	0.11
Color Score[5]	3.4^{a}	3.0^{b}	2.8^{bc}	2.7^{c}	3.1^{a}	2.8^{b}	2.7^{bc}	2.5^{c}	0.11	<0.01	0.01	0.89
Marbling Score[6]	1.9	2.0	1.9	2.2	2.2	2.3	2.2	2.2	0.17	0.73	0.05	0.82
Cook Loss (%)[7]	20.73^{a}	18.25^{b}	20.18^{ax}	19.62^{abx}	21.98^{a}	18.41^{b}	18.37^{by}	16.88^{by}	0.64	<0.01	0.06	<0.01
Hunter L[8]	43.88^{c}	48.62^{b}	48.83^{ab}	50.03^{a}	44.94^{c}	49.20^{b}	49.17^{b}	50.96^{a}	0.47	<0.01	0.03	0.87
Hunter a[8]	11.96^{bx}	13.66^{a}	13.92^{a}	13.73^{a}	13.13^{by}	13.86^{a}	13.78^{a}	14.00^{a}	0.18	<0.01	<0.01	<0.01
Hunter b[8]	2.49^{bx}	3.71^{a}	3.79^{ax}	3.60^{ax}	3.40^{cy}	3.86^{b}	4.29^{ay}	4.59^{ay}	0.13	<0.01	<0.01	<0.01
Hue Angle[9]	11.62^{bx}	15.14^{a}	15.21^{ax}	14.67^{ax}	14.46^{by}	15.55^{b}	17.29^{ay}	18.20^{ay}	0.59	<0.01	<0.01	0.02
Chroma[10]	12.27^{bx}	14.19^{a}	14.46^{a}	14.23^{ax}	13.60^{by}	14.42^{a}	14.47^{a}	14.77^{ay}	0.17	<0.01	<0.01	<0.01
Intact Desmin[11]	1.18^{a}	0.64^{b}	0.50^{c}	0.50^{c}	-	-	-	-	0.06	<0.01	-	-
Degraded Troponin-T[12]	0.00^{d}	0.40^{c}	0.74^{b}	1.08^{a}	-	-	-	-	0.09	<0.01	-	-
SL (µm)[13]	1.83	1.81	1.82	1.82	-	-	-	-	0.03	0.84	-	-

[a, b, c, d] Means with different superscripts within rows are significantly different within treatments (P<0.05).

[x, y] Means with different superscripts within rows and days aged are significantly different between treatments (P<0.05).

[1] A five-point Star Probe (SP) attachment fitted with an Instron was used to assess force needed to compress a chop to 20% of its original height (Carlson et al. 2017).

[2] Warner Bratzler Shear Force (WBS) attachment fitted with an Instron was used to assess force needed to shear through cored samples (Huff-Lonergan et al. 2002).

[3] Percent chop purge = (weight of package with purge- weight of package without purge/chop weight) x 100.

[4] pH measurements were taken at the center of each chop.

[5] National Pork Board standards, 6-point scale (1 = pale pinkish gray/white; 6 = dark purplish red).

[6] National Pork Board standards, 10-point scale (1 = 1% intramuscular fat; 10 = 10 % intramuscular fat).

[7] Chops were cooked to an internal temperature of 68˚C on clamshell grills. Percent cook loss= (raw chop weight – cooked chop weight)/raw chop weight] x 100.

[8] Hunter L, a, and b determined with Minolta Chroma Meter with D65 light source, 50 mm aperture, and 0˚ observer.

9 Hue angle values were calculated using the following equation: arctangent (b/a) (AMSA, 2012).

10 Chroma values were calculated using the following equation: $(a^2+b^2)^{1/2}$ (AMSA, 2012).

[11] Ratio of the densitometry units of the intact 55-kDa band of the sample compared to the 55-kDa band of the reference sample.

[12] Ratio of the densitometry units of the degraded 30-kDa band of the sample compared to the 30-kDa band of the reference sample.

[13] Sarcomere length (SL) was determined using helium-neon laser diffraction (Cross et al., 1981).

Table 2 Summary of fresh pork loin quality attributes, proteolysis of whole muscle protein fraction desmin and troponin-T, sarcoplasmic protein fraction calpain-1 autolysis and peroxiredoxin-2 in pork Longissimus dorsi (LM) of selected star probe groups.

Item	Low Star Probe Group (n=6)					High Star Probe Group (n=6)					*P*-Value		
Days Aged	**1**	**8**	**14**	**21**	**SEM**	**1**	**8**	**14**	**21**	**SEM**	**Days Aged**	**Category**	**Days Age *Categor**
Star Probe (kg)[1]	7.64^{ax}	5.50^{bx}	5.86^{bx}	5.72^{bx}	0.57	10.26^{ay}	8.59^{by}	8.30^{by}	8.76^{by}	0.60	<0.01	<0.01	0.90
Purge (%)[2]	0.14^{c}	1.32^{b}	1.83^{abx}	2.41^{ax}	0.35	0.16^{d}	1.64^{c}	3.27^{by}	4.54^{ay}	0.35	<0.01	<0.01	0.02
pH[3]	5.82^{abx}	5.79^{b}	5.86^{ax}	5.86^{ax}	0.02	5.76^{ay}	5.73^{a}	5.78^{ay}	5.76^{ay}	0.02	0.06	<0.01	0.80
Fabrication pH[4]	5.94	-	-	-	0.02	5.96	-	-	-	0.02	-	0.18	-
Color Score[5]	3.1	2.8	2.5	2.5	0.2	3.1	2.7	2.6	2.6	0.2	0.02	0.88	0.99
Marbling Score[6]	2.0^{bx}	2.3^{abx}	2.0^{bx}	2.5^{ax}	0.2	1.3^{y}	1.5^{y}	1.4^{y}	1.7^{y}	0.2	0.02	<0.01	0.86
Cook Loss (%)[7]	22.40^{a}	17.32^{cy}	20.47^{ab}	18.71^{bcy}	1.00	19.71	20.49^{y}	22.49	22.22^{y}	1.00	0.07	0.04	0.01
Hunter L value[8]	44.96^{b}	49.79^{a}	49.96^{a}	50.55^{a}	0.76	44.34^{b}	49.23^{a}	48.44^{a}	50.25^{a}	0.76	<0.01	0.17	0.87
Hunter a value[8]	11.81^{b}	13.91^{a}	13.87^{a}	13.79^{a}	0.26	11.97^{b}	13.19^{a}	13.93^{a}	13.58^{a}	0.26	<0.01	0.35	0.35
Hunter b value[8]	2.86^{b}	4.24^{a}	4.23^{a}	3.92^{a}	0.19	2.71^{b}	3.89^{a}	3.82^{a}	3.77^{a}	0.20	<0.01	0.04	0.82
Intact Desmin[9]	1.16^{a}	0.58^{b}	0.47^{bx}	0.38^{bx}	0.12	1.32^{a}	0.81^{b}	0.81^{by}	0.93^{by}	0.13	<0.01	<0.01	0.33
Degraded Troponin-T[10]	-	0.60^{cx}	1.04^{bx}	1.53^{ax}	0.13	-	0.05^{b}	0.33^{ab}	0.50^{a}	0.14	<0.01	<0.01	<0.01
Peroxiredoxin-2[11]	1.01^{a}	0.65^{bx}	0.73^{b}	0.58^{bx}	0.06	1.00^{a}	0.95^{aby}	0.78^{bc}	0.77^{cy}	0.06	<0.01	<0.01	0.06
Autolyzed Calpain-1 (%)[12]	53.67	-	-	-	9.14	57.83	-	-	-	9.14	-	0.75	-
Sarcomere Length, µm[13]	1.92^{ax}	1.89^{ax}	1.86^{ab}	1.89^{ax}	0.04	1.78^{by}	1.76^{by}	1.79^{b}	1.78^{by}	0.04	0.86	<0.01	0.77

[1] A five-point Star Probe attachment fitted with an Instron was used to assess force needed to compress a chop to 20% of its original height (Carlson et al. 2017).
[2] Percent chop purge = (weight of package with purge- weight of package without purge/chop weight) x 100.
[3] pH measurements were taken at the center of each chop.
[4] pH measurements taken at the commercial processing plant at approximately 20 hours postmortem.
[5] National Pork Board standards, 6-point scale (1 = pale pinkish gray/white; 6 = dark purplish red)
[6] National Pork Board standards, 10-point scale (1 = 1% intramuscular fat; 10 = 10 % intramuscular fat)
[7] Chops were cooked to an internal temperature of 68˚C on clamshell grills. Percent cook loss= [(raw chop weight – cooked chop weight)/raw chop weight] x 100.
[8] Hunter L, a, and b determined with Minolta Chroma Meter with D65 light source, 50 mm aperture, and 0˚ observer.
[9] Ratio of the densitometry units of the intact 55-kDa band of the sample compared to the 55-kDa band of the reference sample.
[10] Ratio of the densitometry units of the degraded 30-kDa band of the sample compared to the 30-kDa band of the reference sample.
[11] Ratio of the densitometry units of the intact 22-kDa band of the sample compared to the 22-kDa band of the reference sample.

[12] Percentage indicates the percent of autolyzed calpain-1 as a total of calpain-1 in each sample.
[13] Sarcomere length (SL) was determined using helium-neon laser diffraction (Cross et al., 1981).
[14 a, b, c, d] Means within rows and within days of aging with different superscripts are significantly different ($P<0.05$).
[14 x, y] Means within rows and day of aging with different superscripts are significantly different ($P<0.05$).

Table 3 Identified proteins of 2D-DIGE experiment 11 cm immobilized pH gradient strips, pH 3-10 and 4-7.

Spot Number	Immobilized pH gradient	Protein	Ratio[1]	*P*-value
246	3-10	Mitochondrial Aldehyde Dehydrogenase	-2.14	0.14
248	3-10	Pyruvate Kinase	-1.84	0.16
272	3-10	Creatine Kinase M-Type	-1.64	0.12
305	3-10	Glyceraldehyde-3-phosphate dehydrogenase	-2.37	0.11
314	3-10	Glyceraldehyde-3-phosphate dehydrogenase	-1.59	0.15
353	3-10	Triosephosphate Isomerase	-1.54	0.13
355	3-10	Triosephosphate Isomerase	-1.52	0.13
444	3-10	Adenylate Kinase Isoenzyme 1	-2.08	0.08
161	4-7	Heat Shock Cognate 71 kDa protein	1.14	0.09
214	4-7	Myc box-dependent-interacting protein 1	-1.23	0.07
244	4-7	Mitochondrial Dihydrolipoyl Dehydrogenase	-1.19	0.05
256	4-7	Protein Disulfide-Isomerase	-1.41	0.04
264	4-7	Mitochondrial Aldehyde Dehydrogenase	-1.32	0.02
271	4-7	Mitochondrial ATP Synthase Subunit Beta	-1.71	<0.01
272	4-7	Annexin A7	-1.33	0.15
273	4-7	Hsc 70-Interacting Protein	1.14	0.20
310	4-7	Mitochondrial Isocitrate dehydrogenase subunit alpha	-1.25	0.06
332	4-7	Phosphoglycerate Kinase 1	-1.36	0.04
371	4-7	Annexin A5	-1.20	0.11
409	4-7	Peroxiredoxin-2 (Fragment)	-1.25	0.20
462	4-7	Myosin Regulatory Light Chain 2 Isoform	-1.46	0.05

[1]Ratio indicates spot abundance differences between low and high star probe samples. Positive values represent more abundant in the low star probe group. Negative values represent less abundant in the low star probe group.

Discussion:

Consumers are willing to pay a premium for and place the greatest value on eating quality attributes of pork, specifically pork tenderness and flavor (Murphy et al., 2015; Lusk et al., 2018). However, predicting pork tenderness is difficult due to the large variety of factors that impact tenderness including pH decline (Melody et al., 2004; Boler et al., 2010), collagen content (Nishimura et al., 2009), lipid content (Lonergan et al., 2007; Wilson et al., 2017), and postmortem protein changes (Maddock-Carlin et al., 2006; Liu et al., 2016). The current study identified differences in the sarcoplasmic proteome 1 d postmortem to predict aged loin tenderness. Variations in star probe values were attributed to differences in pH, marbling, water holding capacity, proteolysis, and sarcoplasmic protein profile at 1 d aging. The HSP group had greater abundance of metabolic, regulatory, and mitochondrial associated proteins whereas the LSP group had greater abundance of stress response proteins. The sarcoplasmic proteome analysis results confirm a difference in glycolytic metabolism capabilities between star probe groups, thus demonstrating the need to investigate more deeply the role of metabolic and regulatory proteins in the development of pork tenderness. Identification of many mitochondrial proteins in the sarcoplasmic proteome may suggest solubilization of by-products of the mitochondrial electron transport chain (Matarneh et al., 2018), due to rupture of mitochondria. The rupture of mitochondria in muscle may increase the need for stress related proteins or need for antioxidant proteins. Once the status of these proteins is defined, robust protein biomarkers can identify products of differing tenderness.

Aging fresh pork loin for 8 d showed a 22% and 32% improvement in SP and WBS values, respectively. The current results suggest that aging 14 or 21 d did not result in improved SP or WBS values compared 8 d aging. Degradation of desmin and troponin-T were, in general, consistent with instrumental tenderness measurements across days aging, demonstrating that desmin degradation to 14 d and troponin-T degradation to 21 d are key components of fresh pork tenderness. Sarcomere length did not change during the postmortem aging process. Protein degradation data were numerically more highly correlated with instrumental tenderness measurements later postmortem when compared with sarcomere length, suggesting protein degradation may have a greater impact on tenderness values later postmortem. Furthermore, SP and WBS values are highly correlated instrumental tenderness measurements regardless of post-aging storage conditions used in this study. These results demonstrate that post-aging freezing did not have a significant impact on pork quality features of color and marbling score, cook loss, and instrumental tenderness measurements. Lastly, freezing pork at 1 d postmortem will not allow products to improve in SP or WBS values so a recommended best practice is aging pork prior to freezing.

The immediate application of this research is that fresh pork loin should be allowed to age before freezing in order to improve fresh pork quality. This might have immediate considerations for smaller processors. In addition, it is clear that pork aging must be considered as a source of variation in fresh pork loin quality. Immediate researchable questions surround the topic of the heretofore un-reported observation that mitochondrial disruption in post mortem or post rigor pork can be an indication of the progression of proteolysis and improvement in tenderness. The rupture of mitochondria in muscle may increase the need for stress related proteins or need for antioxidant proteins. Once the status of these proteins is defined, robust protein biomarkers can identify products of differing tenderness.

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