The impact of aging time, storage type and early postmortem sarcoplasmic proteome variations on pork loin quality

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The impact of aging time, storage type and early postmortem sarcoplasmic proteome variations on pork loin quality

by

Matthew David Schulte

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Meat Science

Program of Study Committee:
Steven M. Lonergan, Major Professor
Elisabeth Huff-Lonergan
John F. Patience
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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2018

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ABSTRACT

Consumers value and are willing to pay for consistently tender and juicy pork products. However, large variations in pork quality exist in the current marketplace. Predicting pork quality is difficult due to it being a multifactorial issue. To better understand the development of pork quality we performed two studies to analyze the impact of different storage and aging practices as well as variations in the sarcoplasmic proteome between tough and tender pork products. Finding robust and consistent measurements for determining differences in pork quality is essential to identifying variations in pork quality in the current marketplace.

The objective of the first experiment was to observe the aging response of fresh pork loins over 21 days (d) aging, determine the impact of post-aging freezing on pork quality attributes, and document the relationship between two instrumental tenderness (Star probe (SP) and Warner-Bratzler shear force (WBS)) measurements. It was hypothesized that 1) aging to 21 d would impact pork quality features, 2) post-aging freezing would decrease pork quality attributes, and 3) SP and WBS are highly related. Both loins from 20 carcasses were collected one day postmortem from carcasses of Duroc sired crossbred pigs at a commercial harvest facility. Eight loin chops (2.54 cm thick; longissimus muscle) were fabricated from each loin and vacuum packaged. Four chops from each carcass were aged for 1, 8, 14, and 21 d at 4° C and evaluated immediately (Fresh). Four chops adjacent to fresh chops were frozen (-29° C) post-aging for two weeks, thawed, and evaluated for quality attributes (Frozen). At completion of each treatment (Fresh or Frozen), adjacent pairs of chops were evaluated for purge, Hunter L, a, and b value, pH, color and marbling score, cook loss, and SP or WBS.
Desmin degradation was analyzed using whole muscle (myofibrillar and sarcoplasmic) protein extracts from fresh samples at each day of aging. Post-aging freezing had no significant impact on SP, WBS, pH, color score, marbling score, or Hunter L value at any aging period ($P>0.05$). Fresh chop purge increased at each d of aging ($P<0.01$). Greater purge at 1, 8, and 14 d aging resulted from post-aging freezing ($P<0.01$). Post-aging freezing chop purge was not different at 21 d aging compared with 8 d aging ($P>0.05$). Less cook loss was observed in post-aging freezing chops after 1 d aging ($P<0.01$) but was not different after 8 d aging when compared with 14 and 21 d ($P>0.05$). Post-aging freezing chop cook loss was less than fresh chop cook loss at 14 and 21 d aging ($P<0.05$). Chop Hunter L value increased ($P<0.01$) from 1 to 8 and 14 to 21 d aging regardless of treatment. Chop Hunter L value was not different at 8 and 14 d aging regardless of treatment ($P>0.05$). Chop a value was less ($P<0.01$) at 1 d aging than any other aging period in each treatments. Post-aging freezing resulted in greater chop a value at 1 d aging ($P<0.01$). Star probe value was correlated ($r= 0.85; P<0.01$) with WBS values across all aging timepoints and treatments. Intact desmin in whole muscle protein extracts decreased ($P<0.01$) between 1, 8, and 14 (1.17, 0.64, and 0.50 respectively) d aging but was not different from 14 to 21 d aging (0.50 and 0.52, respectively, $P>0.05$). The results demonstrate aging did not improve SP or WBS values after 8 d aging. This result is similar to the observed changes in desmin degradation. Instrumental tenderness measurements of SP and WBS are highly correlated. Post-aging freezing did not impact color, marbling, Hunter L, or instrumental tenderness. Freezing pork prior to aging will not allow significant improvement in SP or WBS values.
Experiment 2 used a subset of samples from experiment 1 to analyze the sarcoplasmic proteome between experimental groups differing extremely in SP value at 21 d aging. The objectives of this study were to determine the extent to which the sarcoplasmic proteome at 1 d postmortem can explain variations in aged pork quality. Twelve pork loins were categorized by differences in Star Probe (SP) (kg) measurements at 21 d postmortem. Pork loin quality attributes (purge, color, marbling score, SP and cook loss) were measured at 1, 8, 14, and 21 d postmortem. Whole muscle (myofibrillar and sarcoplasmic protein extract) desmin degradation and sarcoplasmic calpain-1 autolysis was determined using SDS-PAGE and Western blot techniques. Loins were sorted into two libraries of samples: Low SP group (LSP) (n=6) (SP<5.80 kg at 21 d postmortem), and High SP group (HSP) (n=6), (SP>7.00 kg at 21 d postmortem). Inclusion parameters of marbling score (1.0-3.0) and pH value (5.69-5.98) were set. Two-dimensional difference in gel electrophoresis (2D-DIGE) and mass spectrometry were used to determine sarcoplasmic proteome differences between experimental groups. The LSP group had a lower ($P<0.01$) SP value at each d of aging compared with the HSP group. Chop purge was lower ($P<0.01$) in the LSP group at 14 and 21 d aging but did not differ at 1 and 8 d aging ($P>0.05$) compared with the HSP group. The LSP group had a greater pH at each d of aging ($P<0.05$) compared with the HSP group. Marbling score was also greater in the LSP group compared with the HSP group at each d of aging ($P<0.05$). Cook loss was lower in the LSP group compared with the HSP group at 8 and 21 d aging ($P<0.05$) but did not differ between categories at 1 and 14 d aging ($P>0.05$). Desmin degradation was greater in the LSP chops compared with the HSP chops at 14 and 21 d aging ($P<0.05$). Color score, Hunter L, a, b, and calpain-1 autolysis were not
different between SP groups. Identified protein spots from 2D-DIGE determined that star probe groups had differing abundance of metabolic, mitochondrial associated and regulatory proteins in the sarcoplasmic fraction. The LSP had greater abundance of stress response proteins. Differences in pork quality and proteolysis were observed between experimental groups. Glycolytic, regulatory, and stress response proteins may be used as potential biomarkers at 1 d postmortem to predict aged pork loin quality differences. The results identify that not only proteolysis is important in the development of pork tenderness but also other protein changes, specifically in the sarcoplasmic fraction, have impacts on tenderness development. Further identification of the role of these biomarkers will assist with understanding the development of pork tenderness.
CHAPTER 1. GENERAL INTRODUCTION

Consumers are willing to pay for high quality pork cuts (Lusk et al. 2018) and value the eating quality of pork attributes including pork flavor and tenderness (Murphy et al. 2015). Tenderness is a fundamental part of pork quality (Moeller et al. 2010). Predicting differences in pork tenderness is very challenging due to the multiple factors impacting the development of tenderness. Large variations in pork quality are found in the current pork retail case (Bachmeier et al. 2016). Many attempts have been made to predict pork quality differences, but a robust system has not been defined due to the multifactorial and complexity of postmortem pork quality development. Consumers desire to purchase high quality pork products but the ability to predict pork quality differences is not currently available. One approach to accomplish this is through a greater understanding of how development of pork tenderness development occurs postmortem.

Tenderness development is influenced by a variety of factors including pH (Melody et al. 2004; Lonergan et al. 2007), collagen content (Wheeler et al. 2000; Nishimura et al. 2009), sarcomere length (Wheeler et al. 2000), and protein degradation (Huff-Lonergan et al. 1996; Wheeler et al. 2000; Melody et al. 2004; Carlson et al. 2017). Carlson et al. (2017b) demonstrated that desmin and peroxiredoxin-2 differed in abundance in the sarcoplasmic proteome of aged pork loins classified by star probe (SP) value, possibly explaining variations in SP values. It still remains undefined if these same differences in the sarcoplasmic proteome of early postmortem pork can be used to predict fresh pork loin quality. Identifying early postmortem tenderness biomarkers is critical for quality-based pork marketing.

The impact of proteolysis of myofibrillar, cytoskeletal, and intermediate filament proteins on tenderness development and aging has been well documented (Wheeler et al. 2000).
The benefit of extended aging periods has not been well documented.

Freezing is one of the oldest methods to increase the safety and shelf life of meat products but can negatively impact meat quality compared with meat that has never been subjected to freezing (Leygonie et al. 2012). Water loss (Vieira et al. 2009; Leygonie et al. 2012), protein denaturation (Añón and Calvelo 1980), and oxidation of lipids and proteins (Estévez 2011) may be impacted by the process of freezing and thawing. Studies have observed the impact of freezing and thawing across different periods of time (Kim et al. 2011, 2015, 2018; Leygonie et al. 2012; Coombs et al. 2017) identifying that aging prior to freezing will negate some of the negative impacts of freezing and thawing.

Star probe (SP) and Warner-Bratzler shear force (WBS) are two different methods used to measure instrumental tenderness. Studies have used both measurements separately to determine instrumental tenderness and the relationship of these values to trained sensory panels (Huff-Lonergan et al. 2002; Melody et al. 2004; Arkfeld et al. 2015; Richardson et al. 2017). Identifying the relationship of SP and WBS values in fresh and post-aging freezing pork loins will help to further define their relationship.

One objective of this experiment was to document the aging response of fresh pork over 21 d, determine the impact of post-aging freezing on pork loin quality attributes across aging periods, and determine the relationship between SP and WBS values under different storage conditions. It was hypothesized that extended aging would impact pork quality, SP and WBS are highly correlated, and post-aging freezing would negatively impact quality attributes compared to fresh product. Another objective of this study was to document protein profile differences of pork loins aged 1 d postmortem based on high star probe (HSP) and
low star probe (LSP) values at 21 days (d) postmortem. Results from these experiments will help to increase the understanding of factors influencing pork quality and assist with identifying robust, consistent biomarkers to predict aged pork loin quality variations.

First, a general understanding of the factors that influence pork quality development must be defined. This large variation of factors will help to define some differences observed in our experiments.

**Literature Cited**


Huff-Lonergan E, Mitsuhashi T, Beekman DD, et al (1996) Proteolysis of specific muscle structural proteins by mu-calpain at low pH and temperature is similar to degradation in postmortem bovine muscle. The online version of this article, along with updated information and services, is located on the World W. J Anim Sci 993–1008. doi: 10.2527/1996.745993x


CHAPTER 2. REVIEW OF LITERATURE

Introduction

Pork is the most consumed meat product worldwide. The eating quality and experience of these products is one of the most important purchasing decision factors for repeat purchase of pork products (Aaslyng et al. 2007). Consumers expect a consistently tender, juicy and flavorful product that is also a good value. This makes it imperative to improve efficiency of production as well as quality of the product. High quality pork is becoming increasingly important to the pork industry as demand for high quality products continues to increase (Murphy et al. 2015). In the current U.S. retail case, major variations in quality exist (Bachmeier et al. 2016) further complicating the ability to identify quality differences between pork products and assign proper value. Understanding how to differentiate low and high-quality pork products is essential for the future of the pork industry to meet consumer demands for high quality pork. Currently, no certified tender programs exist for pork products. Identifying robust and repeatable methods to predict low and high-quality pork products is needed for the future of the pork industry. One approach is to target protein biomarkers explaining these differences. Understanding muscle structure and metabolism and their role in the conversion of muscle to meat is essential to identifying robust biomarkers for pork quality. This review is focused on a greater understanding of muscle structure, function and how metabolic changes of muscle impact meat products.

Muscle Structure and Architecture

Muscle is a unique tissue that aids in many bodily functions and processes. Three distinct muscle types exist in mammals: skeletal muscle, cardiac muscle, and smooth muscle. These muscle types assist in a variety of functions including movement, blood flow,
metabolic activity, cardiac activity and structural stability. Smooth muscle is non-striated, single nucleated, and involuntary. Cardiac muscle is single nucleated, striated, and involuntary. Both skeletal muscle and cardiac muscle are striated in appearance; however, skeletal muscle is mostly voluntary and multinucleated compared to the involuntary, uninucleate features of cardiac muscle (Goll et al. 1984; Reece 2015). Meat is primarily composed of skeletal muscle which will be the focus of this literature review.

Skeletal muscle is a tissue comprised primarily of bundles of muscle fibers (cells) surrounded by layers of interconnected intramuscular connective tissue. Intramuscular connective tissue layers are composed of macromolecules of collagens, proteoglycans, and glycoproteins which make up the extracellular matrix (Voermans et al. 2008; Nishimura 2015). The extracellular matrix forms a network of connections to undertake and transmit muscle cell contractile forces (Voermans et al. 2008).

The muscle is surrounded by the thickest connective tissue layer termed the epimysium (Liu et al. 1994; Nishimura 2015). The epimysium is the layer where intermuscular fat is deposited (Goll et al. 1984; Liu et al. 1994; Nishimura 2015; Reece 2015).

The muscle is broken into a collection of muscle bundles, called fascicles, which are encapsulated by another connective tissue layer called the perimysium. The perimysium is a multi-layered, loose network of collagen fibrils (Nishimura et al. 2008; Nishimura 2015). Deposition of adipose tissue around this connective tissue layer, termed intramuscular fat, can have an influence on the palatability and tenderness of different whole muscle cuts due to disruption of muscle structural integrity (Goll et al. 1984; Nishimura et al. 1999, 2008; Nishimura 2015; Reece 2015).
The final connective tissue layer is the endomysium. It is a honeycomb structure that encases the individual muscle fibers (Goll et al. 1984; Nishimura et al. 2008; Nishimura 2015; Reece 2015). This network of connective tissue layers works cohesively to support the formation of and functional integrity of skeletal muscles. These different layers of connective tissue can play a key role in meat tenderness due to connective tissue variability between muscles.

Individual muscle cell diameter can range between 10 and 100 microns or larger (Huxley and Niedergerke 1958). These multinucleated muscle fibers are supported for growth, repair, and regeneration by satellite cells (Frontera and Ochala 2015). Variations in muscle fiber size can impact the textural integrity of meat. Muscles that perform larger movements have larger fibers which results in a coarser texture compared to muscles that perform smaller movements (Goll et al. 1984; Reece 2015).

Muscle fibers are surrounded by a cellular membrane called the sarcolemma (Reece 2015). This approximately 70-100 nm thick membrane plays a major role in transmitting signals such as action potentials throughout the entire muscle fiber (Goll et al. 1984; Reece 2015). Transverse tubules serve as an extension of the sarcolemma into the muscle fiber to aide in transfer of action potentials for contraction and relaxation (Peachey 1965). The sarcolemma and transverse tubules work in conjunction to assist in muscles function by turning a chemical signal into a mechanical action.

Integrated with the sarcolemma, through invaginations of the transverse tubules, the sarcoplasmic reticulum encases individual myofibrils. The sarcoplasmic reticulum plays a key role in both contraction and relaxation as a storage location for calcium, which is needed for muscle contraction. The sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA)
and calsequestrin serve as proteins within the sarcoplasmic reticulum which play different roles in maintaining calcium homeostasis (Frontera and Ochala 2015). Calsequestrin loosely binds to calcium within the sarcoplasmic reticulum while the SERCA pump returns calcium to the sarcoplasmic reticulum after muscle contraction and relaxation (Frontera and Ochala 2015).

Myofibrils are responsible for the contractile functions of muscle fibers. Unlike many other organelles, myofibrils are not surrounded by a membrane due to their insolubility at the cell’s normal ionic strength (Goll et al. 1984; Reece 2015). Myofibrils make up a large portion of a muscle fiber's total volume: approximately 80-87% (Goll et al. 1984; Reece 2015). The striated appearance of myofibrils is due to alternating light and dark bands present in skeletal muscle cells.

The repeating contractile unit of a myofibril is the sarcomere. The sarcomere is the smallest contractile unit of striated muscle. A sarcomere is made of a large array of proteins in a very organized structure (Ertbjerg and Puolanne 2017). This precise structure allows for proper alignment of filaments for contraction.

An individual sarcomere spans from Z-line to Z-line, which is approximately 2.5-2.8 µm in length (Cross et al. 1981). The Z-line plays a critical role as an anchoring location for thin filaments along with titin and nebulin (Ertbjerg and Puolanne 2017). The less dense band, called the I-band, is mostly made of actin (Hanson and Huxley 1953; Clark et al. 2002). The I-band is approximately 1 µm long. The more dense band, termed the A-band, is primarily composed of myosin (Hanson and Huxley 1953; Clark et al. 2002). This darker band is approximately 1.5 µm in length and can contain thin filaments during contraction.
(Huxley and Hanson 1954; Clark et al. 2002). A single A-band and two halves of separate I-bands combine to constitute the length of the sarcomere.

The I-band is intersected by the Z-line. Each sarcomere is formed with one full A-band and two halves of an I-band. Centered in the middle of the A-band lies the M-line. The M-line plays a key role in packing thick filaments (Knappeis and Carlsen 1968) and linking individual thick filaments (Agarkova and Perriard 2005).

Two additional proteins lie in line with the thick and thin filaments, the first being titin, the largest currently identified protein. Titin spans half of a sarcomeres length going from Z-line to M-line. This semi-elastic protein aides in maintaining sarcomeric structure and muscle elasticity (Clark et al. 2002). Nebulin aligns with the thin filament and plays a key role in both contraction stability and governing thin filament length (Littlefield and Fowler 1998).

**Proteins in Muscle**

Muscle proteins are classified based on their solubility in aqueous solutions. Three categories exist: sarcoplasmic, myofibrillar (or contractile) and stromal proteins. Sarcoplasmic proteins are soluble in water, while myofibrillar proteins are soluble when extracted from the myofibril, and stromal proteins are not soluble in neutral aqueous solutions (Goll et al. 1984). The structural arrangement and functional properties of these proteins plays a significant role in both living muscle and meat.

Actin, the primary protein in the thin filament, is found in a G-actin (Globular) or F-actin (Filamentous) form. G-actin is a monomeric structure that can polymerize into F-actin. Two twisted alpha helices of F-actin comprises most of the thin filament (Holmes et al. 1990;
Pollard 1990, 2016; Clark et al. 2002; Sweeney and Hammers 2018). Six thin filaments surround a single thick filament in a hexagonally arranged structure to assist with cross bridge formation during contraction (Hanson and Huxley 1953; Huxley 1969). The thin filament extends from the Z-line to the A-band and is primarily located in the I-band. However, it can be found in portions of the A-band depending on the contractile state of the muscle (Hanson and Huxley 1953). The thin filament is anchored at the Z-line by actin binding proteins such as filamin, α-actinin and Cap Z (Clark et al. 2002). The troponin complex and tropomyosin are closely associated with actin within the thin filament to assist with contraction (Clark et al. 2002; Sweeney and Hammers 2018).

Several hundred myosin molecules make up the majority of the thick filament (Au 2004). The thick filament is surrounded by six thin filaments to assist with the completion of contraction (Huxley 1969; Weeds and Lowey 1971). Myosin is the molecular motor powering contraction (Clark et al. 2002; Au 2004). Myosin is a 520 kDa protein composed of two heavy chains (~220 kDa) and 4 light chains (~20 kDa) making up the head and tail regions of myosin (Lowey et al. 1969; Weeds and Lowey 1971; Clark et al. 2002). This molecule contains two heads per molecule (Huxley 1969; Weeds and Lowey 1971). Each myosin head has an ATP and actin binding site that allows myosin to interact with actin in the presence of ATP, along with the conformational change to tropomyosin in elevated calcium levels. (10⁻⁵ M), allowing access to the actin binding site (Huxley 1969; Prochniewicz et al. 2004). The light chains can be separated into two pairs; the first pair being part of the essential light chain family and the second pair being part of the regulatory light chain family (Weeds and Lowey 1971; Clark et al. 2002; Au 2004). These light chain pairs are essential for ATPase activity (Weeds and Lowey 1971). The regulatory light chains
can be phosphorylated impacting their ability to bind calcium and thus modulating the ATPase cycle (Perrie et al. 1973; Sweeney and Hammers 2018). Many myosin heavy chain (MHC) and myosin light chain (MLC) isoforms exist to accommodate for the diverse range of physical activity rates and time periods needed for differing muscles. These different isoforms are critical to differences in muscle fiber type formation and function (Weeds and Lowey 1971; Perrie et al. 1973; Bowker et al. 2004b; Choi et al. 2007).

Troponin is a key contraction regulatory protein. It is located on the thin filament with close association to actin and tropomyosin (Clark et al. 2002). Troponin is a complex of three subunits (troponin-C, troponin-I, and troponin-T) with individual and unique roles in the contraction process (Greaser and Gergely 1971; Weber and Murray 1973; Zot and Potter 1987; Clark et al. 2002). At low calcium concentrations \(10^{-7}\) M (Huxley 1969), muscle remains in the relaxed state because tropomyosin blocks myosin binding sites under that condition. When calcium concentrations increase \(10^{-5}\) M(Huxley 1969), troponin-C binds calcium causing a conformational change in troponin-I, resulting in the movement of troponin-T (Blumenschein et al. 2005). This series of events causes the movement of tropomyosin off myosin binding sites on the thin filament allowing for interaction between actin and myosin molecules (Blumenschein et al. 2005).

Tropomyosin is the physical regulatory component of actin and myosin crossbridge formation (McKillop and Geeves 1993). Tropomyosin is mainly negatively charged and lies in the positively charged groove of actin (von der Ecken et al. 2015; Henderson et al. 2017). This specific location covers myosin-binding sites on actin. Tropomyosin is composed of seven pseudo repeating units which span over seven actin monomers (von der Ecken et al. 2015; Henderson et al. 2017). The troponin complex also lies in close association with
tropomyosin (Clark et al. 2002; von der Ecken et al. 2015). While at low calcium concentrations \((10^{-7} \text{ M})\) (Huxley 1969), muscle remains in the relaxed state as tropomyosin continues to block myosin binding sites. Increased calcium concentrations cause a conformation change in the troponin complex, thus moving tropomyosin off of myosin binding sites (Huxley 1969; Chandy et al. 1999). However, tropomyosin can appear in three different conformational states on the thin filament allowing no interaction, full interaction or partial interaction with the thick filament (McKillop and Geeves 1993).

Titin is the largest identified protein \((3,000 \text{ kDa})\) found in nature (Wang et al. 1979; Clark et al. 2002). Titin spans half the length of a sarcomere. The N-terminus is anchored within the Z-line; the C-terminus is integrated into the M-line (Clark et al. 2002; Tskhovrebova and Trinick 2010). Overlap of titin at the M-line allows for continuation of its filament through sarcomeres. Within the I-band, titin has elastic properties that allow for governing of myofibril flexibility (Maruyama et al. 1977; Clark et al. 2002; Gautel and Djinovic-Carugo 2016). Additionally, titin plays a key role in thick filament formation and connecting the thick filament to the Z-line (Clark et al. 2002; Au et al. 2004; Tskhovrebova and Trinick 2010).

Nebulin is a large protein \((600-900 \text{ kDa})\) associated with the thin filament (Horowits et al. 1986; Wright et al. 1993; Clark et al. 2002). Nebulin plays a role in the assembly and organization of as well as a ruler for the length of the thin filament (Labeit et al. 1991; Clark et al. 2002). Nebulin is closely associated with actin, troponin and tropomyosin along the thin filament (Labeit et al. 1991). The N terminal region of nebulin interacts with tropomodulin and other capping proteins to regulate the thin filament length (McElhinny et al. 2001, 2003). The C terminal region of nebulin extends into and is anchored at the Z-line (Wright et al.
Nebulin also assists with regulation of contraction, transduction of signals, and linking myofibrils to intermediate filament proteins (McElhinny et al. 2003; Tskhovrebova and Trinick 2017).

Desmin is an intermediate filament protein that integrates myofibrils at the Z-line with surrounding myofibrils, organelles and the sarcolemma within muscle (Lazarides and Hubbard 1976; Clark et al. 2002; Bär et al. 2004; Baron et al. 2004). Desmin also plays a key role in structural integrity and alignment of mature myofibrils (Clark et al. 2002). Several different proteases can degrade desmin. Intact desmin (55 kDa) can be degraded by calpain-1, which occurs in the head and tail regions, and when incubated with cathepsin B, has shown cleavage of peptides but no specific desmin fragments (Baron et al. 2004).

**Skeletal Muscle Contraction**

Contraction and relaxation of living muscle tissue is a highly organized process involving the release and reuptake of calcium ions as well as the hydrolysis of adenosine triphosphate (ATP) for completion. The contraction process is completed by the interaction between the thick and thin filaments within a sarcomere after an electrical signal is received from the brain. This electrical signal is transmitted through muscle fibers by the overlying basement membrane, the sarcolemma, and transverse tubules (Peachey 1965). A triad is formed through the connection of one transverse tubule with two sarcoplasmic reticuli (Porter and Palade 1957). Transverse tubules convert this electrical signal to a chemical signal within the sarcoplasmic reticulum causing a cascade release of calcium. Depolarization of the transverse tubules (Porter and Palade 1957) results in this calcium release into the sarcoplasm from the sarcoplasmic reticulum (Spudich and Watt 1971; Lai et al. 1988). Calcium release is initiated through the ryanodine receptor (Lai et al. 1988). These
sequential events result in the interaction of proteins to complete contraction in living muscle tissue.

Several hundred myosin molecules make up the majority of the thick filament (Au 2004). This protein plays a key role in contraction (Clark et al. 2002; Au 2004). This 520 kDa protein is composed of two heavy chains (~220 kDa) and 4 light chains (~20 kDa) making up the head and tail regions of myosin (Lowey et al. 1969; Weeds and Lowey 1971; Clark et al. 2002). This molecule contains two heads per molecule (Weeds and Lowey 1971). The light chains can be broken into two pairs; the first pair being part of the essential light chain family and the second pair being part of the regulatory light chain family (Weeds and Lowey 1971; Clark et al. 2002; Au 2004; Adkins et al. 2017). These light chain pairs are essential for ATPase activity (Weeds and Lowey 1971). The regulatory light chains can be phosphorylated and thus regulating the ATPase cycle (Perrie et al. 1973; Sweeney and Hammers 2018). Each myosin head has an ATP and actin binding site that allows myosin to interact with actin in the presence of ATP along with elevated calcium levels. (10^{-5} \text{ M}) (Huxley 1969; Prochniewicz et al. 2004). Myosin bound ATP is hydrolyzed to ADP and phosphate. The ATP hydrolysis positions myosin heads to interact with actin creating cross bridges between actin and myosin on thin and thick filaments, respectively. Myosin heads interact in nonsynchronous motions to exert a power stroke which pulls actin filaments towards the center of the sarcomere causing a shortening of the sarcomere (Rayment and Holden 1993; Houdusse and Sweeney 2016; Sweeney and Hammers 2018). Adenosine diphosphate and phosphate produced from hydrolysis of ATP are released from myosin (Irving et al. 1992; Clark et al. 2002). If ATP is present, it will bind to myosin heads and actomyosin crossbridge will be dissociated allowing a return to the relaxed state. When ATP
production ceases and ATP stores are completely depleted, permanent cross bridges will be formed resulting in what is known as rigor mortis.

**Muscle Fiber Type**

Muscle fiber types of individual muscles can vary greatly between animals within a single species as well as across species. These variations in fiber type are due to adaptations of different activities within a muscle. Muscle fibers are classified based on their metabolic capacity, rate of contraction, and fatigue rate. Generally classified as three fiber types exist within muscle: Red (slow twitch; type I), white (fast twitch, type II), and intermediate (characteristics between red and white fibers) (Brooke and Kaiser 1970; Peter et al. 1972; Reece 2015). Animals may have a greater predominance of one fiber type due to differences in physical activity and function, but most skeletal muscle has a mixture of all three types (Close 1972).

Red, slow twitch fibers play an integral role in activities that require endurance. This sustained function is maintained by greater mitochondria concentrations and increased oxidative metabolism capacity compared to white fibers (Bottinelli R 2000; Schiaffino and Reggiani 2011). Increased oxidative metabolism capacity results in increased need for muscle capillary density and size (Schiaffino and Reggiani 2011). The deeper red pigmentation seen in red fibers can be explained by a greater myoglobin content in these fibers. Once again, the increased need for oxygen for metabolism increases the need for myoglobin to transport oxygen to the mitochondria present in this fiber type. Sarcoplasmic Endoplasmic Reticulum Calcium (SERCA) pump isoforms also play a role in the fatigue rate of differing fiber types. The less dense and abundant isoform, SERCA2a, is present in red fibers (Lytton et al. 1992; Allen et al. 2008).
White, fast twitch fibers have more rapid contraction times with greater myosin ATPase activity. ATPase activity provides the energy which is needed for muscle contraction; white fibers have greater ATPase activity resulting in more rapid contraction rates (Barany 1967; Bowker et al. 2004a, b). This fiber type also has a well-established glycolytic metabolism system and increased glycolytic activity (Choe et al. 2008; Schiaffino and Reggiani 2011). This metabolic system requires glycogen for anaerobic respiration (Bottinelli R 2000; Schiaffino and Reggiani 2011). The appearance of these muscle fibers is observed due to the lower myoglobin concentration. Due to the increased need of calcium for more rapid contraction, a greater density of SERCA pumps and quicker uptake of calcium is present in fast twitch fibers (Everts et al. 1989). This SERCA pump isoform (SERCA1a) is also denser than the red fiber isoform (Lytton et al. 1992; Allen et al. 2008).

Differences in fiber type metabolic activity and contraction speed can be classified by differences in myosin heavy chain (MHC) isoforms present within the muscle. Being a major structural component of myosin and the thick filament, MHCs play a key role in contraction speed and attachment to actin and the thin filament. Each isoform has differing ATPase activity levels thus classifying individual fibers based on their contractile and metabolic properties (Choi et al. 2007). The rate of loss of activity postmortem of isoforms is dependent on time, pH and calcium concentrations (Bowker et al. 2004a, b). There are over ten different MHC isoforms identified with four being present in mammalian skeletal muscle: type I, IIa, IIx, and IIb (Schiaffino and Reggiani 2011). These heavy chain isoforms can be grouped based on contractile speed as well as metabolism ability. Red, slow twitch fibers contain mainly types I and IIa while white fibers contain predominately types IIx and I Ib.
Muscle Metabolism

Adenosine triphosphate (ATP) is the primary energy source for muscle contraction and relaxation processes in living muscle. Adenosine triphosphate also plays a significant role during the conversion of muscle to meat. Due to the relatively low ATP concentrations within muscle (8-15 µmol/g of muscle; Goll et al. 1984), aerobic and anaerobic energy production pathways must be used to produce the needed ATP for muscle functions such as contraction and relaxation. Glycolysis can be completed during both aerobic and anaerobic conditions through differing pathways. For anaerobic metabolism, phosphocreatine is degraded and glycogen is broken down to produce ATP (Westerblad et al. 2010). Creatine kinase catalyzes the reversible reaction between phosphocreatine and adenosine diphosphate (ADP). The products of this reaction are creatine and ATP (Goll et al. 1984; Westerblad et al. 2010). During periods of reduced ATP use, rephosphorylation of creatine replenishes the diminished stores of phosphocreatine. A separate reaction that assists with maintaining cellular ATP at a constant level results is the conversion of two ADP to ATP and AMP which is catalyzed by myokinase (Goll et al. 1984; Scheffler and Gerrard 2007).

These reactions work together to produce the high energy needs for muscle function. Glycolysis is essential for muscle function when the consumption of ATP is greater than the production of energy for biological function. Through glycolysis and its pathway of reactions, one molecule of glucose is converted to two molecules of pyruvate while also generating energy molecules as ATP (Tymoczko et al. 2013). Glucose is stored in muscle as a very large, branched polymer known as glycogen. The breakdown of glycogen to glucose monomers is the starting point of the glycolytic pathway and production of three ATP molecules. During anaerobic conditions, pyruvate formed from the breakdown of glucose is
used generate lactate and hydrogen atoms used for ATP generation (Tymoczko et al. 2013).

Muscle glycolytic pathway steps are described below:

1. Glycogen within living muscle is broken down into glucose 1-phosphate by the cooperation of glycogen phosphorylase and glycogen debranching enzyme.

2. Glucose 1-phosphate is converted to glucose 6-phosphate by the shift of a phosphoryl group catalyzed by phosphoglucomutase.

3. Glucose 6-phosphate is converted to fructose 6-phosphate through isomerization by phosphoglucone isomerase.

4. Fructose 6-phosphate is phosphorylated by ATP and phosphofructose to fructose 1,6-bisphosphate.

5. Fructose 1,6-bisphosphate is split into two triosephosphates that are isomers to each other (glyceraldehyde 3-phosphate and dihydroxyacetone phosphate), a reversible reaction catalyzed by aldolase.

6. Glyceraldehyde 3-phosphate is converted to 1,3-bisphosphoglycerate by an oxidation-reduction reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase.

7. 1,3-bisphosphoglycerate is converted to 3-phosphoglycerate through the transfer of a phosphoryl group. This reaction is catalyzed by phosphoglycerate kinase.

8. 3-phosphoglycerate is converted to 2-phosphoglycerate by the shift of a phosphoryl group by phosphoglycerate mutase.

9. 2-phosphoglycerate is then transformed into phosphoenolpyruvate through the dehydration reaction being catalyzed by enolase.

10. Phosphoenolpyruvate is converted to pyruvate through the transfer of a phosphoryl group by pyruvate kinase.
It must be kept in mind that steps 5 through 9 are completed in duplicate due to two triosephosphates being produced from a single fructose 1,6-bisphosphate (Tymoczko et al. 2013).

The aerobic process of cellular respiration is the most efficient energy cycle for ATP production, producing over 90% of the ATP required for daily function (Tymoczko et al. 2013). Pyruvate from glycolysis is unable to enter this energy cycle until decarboxylation by pyruvate dehydrogenase to form acetyl CoA. This molecule can then enter the mitochondria to start cellular respiration, also known as the citric acid cycle. The citric acid cycle only functions in aerobic conditions netting 36 total ATP from a single glucose molecule. The Citric acid cycle steps are described below:

1. Acetyl CoA and oxaloacetate condensate to produce citrate under the catalyzing effect of citrate synthase.
2. Citrate is then isomerized to isocitrate. This step is catalyzed by aconitase resulting in the interchange of a hydrogen and alcohol group.
3. Alpha-ketoglutarate is formed by the oxidative decarboxylation of isocitrate, which is catalyzed by isocitrate dehydrogenase.
4. Another oxidative decarboxylation reaction occurs on alpha-ketoglutarate to form succinyl CoA. This reaction is catalyzed by alpha-ketoglutarate dehydrogenase.
5. The thioester bond of succinyl CoA is cleaved forming succinate. This reaction results in the phosphorylation of an ADP molecule. This reaction is catalyzed by the enzyme, succinyl CoA synthetase.
6. Succinate is oxidized by the catalyst, succinate dehydrogenase to form fumarate.
7. L-malate is formed through the hydration of fumarate by the catalyzing ability of fumarase.

8. Finally, L-malate is oxidized to oxaloacetate by the catalytic abilities of malate dehydrogenase.

Through these different metabolism pathways, muscle is able to meet its energetic needs and properly function. These same processes have a significant impact on the conversion of muscle to meat postmortem.

**Conversion of Muscle to Meat**

Many biochemical and structural changes occur during the conversion of muscle to meat. Immediately following exsanguination, postmortem homeostatic conditions are lost, and a large array of metabolic, energetic and structural attributes change prior to completion of rigor mortis. Rigor mortis occurs in four separate phases: delay, onset, completion and resolution of rigor. Post exsanguination, the loss of homeostasis occurs, and the animal strives to maintain homeostatic conditions through utilization of ATP. During the first phase of rigor mortis, ATP is present in sufficient concentrations for myofibrils to contract as calcium is released from the sarcoplasmic reticulum. Creatine phosphate stores are used to achieve the energy demands of the muscle for the contraction process. The muscle is relatively extensible and elastic at this stage. However, once ATP supplies and oxygen concentrations decrease the muscle must switch over from aerobic ATP production to anaerobic ATP production. This change in energy production marks the beginning of the conversion of muscle to meat and is termed the delay phase of rigor.

The onset of rigor, the second phase of the conversion of muscle to meat typically occurs in less than 3 hours in pork *longissimus* muscle (Briskey et al. 1966). Due to
anaerobic energy production being the source of ATP production, lactate and hydrogen ions accumulate from glycolysis due to the muscle trying to clear pyruvate buildup by lactate dehydrogenase (Huckabee 1958). Due to the body’s inability to effectively eliminate these products, a gradual pH decline is observed within the muscle overtime. The typical pH of living muscle is 7.4 and will decline to approximately 5.7 due to this accumulation (Bate-Smith and Bendall 1946; Briskey and Wismer-Pedersen 1961). During this phase of ATP depletion, actin and myosin bonds are formed similar to the regular contraction process but are unable to release due to decreasing presence of ATP (Bate-Smith and Bendall 1946). The muscle becomes relatively less extensible and elastic compared to its prior state. Once all ATP has depleted from the muscle, it enters the third stage of rigor mortis, the completion of rigor. At the completion of rigor, permeant, irreversible actomyosin cross-bridges form and some shortening of sarcomeres occurs (Wheeler and Koohmaraie 1994; Honikel 2014). This results in meat which is the least tender and extensible.

The final phase of rigor mortis, resolution, is the point at which enzymes cause softening of the muscle tissue due to protein degradation and proteolysis. The release of actomyosin cross-bridges accounts for physical changes occurring during the conversion of muscle to meat but the majority of this change in softening and extensibility is due to protein degradation.

Many factors can influence the rate and extent of pH decline. Rapid pH decline or very low ultimate pH (<5.3) can be a result of the quality defect termed PSE (pale, soft and exudative) (Briskey and Wismer-Pedersen 1961). Increased incidences of PSE pork can occur due to halothane genotype, pre-slaughter handling, and stunning method resulting in increased rates of pH decline, lesser water holding capacity and greater purge loss (Channon
et al. 2000). Pigs heterozygous for the halothane gene are associated with leaner, faster growing pigs with a greater feed conversion compared to pigs without this gene (Sather and Murray 1989). Preharvest stress can influence the rate of pH decline and the resulting quality defects of PSE or DFD (dark, firm and dry) in pork. Induced psychological and physical stressors prior to harvest can result in lower glycogen levels due to the animal directing glycogen stores to cope with stressor resulting in increased pH decline rates. Prolonged antemortem stress will result in meat that is DFD, which will have a pH which does not decrease as much, thus a greater ability to retain water (Monin and Santé-Lhoutellier 2014). However, the color will be much darker (Warriss and Brown 1993; Adzitey and Nurul 2011).

The rate of pH decline will increase when higher chilling temperatures are used (Briskey and Wismer-Pedersen 1961; Kim et al. 2014). Additionally, increased muscle temperature prior to harvest also has a significant impact on the rate of pH decline and ultimately meat quality (Rosenvold and Andersen 2003). All of these factors of the conversion of muscle to meat can have a significant impact on fresh meat quality attributes.

Oxidation of proteins can occur in pre-rigor and early post-rigor muscle and this can adversely impact the proteolytic abilities of proteases, specifically the calpain proteolytic system (Martinaud et al. 1997; Harris et al. 2001; Rowe et al. 2004). Calpain oxidation is dependent on pH. Calpastatins ability to inhibit calpain-1 and calpain-2 is not affected by pH or oxidative conditions (Geesink and Koohmaraie 1999; Carlin et al. 2006). Carlin et al. (2006) demonstrated that inhibition of calpain-1 by calpastatin at pH 6.5 and 7.0 (165 and 295 mM NaCl) was decreased while in the presence of H₂O₂. Stronger inhibition of calpain-1 was obtained when 100 µM of hydrogen peroxide were added to calpain compared with calpain-1 without hydrogen peroxide (Guttmann et al. 1997). When looking at a pH more
closely associated with meat (pH 6.0), higher ionic strength (295 mM NaCl) conditions demonstrated similar results to high pH inhibition results (Carlin et al. 2006). This demonstrates the impact of high ionic strength conditions on calpain-1 activity in muscle during the conversion of muscle to meat. However, similar results were not seen at lower ionic strength conditions (165 mM NaCl) (Carlin et al. 2006). Oxidation of calpain-1 and calpain-2 decreased proteolytic activity (Guttmann et al. 1997; Guttmann and Johnson 1998; Carlin et al. 2006). Calpain activity and calpastatins ability to inhibit the calpains can be dramatically impacted by pH, ionic strength, and oxidation (Carlin et al. 2006) Contrary to calpain results, when oxidized, calpastatin activity is not impacted (Carlin et al. 2006). Clearly, changes in oxidative conditions early postmortem can greatly impact the activity of the calpains and their impact on the development of postmortem tenderization of meat through aging.

Many changes occur immediately postmortem influencing the protein profile of meat. Proteomic studies have been implemented to track and report these changes during the conversion of muscle to meat. Proteomics, the study of proteins and their function, has aided meat scientist in discovering proteome changes that occur from the start of the conversion of muscle to meat; to the end of the aging process. A large array of proteomic analysis technologies exist (Lippolis and Reinhardt 2008). Many studies have been conducted to understand the mechanisms that control postmortem muscle changes ultimately impacting meat quality such as tenderness and color (Lametsch and Bendixen 2001; Lametsch et al. 2003; Jia et al. 2006; Suman et al. 2007; Joseph et al. 2015; Carlson et al. 2017a). Proteomic approaches are also used to identify differences of meat quality defects (Schilling et al. 2017).
Lametsch and Bendixen (2001) identified the 15 most notable changes of the proteome in porcine *Longissimus dorsi* muscle using two-dimensional electrophoresis at 0, 4, 8, 24, and 48 hours postmortem. Their results identified 18 spots which originated from 9 different proteins. Proteins identified included several structural proteins (actin, myosin heavy chain, and troponin-T) and metabolic proteins (glycogen phosphorylase, creatine kinase, phosphopyruvate hydratase, myokinase, pyruvate kinase, and dihydrolipoamide succinyl transferase) (Lametsch et al. 2002). Some of these resulting spots were degradation products of these proteins. These results identify how muscle structure and metabolism change throughout the conversion of muscle to meat can impact meat quality, postmortem aging, and subsequent proteolysis.

Investigating antemortem muscle changes to postmortem meat is essential to understanding biochemical changes that occur postmortem. In bovine muscle, Jia et al. (2006) analyzed the *longissimus thoracis* muscle using two-dimensional gel electrophoresis (pH 4-7) and mass spectroscopy to identify changes in metabolic proteins from live muscle to postmortem samples. 15 different proteins were identified related to metabolism, response to stress and structure (Jia et al. 2006). These results identified shifts in energy metabolism from living muscle to meat specifically related to glycolytic pathways and the TCA cycle (Jia et al. 2006). This was shown by increased abundance of the following enzymes; phosphoglycerate kinase, enolase, aldehyde dehydrogenase, succinyl-CoA synthetase, isocitrate dehydrogenase, and 3-hydroxyisobutyrate dehydrogenase (Jia et al. 2006). From these identified enzymes, these results signify the need to further investigate the role of these metabolic proteins in the development of differences in meat quality.
Fresh Meat Quality

Color

Consumers use the external appearance of meat products to indicate the freshness and wholesomeness of that product (Mancini and Hunt 2005). The degree of red pigmentation of a meat product is primarily determined by the abundance of and the chemical state of myoglobin, the main pigment in meat from skeletal muscle (Hector et al. 1992). Hemoglobin and cytochromes do contribute to meat color as well, however, at much lesser value than myoglobin (Mancini and Hunt 2005). Myoglobin consists of a heme (porphyrin ring plus iron) with six coordination sites that are located in the hydrophobic region of a globin protein. The first four coordination sites are bound to nitrogen, the fifth site is bound by histidine-93, and the final site is available to readily bind oxygen, water, or carbon monoxide (Møller and Skibsted 2006).

Cattle have the darkest colored lean followed by lamb, pig, and poultry in decreasing order of myoglobin concentrations. Variations also occur with age as increasing myoglobin concentrations occur with greater chronological age due to the decrease in affinity of oxygen for myoglobin (Møller and Skibsted 2006; Jeong et al. 2014). Sex of animals can also influence myoglobin concentration and the color of meat. Intact males are shown to have a great concentration of myoglobin and darker lean color than their female and nonintact counterparts (Seideman et al. 1984). Significant differences in myoglobin concentrations and color stability between several different breeds of cattle (King et al. 2010). Myoglobin concentration is highly heritable and can greatly affect meat color based on genetic selection (Jeong et al. 2014). Lastly, red muscle fibers contain more mitochondria for metabolic needs
thus needing a greater amount of myoglobin for oxygen transport resulting in a deeper red pigment.

Because color influences purchasing decisions and because color specifications are set by buyers of pork, variations of color in fresh meat must be analyzed carefully. Two common methods are used to evaluate color, the first being the standardized 6-point scale developed by the National Pork Board (National Pork Board. 2000). This scale evaluates color based on a score of 1 being a pale pigment to a 6 representing a dark purplish red pigment. The second method is the use of colorimeters and spectrophotometers as an objective measurement tool to assess the reflectance of fresh meat products. This measurement method can vary in type of instrument used, color system, illuminants, aperture size, and observer angle which should be taken into consideration for research projects (Jeong et al. 2014). Both methods can obtain valuable information about color measurements but use of one method over another should be dependent upon the goals of the proposed project.

It is currently unknown how to predict meat color changes and what biomarkers could be used to predict changes in fresh meat color across aging times.

**Lipid Content**

Consumer purchasing intent and demands must be considered for fresh pork quality. Visual analysis of fresh products can significantly impact consumer purchasing decisions. In a study evaluating 142 consumers purchasing intent of raw loin chops based on visual appraisal, the results demonstrated that consumers chose to purchase leaner chops compared to highly marbled chops, however, through in home sensory analysis, participants determined
that greater intramuscular fat in chops were more tender, juicy and flavorful compared with the leaner chops (Brewer et al. 2001). This demonstrates that consumer purchasing intent based on visual appraisal is inconsistent with their overall eating experience.

Selection for leaner, more efficient pigs continues to occur in the pork industry due to producer genetic selection and consumer demands (Lonergan et al. 2001). The impacts of lesser lipid content on pork quality must continue to be considered. Lipid content has the potential to impact meat quality factors such as tenderness. Total lipid content of meat from pigs divergently selected for greater feed efficiency and increased carcass composition resulted in a reduction in total intramuscular lipid (1.14% lipid) content in the loin compared with their less efficient counterparts (1.67% lipid) (Smith et al. 2011). Similar results were demonstrated on pigs selected for greater feed efficiency containing 1.30% lipid content in the loin compared with their less efficient counterparts exhibiting 1.70% lipid (Arkfeld et al. 2015). These results demonstrate that genetic selection for greater feed efficiency can negatively impact lipid content (Lonergan et al. 2001).

Breed can also significantly influence intramuscular fat deposition. As greater selection for the Duroc breed has increased in commercial settings, results have shown that Duroc pigs have an increased intramuscular fat content when compared to other breeds (Channon et al. 2004; Zhang et al. 2007). Duroc pigs exhibited significantly greater intramuscular fat content compared to Chester White, Berkshire, Poland China, Spotted, Yorkshire, Landrace, and Hampshire (Zhang et al. 2007). Duroc female and male pigs (100%) showed significantly greater intramuscular fat (1.84 and 1.00%, respectively) compared to 50% Duroc/Large White crosses (50/50%) and Duroc (0%) (Large White) pigs (Channon et al. 2004). In the same study, 100% Duroc pigs showed more juicy loin chops
from female carcasses (Channon et al. 2004). Wood et al. (2004) demonstrated that Duroc and Berkshire breeds showed greater intramuscular fat in the longissimus dorsi and psoas major compared with Large White and Tamworth breeds. The sensory results from the same study showed that breeds with a more intramuscular fat in the longissimus dorsi produced loin chops with greater sensory scores for tenderness, juiciness, and overall liking.

The evidence in the literature demonstrates that the effect of lipid content can be variable on fresh pork sensory quality. Several studies have shown minor contributions of increased lipid content to sensory quality (Fernandez et al. 1999; Channon et al. 2004) while others suggest a threshold must be met to ensure acceptable quality (Fortin et al. 2005; Muringai et al. 2017). Others have found that lipid content has an impact within pH ranges. Within intermediate pH ranges (5.50-5.80) increased lipid content showed decreased chewiness scores assessed by a trained sensory panel and decreased star probe values (kg) (Lonergan et al. 2007). However, differences in lipid content in extreme pH ranges will not consistently improve quality characteristics. In the same study, high pH pork (pH>5.80) demonstrated superior sensory quality, texture and cook loss compared to low pH pork (pH<5.50) regardless of lipid content (Lonergan et al. 2007).

More recently, Wilson et al. (2017) classified loins based on marbling and color score as low-, medium-, or high-quality to determine the predictive ability of both quality traits to sensory quality. Classification based on color and marbling alone were not predictive of sensory quality. When classification based on both traits showed that the lower quality chops were perceived to have more intense pork flavor than the high-quality chops (Wilson et al. 2017). Similarly, when genetics, pH, management, and day of slaughter were controlled, extractable lipid did not correlate with sensory characteristics (Rincker et al. 2008). In both
studies, tenderness values were unaffected by lipid content suggesting that a specific amount of lipid is needed to influence tenderness. This also means other factors are influencing fresh pork sensory quality.

Lipid content has been variable in the literature demonstrating its impact on tenderness, meat quality, and sensory quality. As selection for leaner growth continues, it must be better understood how lower lipid content can impact meat quality and sensory quality factors.

**Collagen Content**

Collagen is the most abundant protein in the body. This triple helical protein is found within the three differing connective layers surrounding muscle; the epimysium, perimysium, and endomysium (Nishimura et al. 1994; Nishimura 2015). Typically, we remove and do not consume the epimysium, the outermost layer of connective tissue. The main connective tissue layer that contributes to variations in tenderness of meat is the perimysium due to the fact that it comprises approximately 90% of the intramuscular connective tissue layers (McCormick 1999). Some collagen is heat soluble and the solubility of this protein changes with chronological age (Hill 1966). Collagen that is not readily solubilized through cooking will add to the background toughness of meat. As animals increase in chronological age, cross-linking of collagen increases and imparts increased mechanical strength to the muscle (McCormick 1994). The triple helical arrangement of collagen forms covalent crosslinks through the condensation of hydroxyproline and hydroxylysine residues and their aldehydes; forming sheets of crosslinks (McCormick 1994). With increases in cross-linking, the heat solubility of collagen decreases (Tanzer and Kefalides 1973; Lepetit 2007). Greater abundance of collagen is found in muscles that are more heavily used compared to muscles
that are used less, such as the psoas major (Nishimura et al. 2009). Nishimura et al. (2009) reported that the total amount of collagen (r=0.717) shear force value of the intramuscular connective tissue (r=0.857), and thickness of the secondary perimysium (r=0.750) were highly and significantly correlated with the shear force value of the *psoas major, longissimus dorsi, semimembranosus, semitendinosus, biceps femoris, and triceps brachii* of raw pork muscles. In a study looking at fresh, uncooked pork muscles (Nishimura et al. 2009), the total amount of collagen (r= 0.717), shear force value of intramuscular connective tissue (r=0.857), and thickness of the secondary perimysium (r= 0.750) of several different pork muscles were significantly correlated with shear-force values. Collagen content also varies significantly between differing muscles (Wheeler et al. 2000). Through postmortem aging, collagen strength decreases linearly after approximately 10 days of aging (Judge and Aberle 1982; Nishimura et al. 1998; Nishimura 2015) demonstrating that postmortem changes are occurring in collagen. Through the combination of total collagen content, heat solubility, and cross-linking, collagen can have a major impact on meat tenderness.

A better understanding of how collagen cross-linking can impact tenderness would assist with fully evaluating how to minimize the negative impacts of background toughening imparted by collagen.

**Sarcomere Length**

Many physical changes are occurring during the conversion of muscle to meat, one of those being muscle contraction and subsequent myofibril shortening. This shortening causes decreases in tenderness of whole muscle products. Several factors can influence the rate and extent of this shortening. Temperature can have a significant impact on sarcomere length if carcasses are chilled before the onset of rigor mortis. The resulting phenomenon is known as
cold shortening (Savell 2005). This shortening results in significant increases in toughness of the muscle. Feldhusen (1992) demonstrated that pork *longissimus dorsi* chilled in ultra-rapid conditions (-5 degrees Celsius) prior to rigor resulted in shortened sarcomere lengths by 33.5% compared to control samples (Feldhusen and Kühne 1992). The shortened sarcomeres (<1.85 µm) also showed significant correlations with Warner-Bratzler shear force values (*r*= -0.76) and ultimately tougher chops with a greater amount of sarcomere shortening. It has also been demonstrated that sarcomere length varies greatly between pork muscles (Wheeler et al. 2000). In this study they demonstrated that 40 percent of the variation in tenderness seen in all muscles combined (*Biceps femoris, Longissimus, Semimembranosus, Semitendinosus*, and *Triceps brachii*) was explained by sarcomere length (Wheeler et al. 2000). Sarcomere length across all muscles has shown to be significantly correlated to tenderness (0.64), total connective tissue (0.62), and juiciness (0.64) demonstrating its impact on tenderness differences between muscles. With these differences in mind, it must be kept in mind that sarcomere length can vary among muscles and impact the overall quality development of meat products.

**Water Holding Capacity**

Water holding capacity of fresh meat is its ability to bind and hold water. Water comprises approximately 75% of muscle and increases economic value of meat with increased water holding capacity. The water holding capacity of meat is important both economically and for product quality. Water holding capacity can also affect the desirability of meat products by influencing the juiciness, appearance, firmness, and tenderness of that product.
Drip loss is a measurement of fresh meats ability to hold water throughout aging. Drip loss is the difference in weight of a meat product at two separate time points. This loss of weight typically includes mostly water, being that muscle is comprised of approximately 75% water, but must be noted that sarcoplasmic proteins (water soluble) and degradation products of myofibrillar proteins are also found in this fluid. This fluid is cellular or sarcoplasmic fluid. This fluid is red or pink in color due to the sarcoplasmic protein, myoglobin, being lost from the muscle.

The rate and extent of pH decline are instrumental to the water holding capacity of pork. Early postmortem changes in pH can greatly influence the extent of drip loss due to the dipolar characteristics of water (Melody et al. 2004). During the most extreme changes in pH, for example PSE meat, proteins can become denatured and their ability to bind water will decrease greatly. In contrast, meat that maintains a high pH, for example DFD meat, manages to keep a greater ability to retain water due to its pH being closer to living physiological levels.

Contraction of the sarcomere, specifically rigor shortening, is another facet to explain differences in drip loss. It has been estimated that approximately 85% of the water in muscle is held in the myofibrils (Offer and Cousins 1992). During postmortem contraction and rigor onset, muscles that have greater shortening of sarcomeres resulted in greater drip loss (Honikel et al. 1986).

It is also well documented that protein degradation can play a significant role in determining water holding capacity (Kristensen and Purslow 2001; Melody et al. 2004; Zhang et al. 2006). Specifically, degradation of cytoskeletal proteins that link muscle cells together, such as desmin, has been significantly correlated to increased water holding
capacity (Melody et al. 2004; Zhang et al. 2006). However, Zhang (2006) showed that degradation of integrin, a membrane-associated protein that plays an adhesion role for the extracellular matrix, resulted in lower water holding capacity. Difference in water holding capacity show that degradation of different proteins can result in different changes to the muscle structure and the available space for fluid retention.

Several biomarkers for increased tenderness have been identified. However, it is still uncertain how these biomarkers impact this quality factor and how they may be measured at line speeds in the industry.

**Postmortem Proteolysis**

Following rigor development, most pork goes through an aging period under refrigerated conditions. Through aging pork undergoes chemical changes and reactions impacting the taste, texture and aroma of the product. Aging has shown to have a significant impact on the tenderness of pork (Channon et al. 2004; Melody et al. 2004).

Changes in the meat proteome during postmortem aging can influence many pork quality factors such as water holding capacity and tenderness. Di Luca et al. (2013) examined exudate of pork *longissimus* muscle using 2-dimensional Difference in Gel Electrophoresis (2D-DIGE), mass spectrometry and Western blot analysis. 2D-DIGE uses protein labeling techniques to compare spots separated by both isoelectric point (pH 4-7) and molecular weight to identify proteins significantly different between treatments. This within gel analysis method was used to identify proteins differently expressed between samples expressing high (6%), intermediate (4%), or low (2.5%) drip loss through postmortem aging (Di Luca et al. 2013). Results of this analysis identified differences in abundance of proteins
with biological processes related to structure, metabolism, response to stress, and transporter function (Di Luca et al. 2013). Between extremes in drip loss, high drip loss samples expressed significantly less abundance of triosephosphate isomerase, creatine kinase M-type, serum albumin, and transferrin (Di Luca et al. 2013). In contrast, the high drip loss group expressed significantly greater abundance of β tropomyosin. When comparing the high to intermediate drip loss group, lesser abundance of annexin A7, titin, heat shock protein 70, and heat shock cognate 71 were found in the high drip loss group (Di Luca et al. 2013). Results from this study demonstrated significantly lower abundance of stress-induced phosphoprotein, β tropomyosin, and filaggrin 2 in low drip loss samples compared with intermediate drip loss samples (Di Luca et al. 2013). These differences in protein abundance, specifically heat stress related proteins, may be related to localization within the nucleus or myofibrillar proteins. These stress related proteins may also defer protein modifications. Lastly, this study suggested triosephosphate isomerase and transferrin as potential biomarkers for predictive drip loss at 1 d postmortem (Di Luca et al. 2013). These results may be indicative of differences in protein degradation and differences in muscle response to hypoxic conditions.

Proteolysis, or protein degradation, has a significant impact on the aging of meat. Proteolysis is the breakdown of proteins to peptides or amino acids. Proteolysis of many structural proteins including desmin, titin, and nebulin have been observed through aging (Wheeler and Koohmaraie 1994; Taylor et al. 1995; Carlson et al. 2017b). Degradation of some myofibrillar proteins, like troponin-T, are clear indicators of postmortem proteolysis. Proteolysis of these different proteins is accomplished by the enzymatic function of endogenous proteases found in muscle.
Degradation of specific proteins has shown to account for variations in tenderness. In pork *semimembranosus* and *biceps femoris* muscles, desmin degradation was associated with 26 and 38% of the variation in tenderness value of these muscles (Wheeler et al. 2000). In another study examining influences on tenderness, Melody et al. examined desmin, nebulin, and titin degradation three different muscles (*longissimus dorsi*, *semimembranosus*, and *psoas major*) at 45 min, 6, 24, 48, and 120 h postmortem. The *longissimus dorsi* demonstrated greater desmin, nebulin, and troponin-T degradation at 48 and 120 h postmortem than the *semimembranosus* indicating a possible explanation for differences in Warner-Bratzler shear force values at 48 and 120 h postmortem (Melody et al. 2004). The *psoas major* demonstrated a more rapid rate of pH decline demonstrating earlier calpain-1 activation and autolysis compared with the other two muscles (Melody et al. 2004). These early postmortem changes resulted in earlier degradation of titin and desmin, and a faster appearance of calpain-1 bound to myofibrils showing the impact of pH on proteolysis (Melody et al. 2004).

A more recent study analyzing differences in aged pork loin tenderness values, with constrained variations in ultimate pH and color, examined protein degradation of desmin, filamin, titin, and troponin-T (Carlson et al. 2017b). Results indicated significantly less intact troponin-T, filamin, and desmin (Carlson et al. 2017b). Intact titin was also visible in some of the less tender samples but was not found in the more tender samples (Carlson et al. 2017b). These results demonstrate that postmortem proteolysis can vary greatly regardless of the pH.

The calpain system is the primary protease system and is essential to degrade cytoskeletal and sarcomeric proteins. Muscle specifically contains calpain-1, calpain-2, calpain-3 and their endogenous inhibitor, calpastatin (Goll et al. 1998; Koohmaraie and Geesink 2006).
Calpain-1 and calpain-2 are calcium dependent cysteine proteases primarily associated with postmortem protein degradation of proteins (Busch et al. 1972; Geesink et al. 2006). Calpain-2 requires millimolar calcium levels to become activated while calpain-1 requires micromolar calcium levels (Goll et al. 1998). Due to this greater need for calcium to be active, calpain-1 is the primary contributor to postmortem proteolysis of muscle proteins early postmortem (Geesink et al. 2006; Koohmaraie and Geesink 2006) whereas calpain-2 has been shown to be responsible for tenderization of beef muscle aged for longer periods of time (Phelps et al. 2016; Colle and Doumit 2017).

Calpain-1 and calpain-2 are heterodimers composed of two dynamic subunits, an 80-kDa catalytic subunit and a 28-kDa subunit (Dayton et al. 1976; Koohmaraie and Geesink 2006). The 80 kDa subunit is the catalytic subunit used for the process of protein degradation in postmortem muscle. This subunit is composed of 4 domains (Domain I, II, III, IV) (Suzuki 1990; Goll et al. 2003; Huff-Lonergan et al. 2010). The first domain contains the N-terminal end of the protein. Domain II contains the cysteine and histidine residues found in all cysteine proteases making it the catalytic domain. Domain III and IV have sequences for predicting E-F hand binding cites of calcium (Suzuki 1990; Goll et al. 2003; Huff-Lonergan et al. 2010).

Different calcium concentrations are needed for the proteolytic activity of both calpain-1 (3-5 μM calcium concentration for half-maximal activity) and calpain-2 (400-800 μM calcium concentration for half-maximal activity) (Kubota et al. 1981; Suzuki et al. 1981; Goll et al. 2003). Calcium is also required for the inhibitory function of calpastatin on the calpains. Following rigor, calcium enters the sarcoplasm of muscle fibers activating the calpains by binding to them. Incubation with calcium will cause the calpains to autolyze.
Autolysis of calpain-1 reduces its mass from 80 kDa to 78 or 76 kDa while calpain-2 autolyses to a mass of 78 kDa due to the removal of the N-terminal of the protein and other amino acids (Dayton 1982; Goll et al. 2003; Li et al. 2004). Through autolysis, the calcium concentration needed for half maximal activity is reduced (Saido et al. 1994; Goll et al. 2003; Huff-Lonergan et al. 2010).

Autolysis products of calpain play a significant role in the proteolytic activity of the calpains. Following degradation, these degradation products form dimers that have no proteolytic activity which could explain why autolysis can occur but proteolysis may not (Li et al. 2004). Many biological factors can influence the rate of autolysis, playing a significant role in the rate of proteolysis.

Muscle pH, oxidative conditions, and phosphorylation state of the calpains and calpastatin can significantly impact their function. Early postmortem pH and rate of pH decline can influence calpain activity and the rate of autolysis (Melody et al. 2004; Maddock et al. 2005; Carlin et al. 2006; Bee et al. 2007; Pomponio et al. 2010). Through in vitro analysis, calpain-1 activity was the greatest at pH 6.5 rather than pH 7.5 or 6.0. Calpain-2 activity was greatest at pH 7.5. Additionally, the inhibition ability of calpastatin on the calpains seemed unaffected by pH under non-oxidized conditions (Maddock et al. 2005; Carlin et al. 2006). Pomponio et al. (2010) and Bee et al. (2006) observed that an increased rate of pH decline resulted in decreased calpain-1 activity due to an increased rate of autolysis and loss of function. Melody et al. (2004) demonstrated that the Psoas major had the greatest rate of pH decline and calpain-1 autolysis within 45 minutes postmortem compared to the Longissimus dorsi and Semimembranosus, which showed a slower rate of pH decline and no calpain-1 autolysis (Melody et al. 2004).
Carlin et al. (2006) used hydrogen peroxide to induce oxidation of purified calpains resulting in decreased activity. Oxidation of the calpains resulted in decreased degradation of desmin in myofibrils (Carlin et al. 2006). An *in vitro* study examining the specific site of oxidation of calpain-1 showed that a disulfide bond is formed between Cys115 and Cys108 of the active site (Lametsch et al. 2008) explaining why oxidation inhibits calpain-1 activity. Calpain-2 was not impacted because it does not have a nearby SH group. Interestingly, oxidation in the presence of both calpain-1 and calpastatin resulted in an increase in desmin degradation. This observation can be explained by a complex forming between the protease and its inhibitor (Carlin et al. 2006).

Another significant player in regulation of calpain and calpastatin activity is nitric oxide (NO). Nitric oxide is an endogenous free radical molecule that could regulate many muscular functions including muscle contraction and energy metabolism (Stamler and Meissner 2001). Nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to L-citrulline generating nitric oxide. Neuronal nitric oxide synthase is the primary isoform found in skeletal muscle (Samengo et al. 2012). Neuronal nitric oxide synthase activity was greater and lasted for a longer duration of time in the *seminembranosus* compared to the *longissimus dorsi* and *psoas major* demonstrating differences in activity levels within differing fiber types (Liu et al. 2014). This decreased intensity is thought to be due to degradation by calpain. Neuronal nitric oxide synthase has also been shown to inhibit calpain-1 autolysis and decrease protein degradation (Li et al. 2014; Liu et al. 2016; Zhang et al. 2018). Liu et al. (2016) identified 4 cysteine residues in the catalytic subunit, and one peptide from the small subunit that were identified as being nitrosylated. Specifically, site 49, 351, 384, and 592 in the catalytic subunit and site 142 in the small subunit of calpain-1 were nitrosylated (Liu et
al. 2016). Even under nitrosylated conditions, some proteolysis occurred indicating autolysis of calpain-1 is not fully inhibited but is only partially blocked. The role of nitric oxide in regulating calpain activity and proteolysis exemplifies the importance of nitrosylation to protease function.

Many proteins degraded by calpain-1 are important for muscle structure and integrity. Degradation analysis of aged and unaged myofibrils that were incubated with calpain-1 demonstrated degradation of cytoskeletal proteins including desmin, filamin, nebulin, titin, and troponin-T (Huff-Lonergan et al. 1996). Lametsch et al. (2004) showed consistent results in porcine myofibrils incubated with calpain-1. Actin, desmin, myosin heavy chain, myosin light chain I, troponin-T, and tropomyosin isoforms were shown to be degraded by \textit{in vitro} calpain-1 (Lametsch et al. 2004). These observations provide further evidence that calpain-1 plays a significant role in postmortem proteolysis and aging of meat.

Other proteases can also impact protein degradation. Intact desmin (55-kDa) is degraded by calpain-1 in the head and tail regions. When desmin is incubated with cathepsin B, cleavage of peptides occurs but no specific desmin fragments are formed (Baron et al. 2004).

Use of proteomic technologies has been implemented in varied species to identify biomarkers for tenderness. Variations in beef tenderness have been analyzed in the sarcoplastic fraction of the \textit{longissimus thoracis} (Jia et al. 2009) and \textit{longissimus dorsi} (Anderson et al. 2012). Jia et al. (2009) examined the sarcoplastic protein profile using two-dimensional gel electrophoresis (pH 5-8) and mass spectroscopy to identify biomarkers of tenderness between tough and tender samples based on Warner-Bratzler shear force values. 7 total spots were identified to be significantly different between tender and tough samples.
Malate dehydrogenase, peroxiredoxin-6, and myosin light chain 1 were more abundant in the more tender samples while histidine triad nucleotide-binding protein 1, stress-70 protein, UPF0366 protein C11orf67 homolog and protein DJ-1 were more abundant in the tough samples (Jia et al. 2009). Peroxiredoxin-6 was further investigated between the tough and tender samples identifying oxidative differences between the samples indicating differences in oxidative stress and its potential role as a biomarker for meat tenderness (Jia et al. 2009).

In a similar study, Anderson et al. (2012) also used 2D-DIGE to compare the sarcoplasmic protein profile of beef *longissimus dorsi* muscle to identify potential proteins related to tenderness. Through mass spectroscopy, seven different proteins were identified as being significantly ($P<0.10$) different in abundance between groups (Anderson et al. 2012). These proteins included phosphoglucomutase 1, myosin light chain 1, actin, tropomyosin alpha-1 chain, myomesin-2, and KBTBD10 protein which were all found to be more abundant in the low star probe group (Anderson et al. 2012). However, the most alkaline isoform of phosphoglucomutase-1 was found to be significantly more abundant in the high star probe group (Anderson et al. 2012). Additionally, these results are consistent with Jia et al. (2009) demonstrating greater abundance of myosin light chain 1 in the more tender samples and its potential role as a biomarker for tenderness (Anderson et al. 2012). These results identify that changes in phosphorylation change can significantly impact protein functionality.

In a study analyzing changes of the whole muscle (myofibrillar and sarcoplasmic) proteome of porcine muscle from harvest to 3 days postmortem (using 2-dimensional electrophoresis (pH 4-7) and MALDI-TOF MS) identified 27 changes related to Warner-Bratzler shear force. 11 changes were identified as actin fragments along with other myofibril
proteins including myosin heavy and light chain, Cap Z and titin (Lametsch et al. 2003). Sarcoplasmic protein changes included the following proteins: enolase 1, phosphoglycerate kinase, enolase 3, pyruvate dehydrogenase, 2 spots of glycogen phosphorylase, triosephosphate isomerase, myokinase and eIF-5a (Lametsch et al. 2003). Of these proteins, actin and myosin heavy chain degradation was significantly correlated to tenderness. Additionally, myosin light chain II and triosephosphate isomerase I were significantly correlated to tenderness (Lametsch et al. 2003). These results indicative the possible role of fiber type and differences in glycolytic metabolism rate in the aging process of pork.

In another study analyzing variations in pork tenderness of aged loins (11-16 days) with high and low star probe values were compared to identify potential biomarkers of pork loin tenderness using the sarcoplasmic protein profile. Carlson et al. (2017) used 2D-DIGE analysis (pH 4-7) and mass spectroscopy on aged pork loins (11-16 days) sarcoplasmic extracts to identify proteins significantly different between high and low star probe samples not significantly different in pH, lipid or color characteristics (Carlson et al. 2017a). The results identified 14 different proteins that were significantly (P<0.10) between star probe groups. More tender samples expressed greater abundance of desmin (Carlson et al. 2017a). In contrast, less tender samples expressed greater abundance of mitochondrial aldehyde dehydrogenase, ATP synthase subunit β, pyruvate kinase, creatine kinase M-type, glycerol-3-phosphate dehydrogenase, cytoplasmic malate dehydrogenase, tropomyosin alpha-1 chain, peroxiredoxin-2 and -6, triosephosphate isomerase, protein deglycase DJ-1, myosin light chain 1, and eukaryotic translation initiation factor 5A-1 (Carlson et al. 2017a). These results identify that the high star probe group had greater abundance of metabolic, stress response, and regulatory proteins while the low star probe group showed a greater abundance of
desmin, a structural protein. From these results, desmin and peroxiredoxin-2 may be potential biomarkers located in the sarcoplasmic protein profile of pork loin (Carlson et al. 2017a).

A large array of factors can influence postmortem proteolysis and tenderness through the aging of pork products. A greater understanding of the proteins and postmortem events that impact these factors will allow us to predict quality differences based on the protein profile of pork. Many different proteomic technologies are used to identify these potential biomarkers; 2D electrophoresis, 2D DIGE, mass spectroscopy, SDS PAGE, and western blots to name a few. Through the use of proteomic technologies, we can identify consistent, robust biomarkers that can be used to determine differences in quality of tough and tender pork products. This will ultimately allow the industry to clarify pork quality differences for marketing of high quality pork. However, it must be apparent that these biomarkers must be identifiable, measurable early postmortem, and measurable at production speeds of the industry. Currently it is unknown why extreme variations in proteolysis occur. Changes during the conversion of muscle to meat may be impacting this and must be identified to obtain a greater understanding of why these extreme variations in proteolysis and tenderness exist.

**Post-Harvest Processing Interventions:**

Many meat products are frozen post-aging to maintain meat quality and improve shelf life. However, freezing can result in quality deterioration caused by ice crystal formation, ultimately impacting tissue structure through mechanical damage and denaturation of proteins (Jeong et al. 2011). Jeong et al. (2011) demonstrated that meat subjected to multiple freeze-thaw cycles can damage muscle fibers. From this same study, it was demonstrated that greater myoglobin oxidation occurred and could be explained by reduced metmyoglobin (the
oxidized form of myoglobin) reductase activity due to the freeze-thaw process (Jeong et al. 2011).

In an extended aging and freezing study, beef *longissimus dorsi* muscle was frozen after 0, 20 or 40 days of aging. The 0 days aged sample was frozen for 90 days, the 20 day aged sample was frozen for 70 days, and the 40 day aged sample was frozen for 50 days (Kim et al. 2011a). Their results identified that aging prior to freezing significantly increased sensory tenderness and instrumental tenderness values (Kim et al. 2011a).

Kim et al. (2010) conducted a similar study looking at lamb *longissimus dorsi* aged for 0, 2, 3, or 9 weeks at -1.5° C prior to freezing for 9, 7, 6, or 0 weeks at -18° C, respectively. Chops (6-cm thick) were removed for each aging by freezing period (Kim et al. 2011b). Their results determined aging prior to freezing improved instrumental tenderness and color stability (Kim et al. 2011b). These results are consistent with Kim et al. (2011) emphasizing that products not aged before freezing will not improve in tenderness.

Freezing prior to aging can also significantly impact meat quality attributes. In a study looking at the sequence of freezing and aging, pork loin sections frozen, thawed, and then aged demonstrated the greatest purge loss compared with chops that were aged, frozen, and then thawed or frozen for the entire aging period (Kim et al. 2018). However, no differences in cook loss were found between treatments. In the same study, drip loss was the greatest in the section frozen without any aging period (Kim et al. 2018).

From the prior studies, it is clear that meat quality attributes of lamb, beef, and pork is impacted by the length of postmortem aging as well post-aging freezing. However, it is not clearly defined what the impact of post-aging freezing on multiple days of aging may have
on pork quality attributes. A better understanding of the impact of these practices is important for the future of the pork industry as it is integral to maintain high quality standards. As pork products continue to be transported worldwide, the impact of storage interventions on quality attributes must be understood to maintain consumer and customer satisfaction.

**Summary**

Skeletal muscle is a very intricate tissue with diverse roles in bodily function and the conversion of muscle to meat. Understanding these biological functions will assist us in further identifying what factors influence meat quality attributes and the development of tenderness. With this knowledge of muscle biochemistry, we can identify and further understand potential biomarkers that impact the rate of proteolysis and subsequent tenderness development. These findings will allow the pork industry to implement a value-based marketing system to meet consumer demands for high quality pork. The objectives of this study were to determine differences in the sarcoplasmic protein profile between samples significantly different in instrumental tenderness measurements.

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CHAPTER 3. INFLUENCE OF EXTENDED POSTMORTEM AGING AND POST-AGING FREEZING ON PORK LOIN QUALITY ATTRIBUTES

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Abstract: The objectives were to 1) document the aging response of fresh pork over 21 d aging, 2) determine the impact of post-aging freezing on pork quality attributes across different aging periods, and 3) determine the relationship between star probe (SP) and Warner-Bratzler shear force (WBS) under fresh and post-aging freezing conditions. Both loins from 20 carcasses (from Duroc sired crossbred pigs) were collected one day postmortem. Loin chops (n=8; 2.54 cm thick) were fabricated from each loin and vacuum packaged; four chops from each carcass were aged at 4° C for 1, 8, 14, and 21 d and immediately evaluated (Fresh). Four adjacent chops (2.54 cm thick) were frozen (-29° C) post-aging for two weeks and then thawed for quality evaluation (Frozen). Purge, Hunter L, a, and b, pH, color and marbling score, cook loss, and SP or WBS were evaluated at each aging period. Desmin degradation was determined using protein extracts from each fresh sample at each day of aging. Post-aging freezing had no significant impact on SP, WBS, pH, color score, marbling score, or Hunter L at any aging period. Fresh chop purge increased
significantly 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 significantly 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. 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 significantly ($P<0.01$) between 1, 8, and 14 d aging, but did not decrease from 14 to 21 d aging ($P>0.05$). Aging past 8 d postmortem did not improve SP or WBS, corresponding with changes in desmin degradation.

**Key words:** pork quality, post-aging freezing, star probe, aging, proteolysis, desmin.

**Introduction**

It is well documented that aging fresh pork loins improves tenderness through proteolysis of myofibrillar, cytoskeletal, and intermediate filament proteins (Wheeler et al. 2000; Melody et al. 2004; Carlson et al. 2017). However, the benefit of aging pork past 10-14 days (d) has not been well documented.

Freezing is one of the oldest and most common methods to increase the safety and shelf life of meat products. Freezing and thawing, however, can negatively impact product quality compared to meat that is never frozen (Leygonie et al. 2012a). Major meat quality attributes affected by the freezing and thawing process include water loss (Vieira et al. 2009; Leygonie et al. 2012a), protein denaturation causing discoloration (Añón and Calvelo 1980), as well as oxidation of lipids and proteins (Estévez 2011).

Many studies have observed the impact of freezing and thawing across different periods of time (Kim et al. 2011a, 2015, 2018; Leygonie et al. 2012a; Coombs et al. 2017). Results from these studies have determined that proper aging prior to freezing will negate some of the negative factors associated with the process of freezing and thawing. Additionally, freezing
prior to aging will result in meat that is not allowed to age (Lagerstedt et al. 2008).

Star probe (SP) and Warner-Bratzler shear force (WBS) are two different instrumental
tenderness measurement devices. Many studies have used these measurements separately to
determine tenderness values and their relationship to trained sensory panels (Huff-Lonergan
et al. 2002; Melody et al. 2004; Arkfeld et al. 2015; Richardson et al. 2017). Observations of
SP and WBS in fresh and post-aging freezing pork will help to further define their
relationship.

Documenting the aging response of pork is essential for tenderness development.
Additionally, understanding how freezing and thawing across different aging period is
needed to further define the impact of freezing and thawing on pork quality attributes. The
relationship of SP and WBS in fresh and post-aging freezing pork is not well defined. It was
hypothesized that 1) aging for 21 d would improve pork quality features, 2) post-aging
freezing would decrease pork quality attributes and 3) SP and WBS are highly related
instrumental tenderness measurements. Therefore the objectives of this study were to
document the aging response of fresh pork over 21 d, determine the impact of post-aging
freezing on pork quality attributes across different aging periods, and determine the
relationship between star probe (SP) and Warner-Bratzler shear force values under fresh and
post-aging freezing conditions.

**Materials and Methods**

**Pork Loin Quality Data Collection**

Paired sides of fresh pork loins of similar genetics, management, diets, harvest, and chilling
method were collected one day postmortem from 20 carcasses (Duroc sired crossbreds)
harvested at a commercial processing facility. Loins were vacuum packaged and transported
to the Iowa State University Meat Laboratory for fabrication on day 1. Each pair of loins were fabricated into 8 chops after removal of the sirloin end (approximately 10 cm). See Figure 3.1 for chop fabrication layout. Loin chops (2.50 cm), 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 days 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 days 4° C), individually placed on racks, frozen (-29° C) post-aging for two weeks and then thawed (2° C) for quality evaluation (Frozen). At each day of aging, a small portion (~100 grams) of loin muscle 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. 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 by 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). Visual 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 (D65 light source, 50 mm aperture, and 0-degree observer angle). Each chop was cooked to an internal temperature of 68 ° C on clamshell grills (Cuisinart, East Windsor,
Cook loss was calculated by using the following formula: \[
\frac{(\text{raw chop weight} - \text{cooked chop weight})}{\text{raw chop weight}} \times 100
\]
An Instron (Instron, Norwood, MA) fitted with a five-point star probe attachment was used to measure instrumental tenderness on one pair of cooked chops (Arkfeld et al. 2015; Carlson et al. 2017). Three replicate compressions were made on each chop and averaged for a final tenderness value. Star probe punctures and compresses the chop to 20% of its original height. Values from this analysis are similar to the nature of chewing (Huff-Lonergan et al. 2002). Adjacent chops were cooked in the same manner and analyzed with a Warner-Bratzler shear 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 (Wheeler and Koohmaraie 1994).

**Whole Muscle Protein Extraction**

Frozen meat (*longissimus dorsi*; 100 g) was homogenized in liquid nitrogen. Samples from each aging time (fresh) (0.5 g) were homogenized in 10 mL of whole muscle solubilizing buffer (10mM sodium phosphate, pH 7.0 and 2% wt/vol sodium dodecyl sulfate (SDS)). A Dounce homogenizer attachment on an overhead stirrer (Heidolph, type: RZR 1, No: 501-11000-04-1) was used to mix samples. Samples were clarified (1,500 x g) for 20 min, at 21°C. Sample protein concentrations were determined (Lowry et al. 1951) using premixed reagents (Bio-Rad Laboratories, Hercules, CA). Protein concentrations in each sample was diluted with whole muscle solubilizing buffer to 6.4 mg/ml. Protein concentrations were adjusted to 4 mg/ml with 0.5 mL of Wang’s tracking dye (3 mM EDTA, 3% [wt/vol] SDS, 30% [vol/vol] glycerol, 0.001% [wt/vol] pyronin Y, and 30 mM Tris-HCl, pH 8.0) and 0.1 mL of 2-mercaptoethanol. Samples were then vortexed and heated for 15 min at approximately 50°C before storage at -80°C. 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).

**Running Conditions**

Desmin degradation was determined using one-dimensional SDS-PAGE gel electrophoresis. Whole muscle protein samples were used to quantify desmin degradation. Whole muscle protein samples (40 µg) were loaded onto a 15% polyacrylamide separating gels (10 cm x 10 cm; acrylamide: N,N’-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.05% [vol/vol] tetramethylenediamine (TEMED), 0.05% 9 [wt/vol] ammonium persulfate (AMPER), 0.5 M Tris-HCl pH 8.8) with a 5% stacking gel (10 cm x 3 cm; acrylamide: N,N’-bis-methylene acrylamide= 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.125% [vol/vol] tetramethylenediamine (TEMED), 0.075% [wt/vol] ammonium persulfate (AMPER), 0.125 M Tris-HCl pH 6.8). Protein was extracted from porcine *longissimus dorsi* (aged 0 d) with the identical protocol to generate a reference sample (4 mg/ml of protein) that was included in one well on each gel. SE 260 Hoefer Mighty Small II electrophoresis units (Hoefer, Inc., Holliston, MA) were used to run the 15% gels. The running buffer consisted of 25 mM Tris, 192 mM Glycine, 2 mM EDTA, and 0.1% [wt/vol] SDS. Gels for whole muscle desmin Westerns were run at a constant 120 V for approximately 360 V-h.

**Transferring Conditions**

At completion of running the SDS-PAGE gels, the gels were transferred to polyvinylidene difluoride (PVDF) membranes with pore sizes of 0.2 µm (Immobilon-PSQ, 26.5 by 3.75 M RL, VCAT#ISEQ00010, Millipore Corporation, Billerica, MA). Membranes were soaked in methanol for less than 1 min for activation prior to transferring. Gels were transferred using a TE-22 Mighty Small Transphor unit (Hoefer, Inc., Holliston, MA). The unit ran at a constant
voltage of 90 V for 90 min at 4° C. Transfer buffer was composed of 25 mM Tris, 192 mM Glycine, 2 mM EDTA and 15% [vol/vol] methanol (Carlson et al. 2017).

**Western Blot Analysis**

Once transfer was complete, the gel was discarded, and the membrane was blocked for 60 min at 22° C in PBS-Tween (80 mM NaH$_2$PO$_4$, anhydrous, 20 mM NaH$_2$PO$_4$, 100 mM NaCl, 0.1% [vol/vol] poloxyethylene sorbitan monolaurate [Tween-20]) mixed with 5% non-fat dry milk (NFDM). Immediately following, primary antibodies were added to the blots after dilution in PBS-Tween. Desmin primary antibody dilution contained 1:40,000 using polyclonal rabbit anti-desmin antibody produced at Iowa State University (Huff-Lonergan et al. 1996; Carlson et al. 2017). Blots were incubated in primary antibodies overnight (Approximately 15-20 hours) at 4° C. After incubation with primary antibody, desmin 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 dilutions: desmin- 1:20,000 goat anti-rabbit-HRP antibody (31460, Thermo Scientific, Rockford, IL). Following incubation with secondary antibodies, desmin 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 densitometry of the 55-kDa intact protein band was quantified as a comparative ratio of the sample protein band to the internal reference protein band. See Figure 3.2 for a representative Western blot of desmin analysis. Western blots were completed in duplicate.
**Statistical Analysis**

All quality data measurements (cook loss, pH, purge, subjective color and marbling, Hunter L, a, and b, Star Probe and Warner-Bratzler Shear Force values) 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). Random effects of pig and side were used in the model.

Intact desmin was analyzed using the mixed procedure of SAS version 9.4 (v9.4, SAS Inst., Cary, NC) with fixed effects of days aged and loin. Gel was used as a random effect in the model. Least squares means, and standard errors were reported for all measured attributes. Significance levels were denoted with a $P < 0.05$. Pearson correlations were generated using PROC CORR. Correlations were considered significant when $P < 0.05$.

**Results and Discussion**

Understanding the impact of post-aging freezing on pork quality attributes was a key feature of this experiment. Freezing meat is a practice used to prolong the shelf-life of meat that has been practiced for thousands of years. However, freezing and thawing whole muscle cuts has significant impacts on meat quality attributes, specifically moisture loss (Leygonie et al. 2012b). The results of the current experiment demonstrate the impact of post-aging freezing of pork loins at different aging periods. Table 3.1 summarizes the effects of aging and post-aging freezing on meat characteristics.

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 3.1; $P>0.05$). Chop SP and WBS values were greatest at 1 d aging than any other aging period and did not change after 8 d aging regardless of treatment. These results align with previous data (Kim et al. 2011a, b, 2015, 2018) that demonstrate freezing prior to
aging does not allow product to tenderize if cooked immediately after freezing and thawing. Star Probe and Warner-Bratzler shear force values decreased significantly from 1 to 8 d aging ($P<0.01$). Interestingly, SP 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 ($P<0.01$). This change in WBS value from 14 to 21 d aging is in alignment with other studies that observed decreased WBS values from freezing and thawing (Wheeler et al. 1990; Lagerstedt et al. 2008; Vieira et al. 2009; Kim et al. 2015, 2018). This decrease in WBS value is thought to be caused by ice crystal formation between myofibrils (Leygonie et al. 2012b). From these results, it is understood that post-aging freezing does not significantly impact tenderness values if product is aged prior to freezing and chops must be properly aged prior to freezing to allow proper tenderization.

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. 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 aging across all frozen samples ($P<0.05$), indicating a similar response. The demonstrated numerical difference in percentage change between fresh and frozen WBS values from 1 to 21 d aging are consistent with previous research demonstrating that post-aging freezing resulted in decreased WBS values of beef loins (Kim et al. 2015). Kim et al. (2015) determined that this lesser WBS value from post-aging freezing steer *longissimus* muscle was not due to increased protein degradation but most likely loss of structural integrity caused by the formation of ice crystals between degraded myofibrils (Leygonie et al. 2012b).
In general, freezing and thawing contributed to decreased water holding capacity. This loss of water is caused by multiple factors including protein denaturation and protein modifications as well as disruption of the muscle fiber structure (Leygonie et al. 2012b). The current results demonstrate that fresh chop purge significantly increased at each aging (Table 3.1; \( P<0.01 \)). Post-aging freezing chop purge also increased from 1 to 8 and 8 to 14 d aging \( (P<0.01) \) but decreased from 14 to 21 d aging \( (P<0.01) \). Additionally, post-aging chop purge was greater than fresh chop purge in samples aged 1, 8 and 14 d \( (P<0.01) \) but were not different at 21 d aging \( (P>0.05) \). As expected, fresh chops had the least purge. This observation is consistent with previous research demonstrating that improved water holding capacity is obtained when chops are not frozen (Lagerstedt et al. 2008; Kim et al. 2015; Coombs et al. 2017). Additionally, chop purge increased through aging of fresh chops and generally increased in frozen chops as demonstrated by others looking at beef loin muscle (Lagerstedt et al. 2008; Vieira et al. 2009). Increased purge and water loss in general may be due to protein denaturation, chilling rate, proteolysis during aging, and protein oxidation (Kristensen and Purslow 2001; Rowe et al. 2004; Huff-Lonergan and Lonergan 2005).

Several studies have shown a significant difference in cook loss between fresh and frozen meat samples (Vieira et al. 2009; Leygonie et al. 2012a; Kim et al. 2015). Weight lost during cooking is believed to originate from the melting of fat and the denaturation of proteins which are bound to water causing subsequent cooking losses of both fat and moisture (King et al. 2003; Vieira et al. 2009). However, the current results demonstrate that fresh chops had greater cook loss than post-aging freezing chop cook loss at 14 (Table 3.1; \( P<0.03 \)) and 21 \( (P<0.01) \) d aging. These results are not consistent with past observations identifying significantly greater cook loss in frozen chops compared with fresh chops (Vieira
et al. 2009; Kim et al. 2011a; Kim and Kim 2017). This difference could be due to a variety of factors including rate of pH decline (Melody et al. 2004) and purge loss. It is possible that increased purge loss during aging and freezing could decrease the total moisture available to be lost during cooking in the post-aging freezing samples. The current results also demonstrate that post-aging freezing chops aged 1 d \((P<0.05)\) had the greatest percent cook loss compared with all other frozen chops. This result is also inconsistent with other studies demonstrating that chops frozen before aging demonstrated greater cook loss than chops aged and then frozen (Vieira et al. 2009; Kim et al. 2015). This difference is likely due to the observation of less purge in the d 1 post-aging freezing chops.

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 greater redness values at 1 d aging \((P<0.01)\) than chops aged 8, 14, and 21 d but were not different after 8 d aging \((P>0.05)\) regardless of treatment. 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, less 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 (Mancini and Hunt 2005). Meat color stability is also impacted by display time (Jones-Hamlow et al. 2015), loss of water (Kim et al. 2018), and freezing rate (Kim et al. 2018).

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 discoloration was 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 (MacDougall 1982). 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 to fresh chops (Kim et al. 2011b, 2015, 2018). 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. 2011b). 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). The fresh samples may have had mitochondria that were continuing to respire and thus decreasing the blooming ability of myoglobin due to a lower partial pressure of oxygen within samples aged 1 d compared to post-aging freezing samples aged 1 d (Vestergaard et al. 2000).

The rate of freezing 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 our freezing method. However, this difference is also impacted by decreases in metmyoglobin reducing agents activity and alterations of mitochondrial function (Kim et al. 2011b).
Post-aging freezing had no significant effect on pH, color score, marbling score, or Hunter L value at any aging period (Table 3.1; \( P > 0.05 \)). 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. 2011b, 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. 2011b). This decrease in pH of frozen and thawed meat might be due to the loss of hydrogen ions from denatured proteins within water losses and an increased concentration of hydrogen ions due to less water to dilute ions (Leygonie et al. 2011, 2012b).

Lightness of meat has also shown to vary greatly due to differences in sequence and rate of freezing and thawing. Kim et al. (2018) determined that samples which were frozen quickly prior to aging resulted in significantly darker pork longissimus sections compared to slow freezing sections of pork loin. However, no difference was observed between chops that were subjected to post-aging freezing regardless of freezing rate (Kim et al. 2018). In previous studies, \( L^* \) values demonstrated significantly lighter beef loin steaks at 1 and 7 d aging but were not different at 4 d aging (Kim et al. 2015). Although pork loin chops in this
study were frozen quickly, we did not observe differences in L value at any aging timepoint. This could be due to the freezing temperature applied in this study (-29° C) as well as the storage conditions of chops.

Previous studies have shown a moderate correlation between Star Probe (SP) and a trained sensory panel tenderness score (Huff-Lonergan et al. 2002; Lonergan et al. 2007). Linear regression analysis across all aging periods and treatments can be found in Figure 3.3. Figure 3.3 explains the relationship between SP and WBS value across all days aging and all treatments. 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 measurements (r= 0.89) were numerically more highly correlated than frozen measurements (r= 0.82) across all aging periods. These results demonstrate that SP and WBS are two similar instrumental tenderness measurement devices that can be utilized to assess texture regardless of aging time or conditions.

Postmortem protein degradation is one factor that has an impact on the development of meat tenderness (Taylor et al. 1995; Melody et al. 2004; Carlson et al. 2017). Specifically, desmin is an intermediate filament that connects the myofibril with other myofibrils and integrates surrounding organelles (Clark et al. 2002). 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. Intact desmin abundance is summarized in Figure 3.4. 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 abundance. Additionally, intact desmin decreased by 56% from 1 to 21 d aging across all fresh samples (P<0.05). Intact desmin was also highly
correlated ($P<0.01$) to SP ($r=0.61$) and WBS ($r=0.66$) across all days of aging in the fresh samples. Degradation of desmin 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 this protein can ultimately alter myofibril alignment and connection to conjoining structures and resulting in differences in tenderness. 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.

**Conclusions**

Aging of commodity pork loin at least 8 days showed a 22% and 32% improvement in SP and WBS values, respectively. The results demonstrate that aging commodity pork loin for at least 8 days is needed to improve tenderness. Aging beyond this period may have no effect on improving SP or WBS values. Degradation of desmin was, in general, consistent with this observation, suggesting that desmin degradation is a key component of fresh pork tenderness. 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.
Acknowledgements

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Literature Cited


Huff-Lonergan E, Mitsuhashi T, Beekman DD, et al (1996) Proteolysis of specific muscle structural proteins by mu-calpain at low pH and temperature is similar to degradation in postmortem bovine muscle. The online version of this article, along with updated information and services, is located on the World W. J Anim Sci 993–1008. doi: 10.2527/1996.745993x


### Table 3.1. Summary of effects of aging and post-aging freezing on pork loin chop (n=20) quality characteristics.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days Aged</th>
<th>Fresh</th>
<th></th>
<th></th>
<th></th>
<th>Frozen</th>
<th></th>
<th></th>
<th></th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>8</td>
<td>14</td>
<td>21</td>
<td>1</td>
<td>8</td>
<td>14</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP (kg)¹</td>
<td>8.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.82&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>WBS (kg)²</td>
<td>5.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.36&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>4.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.53&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Purge (%)³</td>
<td>0.15&lt;sup&gt;d&lt;/sup&gt;x</td>
<td>1.31&lt;sup&gt;c&lt;/sup&gt;x</td>
<td>2.23&lt;sup&gt;b&lt;/sup&gt;x</td>
<td>2.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.81&lt;sup&gt;d&lt;/sup&gt;y</td>
<td>2.56&lt;sup&gt;bc&lt;/sup&gt;y</td>
<td>3.83&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>3.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>pH⁴</td>
<td>5.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.82&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.90&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>5.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04</td>
<td></td>
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<tr>
<td>Color Score⁵</td>
<td>3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.7&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>2.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.11</td>
<td></td>
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<tr>
<td>Marbling Score⁶</td>
<td>1.9</td>
<td>2.0</td>
<td>1.9</td>
<td>2.2</td>
<td>2.2</td>
<td>2.3</td>
<td>2.2</td>
<td>2.2</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Cook Loss (%)⁷</td>
<td>20.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20.18&lt;sup&gt;ab&lt;/sup&gt;x</td>
<td>19.62&lt;sup&gt;bc&lt;/sup&gt;x</td>
<td>21.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.37&lt;sup&gt;by&lt;/sup&gt;</td>
<td>16.88&lt;sup&gt;by&lt;/sup&gt;</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>Hunter L value⁸</td>
<td>43.88&lt;sup&gt;d&lt;/sup&gt;</td>
<td>48.62&lt;sup&gt;c&lt;/sup&gt;</td>
<td>48.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>44.94&lt;sup&gt;c&lt;/sup&gt;</td>
<td>49.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.47</td>
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</tr>
<tr>
<td>Hunter a value⁸</td>
<td>11.96&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>13.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.13&lt;sup&gt;by&lt;/sup&gt;</td>
<td>13.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Hunter b value⁸</td>
<td>2.49&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>3.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.79&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>3.60&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>3.40&lt;sup&gt;y&lt;/sup&gt;</td>
<td>3.86&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.29&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>4.59&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>0.13</td>
<td></td>
</tr>
</tbody>
</table>

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

<sup>x, y</sup> 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.
Figure 3.1 Chop fabrication layout.

Side was randomly assigned.
Figure 3.2 Representative Western blot of intact and degraded desmin in pork 
*Longissimus dorsi* (LM) whole muscle samples aged over time. Intact bands (55-kDa) 
and degradation bands (38-kDa) were compared to corresponding bands of a 0 d aged 
pork LM sample (Ref). Star probe (kg) values are provided for the samples across each 
aging period (1, 8, 14, and 21 d).
Figure 3.3 Linear regression showing Star Probe (SP) and Warner-Bratzler Shear Force (WBS) values of *Longissimus Dorsi* (LM) chops across all days aged and treatments (n=158). Coefficient of Determination and slope for star probe values are denoted.

\[ y = 0.8143x + 3.4416 \]

\[ R^2 = 0.7136 \]
*a,b,c* Means with different superscripts are significantly different ($P<0.05$).

**Figure 3.4** Proteolysis of desmin in pork Longissimus dorsi (LM) whole muscle samples. Densitometry of the 55-kDa Intact Band was used to analyze abundance of protein bands corresponding to an internal reference sample (day 0 whole muscle LM sample).
CHAPTER 4. INVESTIGATION OF SARCOPLASMIC PROTEOME CONTRIBUTION TO THE DEVELOPMENT OF PORK LOIN TENDERNESS

A manuscript prepared for submission to Meat and Muscle Biology
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Abstract: The objectives were to determine the extent to which the sarcoplasmic proteome can explain variations in aged pork quality, specifically star probe (SP) value. Pork loins (total n=12) were categorized by differences in SP at 21 d postmortem from a larger group of twenty pork loins. Loins were sorted into the Low SP group (LSP) (n=6), (SP<5.80 kg), and High SP group (HSP) (n=6), (SP>7.00 kg) with inclusion criteria of marbling score (1.0-3.0) and 24 hour pH (5.69-5.98). Pork loin quality (purge, color, marbling score, SP and cook loss) was measured at 1, 8, 14, and 21 d postmortem. Desmin degradation and calpain-1 autolysis were determined using Western blot analysis. Two-dimensional difference in gel electrophoresis (2D-DIGE) and mass spectrometry were used to determine sarcoplasmic proteome differences in abundance and potential modifications of proteins. Star Probe values were lower (P<0.01) in the LSP at each day of aging compared with the HSP. Chop purge was lower (P<0.01) in the LSP compared with the HSP at 14 and 21 d aging but did not
differ at 1 and 8 d aging ($P>0.05$). pH was greater ($P<0.05$) in the LSP compared with the HSP at each d of aging. Marbling score was greater ($P<0.05$) in the LSP compared with the HSP at each d of aging. Greater desmin degradation ($P<0.05$) was detected in LSP chops compared with HSP chops at 14 and 21 d aging. Color score, Hunter L, a, b, and calpain-1 autolysis were not different between SP groups ($P>0.05$). Results from 2D-DIGE identified sarcoplasmic proteins from HSP chops had greater abundance ($P<0.10$) of metabolic and regulatory proteins while the LSP had greater abundance ($P<0.10$) of stress response proteins. Early postmortem glycolytic, regulatory, and stress response proteins may be used as potential biomarkers for aged pork loin tenderness differences.

Key words: pork, desmin, proteolysis, two-dimensional difference gel electrophoresis, tenderness, glycolytic proteins

**Introduction**

Tenderness is fundamental to pork quality (Moeller et al. 2010). Predicting pork tenderness is challenging due to the variety of quality factors that influence tenderness. In the current retail case, large variations in pork quality exist (Bachmeier et al. 2016). Consumers in the U.S. are willing to pay a premium for high quality, third party quality grade assigned pork cuts (Lusk et al. 2018). International customers place greatest value on eating quality attributes such as tenderness and pork flavor in fresh pork products (Murphy et al. 2015). There have been many efforts to predict pork quality, but a robust system has not been defined, demonstrating that the problem is multifactorial and complex. Consumers desire to purchase high quality pork products but the ability to predict pork quality differences is not currently available. One approach to accomplish this is through a greater understanding of how development of pork tenderness occurs postmortem.
Postmortem tenderness development is influenced by a multitude of factors including pH (Melody et al. 2004; Lonergan et al. 2007), collagen content (Wheeler et al. 2000; Nishimura et al. 2009), sarcomere length (Wheeler et al. 2000), and protein degradation (Huff-Lonergan et al. 1996; Wheeler et al. 2000; Melody et al. 2004; Carlson et al. 2017a). Carlson et al. (2017b) demonstrated that abundance of desmin and peroxiredoxin-2 in aged pork loin sarcoplasmic proteome could explain variations in SP values. What remains undefined is how the sarcoplasmic proteome in early postmortem pork can be used to predict fresh pork loin quality after aging. It was hypothesized that SP groups would have differences in pork quality attributes and sarcoplasmic protein profiles at each day of aging. Therefore, the objective of this study was to document protein profile differences of pork loins aged 1 d postmortem based on high and low Star Probe (SP) values at 21 days (d) postmortem. Identifying early postmortem tenderness biomarkers is critical for quality-based pork marketing.

**Materials and Methods**

Twenty pork loins, previously described (Schulte et al., 2018), were sorted based on 21 d aged pork loin star probe values to obtain high and low star probe groups. Loins with high (star probe>7.0) and low (star probe<5.8) star probe values at 21 d postmortem were chosen to represent extreme differences in star probe value. 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 5.98. This classification narrowed the sample set to a balanced experiment of n=6 for high and low star probe categories.
Whole Muscle Protein Extraction

Whole muscle protein extractions were completed as previously described (Schulte et al. 2018). Briefly, meat containing only the *longissimus dorsi* (100 g) was pulverized and homogenized in liquid nitrogen. Samples from each aging time (0.5 g) were homogenized in 10 mL of whole muscle solubilizing buffer (10 mM sodium phosphate, pH 7.0 and 2% wt/vol sodium dodecyl sulfate (SDS)) with a Dounce homogenizer attachment on an overhead stirrer (Heidolph, type: RZR 1, No: 501-11000-04-1), and clarified (1,500 x g) for 20 min at 21°C. Protein concentrations were determined (Lowry et al. 1951) using premixed reagents (Bio-Rad Laboratories, Hercules, CA). Proteins were diluted with whole muscle solubilizing buffer to 6.4 mg/ml and adjusted to a final protein concentration of 4 mg/ml with 0.5 mL of Wang’s tracking dye (3 mM EDTA, 3% [wt/vol] SDS, 30% [vol/vol] glycerol, 0.001% [wt/vol] pyronin Y, and 30 mM Tris-HCl, pH 8.0) and 0.1 mL of 2-mercaptoethanol. Samples were then vortexed, heated for 15 min at approximately 50°C, and stored at -80°C. Protein concentration consistency was 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).

Sarcoplasmic Protein Extraction

Meat containing only the *longissimus dorsi* (100 g) was pulverized and homogenized in liquid nitrogen. Samples from each aging time (3 g) were homogenized with 10 mL of cold sarcoplasmic extraction buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.0) using a Polytron PT 3100 (Polytron, Lucerne, Switzerland). Homogenized samples were clarified by centrifugation at 40,000 times g for 20 min at 4°C. The supernatant was filtered through cheesecloth. The protein content of each sarcoplasmic protein preparation was determined
(Lowry et al. 1951) using premixed reagents (Bio-Rad, Hercules, CA). Final protein concentration for one-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analyses samples were diluted with cold sarcoplasmic extraction buffer to 6.4 mg/ml. Final protein concentrations were diluted to 4 mg/ml using steps as described in whole muscle sample preparation. Samples were vortexed and heated for 15 min at approximately 50° C and stored at -80° C. Samples to be used for two-dimensional difference gel electrophoresis (2D-DIGE) were diluted to 10 mg/ml using cold sarcoplasmic extraction buffer and frozen. Consistency of protein concentrations of all samples were monitored using 15% SDS-PAGE gels and Colloidal Coomassie Blue staining.

Intact desmin in the whole muscle protein extracts (1, 8, 14, and 21 d aged) and sarcoplasmic calpain-1 autolysis at 1 d postmortem were determined using one-dimensional SDS-PAGE gel electrophoresis. Protein (40 µg) for whole muscle intact desmin analysis was loaded onto a 15% polyacrylamide separating gel (10 cm x 10 cm; acrylamide: N,N’-bis-methylene acrylamide = 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.05% [vol/vol] tetramethylenediamine (TEMED), 0.05% 9 [wt/vol] ammonium persulfate (AMPER), 0.5 M Tris-HCl pH 8.8) with a 5% stacking gel (10 cm x 3 cm; acrylamide: N,N’-bis-methylene acrylamide= 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.125% [vol/vol] tetramethylenediamine (TEMED), 0.075% [wt/vol] ammonium persulfate (AMPER), 0.125 M Tris-HCl pH 6.8). Protein (40 µg) for sarcoplasmic calpain-1 autolysis was loaded onto an 8% polyacrylamide separating gel with a 5% stacking gel. A reference sample generated from the whole muscle (myofibrillar and sarcoplasmic) protein extract (4 mg/ml) from a porcine longissimus dorsi (aged 0 d) muscle was included in one well on each gel. SE 260 Hoefer Mighty Small II electrophoresis units (Hoefer, Inc., Holliston, MA) were used to run the gels. The running buffer consisted of 25
mM Tris, 192 mM Glycine, 2 mM EDTA, and 0.1% [wt/vol] SDS. Gels for whole muscle intact desmin Westerns were run at a constant 120 V for approximately 360 V-h. Gels for sarcoplasmic calpain-1 Westerns were run at a constant 120 V for approximately 300 V-h. At completion of running the SDS-PAGE gel, the gels were transferred to polyvinylidene difluoride (PVDF) membranes with pore sizes of 0.2 μm (Immobilon-PSQ, 26.5 by 3.75 M RL, VCAT#ISEQ00010, Millipore Corporation, Billerica, MA). Membranes were soaked in methanol for less than 1 min for activation of the PVDF membrane prior to transferring. Gels were transferred using a TE-22 Mighty Small Transhpor unit (Hoefer, Inc., Holliston, MA). Transfer was run at a constant voltage of 90 V for 90 min at 4° C. Transfer buffer was composed of 25 mM Tris, 192 mM Glycine, 2 mM EDTA and 15% [vol/vol] methanol (Carlson et al. 2017a). Once transfer was complete, the gel was discarded, and the membrane was blocked in PBS-Tween for 60 min at 22° C (80 mM NaH₂PO₄, anhydrous, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% [vol/vol] polyoxyethylene sorbitan monolaurate [Tween-20]) mixed with 5% non-fat dry milk (NFDM). Immediately following, primary antibody was added to the blot after dilution in PBS-Tween. Desmin primary antibody dilution contained 1:40,000 using polyclonal rabbit anti-desmin antibody produced at Iowa State University (Huff-Lonergan et al. 1996; Carlson et al. 2017a). The primary antibody dilution for calpain-1 was 1:5,000 using monoclonal mouse anti-calpain-1 (MA3-940, Thermo Scientific, Rockford, IL). Blots were incubated in primary antibody overnight (approximately 15-20 hours) at 4° C. After incubation with primary antibody, desmin and calpain-1 blots were washed with PBS-Tween 3 times for 10 min. Secondary antibodies were diluted with PBS-Tween and incubated with each blot for 60 min at 22° C. Secondary antibody dilution for desmin was 1:20,000 goat anti-rabbit-HRP antibody (31460, Thermo Scientific, Rockford,
IL) and for calpain-1 was 1:10,000 goat anti-mouse-HRP antibody (A2554, Sigma Aldrich, St. Louis, MO). Following incubation with secondary antibody, desmin blots were washed with PBS-Tween 3 times for 10 min. Calpain-1 blots were washed with PBS-Tween 5 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 densitometry of the 55-kDa intact protein band was quantified as a comparative ratio of the sample protein band to the internal reference protein band. Calpain-1 autolysis was analyzed as a percentage of autolyzed calpain-1 (76-kDa) to total calpain-1 (76, 78, and 80-kDa) within each sample. Western blots were completed in duplicate.

**Two-Dimensional Difference in Gel Electrophoresis**

Sarcoplasmic protein (50 µg) extracts from each sample of each experimental group were labeled alternatively with CyDye3 and CyDye 5 (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 picking gels. Three aliquots of the pooled reference sample (100 µg) 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.

**pH 3-10**

Labeled samples were prepared for running on 11-centimeter pH 3-10 immobilized pH gradient (IPG) strips (GE Healthcare, Piscataway, NJ). Fifteen µg of sample from each experimental group along with the pooled reference sample were combined for a total protein
concentration of 45 µg for each strip (Carlson et al. 2017b). Rehydration solution (DeStreak, GE Healthcare, Piscataway, NJ) was prepared as directed by the manufacturer by combining 2% IPG buffer, pH 3-10 (17-6000-87, GE Healthcare, Piscataway, NJ) and 20 mM 1, 4-Dithiolthreitol (DTT). The rehydration solution was mixed with the prepared proteins. Protein solutions and strips were placed in an individual well of a humidified rehydration chamber. Strips rehydrated overnight (approximately 17 hours) in the dark at room temperature (24° C) prior to being run in the first dimension on an Ettan IPGphor isoelectric focusing system (GE Healthcare, Piscataway, NJ). Strips were run for a total of 11,500 V-h. Strips were stored in tubes at -80° C until equilibration for the second dimension was completed. Equilibration for the second dimension was completed by two 15 min washes. The first wash contained equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and trace amounts of bromophenol blue) and 65 mM DTT. The second wash contained equilibration buffer and 135 mM iodoacetamide. After equilibration, strips were loaded onto 12.5% preparative gels (25.5x20.5 cm, 1.5 mm thick; acrylamide: N,N’-bis-methylene acrylamide= 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.05% [vol/vol] tetramethylenediamine (TEMED), 0.05% [wt/vol] ammonium persulfate (AMPER), 0.5 M Tris-HCl pH 8.8) and run on an Ettan DALT SIX system (GE Healthcare, Piscataway, NJ). An overlay agarose with trace amounts of bromophenol blue was used to solidify strips in place for second dimension separation (two strips per gel). Gels were run until the overlay solution was off the bottom of each gel (approximately 2,500 volt-hours). All gels were run in a running buffer solution consisting of 25 mM Tris, 192 mM Glycine, 2 mM EDTA, and 0.1% [wt/vol] SDS. Duplicates were run for each sample. Once the second dimension was completed, gels were imaged using an Ettan DIGE Imager (GE Healthcare, Piscataway, NJ)
and analyzed using DeCyder™ 2D software version 6.5 (GE Healthcare, Piscataway, NJ) to identify differences in protein abundance between experimental groups. See Figure 4.1 for a representative 11 cm, pH 3-10 2D-DIGE gel.

**pH 4-7**

Labeled samples were prepared for running on 11-centimeter pH 4-7 IPG strips (GE Healthcare, Piscataway, NJ) as described previously with minor adjustments. Briefly, 15 µg of sample from each experimental group along with the pooled reference sample were combined for a total protein concentration of 45 µg for each strip. Rehydration solution (DeStreak, GE Healthcare, Piscataway, NJ) was prepared as directed by the manufacturer by combining 2% IPG buffer, pH 4-7 (17-6000-86, GE Healthcare, Piscataway, NJ) and 20 mM 1, 4-Dithiolthreitol (DTT). The rehydration solution was mixed with the prepared proteins. Protein solutions and strips were placed in an individual well of a humidified rehydration chamber. Strips rehydrated overnight (approximately 17 hours) in the dark at room temperature (23° C) prior to being run as described above for a total of 14,000 V-h. Strips were stored, equilibrated, loaded, run, imaged and analyzed as described above for the 3-10 pH strip.

DeCyder™ 2D software version 6.5 was used to analyze all 2D-DIGE gels for spot identification. Paired t-test were used to determine differences in protein spot abundance between LSP and HSP with significance set at $P \leq 0.10$. Figure 4.2 is a representative 11 cm, pH 4-7 2D-DIGE gel.

**Spot identification**

Spots of interest between experimental groups were chosen for identification ($P<0.10$). The pooled reference sample was used for protein spot identification. For spot identification, gels
were run as described with the corresponding pH range to isolate spots of interest. Picked spots were excised from gels, sent to the Iowa State University Protein Facility, and 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, Star Probe and Warner Bratzler Shear Force values) were analyzed using the mixed procedure of SAS version 9.4 (v.9.4, SAS Inst., Cary, NC). Fixed effects included days aged and category (High or Low Star Probe). Random effect of pig and side was used in the model.

Whole muscle intact desmin and autolyzed sarcoplasmic calpain-1 data were analyzed using the mixed procedure of SAS version 9.4 (v9.4, SAS Inst., Cary, NC) with fixed effects of
days aged and category. Gel was used as a random effect in the model. Least squares means, and standard errors were reported for all measured attributes. Significance levels were denoted with a $P < 0.05$ and trends were signified with a $P < 0.15$.

**Results and Discussion**

**Fresh Quality Attribute Comparison**

Fresh pork characteristics of both experimental groups are summarized in Table 4.1. Loins were sorted based on their 21 d aged star probe value. Star probe values (Figure 4.3) 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 27% decrease in star probe value from 1 to 21 d aging while the HSP group only had a 15% decrease in star probe value. The LSP group also demonstrated less variation in star probe value as aging progressed compared to the HSP group (Figure 4.3).

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 partially be accounted for by significant differences in pH, marbling, and desmin degradation. Postmortem proteolysis not only impacts meat tenderness, but also impacts the ability of meat to retain water (Kristensen and Purslow 2001; Huff-Lonergan and Lonergan 2005). Bee et al. 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) (Bee et al. 2007). In pork, early and ultimate pH is negatively correlated with water holding capacity (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). 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, consumers purchasing intent has shifted towards the desire to purchase pork with less intramuscular fat content (Brewer et al. 2001). 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 1, 8, 14, and 21 d aging ($P<0.05$) 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 ($P>0.05$).

Figure 4.4 and 4.5 show representative Western blots of desmin and calpain-1 analysis, respectively. Calpain-1 is known to play 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; Koohmarai and Geesink 2006). Activation, and subsequent autolysis, of calpain-1 requires a micromolar concentration of calcium and thus autolysis
lowers the calcium concentration needed for activation (Suzuki et al. 1981; Baki et al. 1996). The 76-kDa autolysis product of calpain-1 has been shown to be 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 (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 how much proteolysis 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 day 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 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.

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). Degradation of desmin has consistently shown to relate 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 influencing meat tenderness. The results demonstrate that abundance of intact desmin decreased from 1 to 8 and 8 to 14 d aging (Table 4.1; \( P<0.01 \)) in the LSP group. Intact desmin decreased from 1 to 8 d aging in the HSP group but was not different after 8 d aging (\( P>0.05 \)). 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 73% from 1 to 21 d aging whereas the HSP group only decreased by 31% during the entire aging period.

**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. These proteins differ between star probe groups in abundance. In the corresponding 3-10 pH range, a total of 444 spots were found in the sarcoplasmic protein fraction of 12 samples 1 d postmortem. There was a tendency for classification group to affect abundance of 23 spots (\( P\leq0.15 \)). Of these spots, 7 of the most prevalent spots were chosen for identification (Figure 4.1 and Table 4.2). Spot 248 was also picked due to prevalence and significance (\( P=0.16 \)).

In the refined pH range (4-7), 462 spots were resolved in the sarcoplasmic protein fraction of the LM at 1 d of aging. Abundance of 32 spots were different or tended to be different (\( P\leq0.10 \)) due to aged loin star probe value classification. From these spots, the 13 most prevalent were chose for identification (Figure 4.2 and Table 4.3). These identified protein spots included groups of structural, metabolic, and stress response proteins. Spots 272, 273, 371, and 409 were also picked due to prevalence between experimental groups.
(P≤0.20) 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; experiment 2). Pyruvate kinase was numerically (84%) more abundant in the HSP samples, but not trending or significant (Figure 4.1, Table 4.2; P=0.16). Pyruvate kinase catalyzes the transfer of a phosphoryl group from phosphoenolpyruvate to an ADP molecule resulting in two molecules of pyruvate and ATP (Tymoczko et al. 2013). Phosphorylation results in a shift of isoelectric point towards a more acidic molecule (Julien and Mushynski 1982). When the isoelectric point of pyruvate kinase is shifted due to reversible phosphorylation, pyruvate kinase activity increases (Cowan and Storey 1999). Pyruvate kinase in this study could have been phosphorylated and more acidic demonstrated by a shift in its biological isoelectric point (7.53). However, this difference is unknown currently. In PSE pork, several phosphorylated forms of pyruvate kinase have been identified (Schwagele et al. 1996). In this phosphorylated form, pyruvate kinase exhibited greater activity at the low pH conditions of PSE pork (Schwagele et al. 1996) The chops in the LSP group had a greater pH at each day of aging compared with the HSP group, demonstrating that a greater extent of pH decline could have influenced the greater abundance of pyruvate kinase observed in the HSP group.

Two spots identified as triosephosphate isomerase (Spot 353 and 355) were numerically more abundant (54% and 52%, respectively; Figure 4.1, Table 4.2; 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). Dihydroxyacetone phosphate and glyceraldehyde 3-phosphate are the products of the splitting of Fructose 1, 6-bisphosphate (Ohlendieck 2010; Tymoczko et al. 2013). However, dihydroxyacetone phosphate is quickly isomerized to glyceraldehyde 3-phosphate through the reversible function of triosephosphate isomerase (Tymoczko et al. 2013). 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. However, these results conflict with Lametsch et al. (2003) who demonstrated a strong negative correlation with Warner-Bratzler shear force value at 1 d (-0.46) and 4 d postmortem (-0.64) in whole muscle pork samples. This difference could be due to fiber type demonstrating a different preference for production of ATP. The results could also be identifying posttranslational modifications to the proteins causing the shift in isoelectric point seen in Figure 4.1.

Two spots identified as glyceraldehyde-3-phosphate dehydrogenase (Spots 305 and 314) tended (Figure 4.1, Table 4.2; \( P = 0.11 \) 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). Generally, this protein is soluble during the first phase of aging and may quickly move into the sarcoplasmic protein fraction. However, under increased rates of pH decline
glyceraldehyde-3-phosphate dehydrogenase and other metabolic proteins were less soluble during the early post-mortem pH decline period during anaerobic glycogenolysis (Boles et al. 1992; Laville et al. 2009; Zapata et al. 2009). An increased temperature in pre-rigor meat is known to be accompanied by a quicker rate of pH decline in muscle (Kim et al. 2014). A faster rate of pH decline results in denaturation of proteins and decreased solubility of many sarcoplasmic proteins (Bendall and Wismer-Pedersen 1962; Boles et al. 1992). An increased temperature during early postmortem pH decline may have caused denaturation of this glycolytic enzymes due to its highly reactive cysteine residues. Additionally, this protein was very likely to be impacted by posttranslational modifications and degraded due to the difference in appearance of the two spots.

The only protein identified in the 4-7 pH range directly related to glycolytic energy production was phosphoglycerate kinase 1 (Spot 332; Figure 4.2, Table 4.3). 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). Phosphoglycerate kinase 1 was previously found to be related to water holding capacity with a significantly greater abundance in sarcoplasmic proteome samples that demonstrated an intermediate (4%) drip loss compared to low (2.5%) drip loss samples (Di Luca et al. 2013). The current experiments samples demonstrated similar purge loss values to Di Luca et al. (2013) intermediate and low drip loss values indicating that this protein may be associated with the water holding capacity of pork muscles. This combination of glycolytic enzymes indicates that glycolytic proteins are impacting the extent of and possibly rate of pH
decline in experimental groups, thus, inadvertently impacting proteolytic enzyme activity and subsequent development of tenderness.

**Other Energy Metabolism**

Several other energy metabolic proteins were found to express differential abundance between experimental groups; creatine kinase M-type (Spot 272; Figure 4.1, Table 4.2) as well as mitochondrial ATP synthase subunit beta (Spot 271; Figure 4.2, Table 4.3) and mitochondrial isocitrate dehydrogenase subunit alpha (Spot 310; Figure 4.2, Table 4.3). Creatine kinase M-type tended to be more abundant (64%; Figure 4.1, Table 4.2; \( P=0.12 \)) 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). Similar to glyceraldehyde-3-phosphate dehydrogenase, this protein is soluble under normal pH decline conditions (Boles et al. 1992; Laville et al. 2009; Zapata et al. 2009). However, when rapid rates of pH decline occur, solubility is lost due to denaturation and possibly an increased muscle temperature during postmortem conditions (Joo et al. 1999). Creatine kinase M-type fragments have been found in the soluble fraction of beef LM that was stored for 24 hours postmortem (Jia et al. 2007) as well as whole muscle protein fractions of pork LM at 48 hours of storage (Lametsch et al. 2002). Creatine kinase is readily degraded by the calpains in skeletal muscle (Purintrapiban et al. 2001) and could indicate greater utilization of ATP in postmortem muscle of the HSP group.

In the refined pH range, 71% greater abundance (Figure 4.2, Table 4.3; \( P<0.01 \)) 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). This protein was more abundant in the sarcoplasmic protein fraction of the semitendinosus of pigs subjected to heat stress indicating greater ATP production through oxidative phosphorylation (Cruzen et al. 2015). This protein could also be related to fiber type and the need for more ATP production through mitochondrial function. This would mean the HSP group may have greater mitochondria concentrations and greater proportions of red, slow twitch fibers or white, fast twitch fibers. Additionally, identification of this protein in the sarcoplasm may indicate leakage from the mitochondria. This could be caused by postmortem rupture of the mitochondria caused by pH decline differences (Dutson et al. 1974).

Mitochondrial isocitrate dehydrogenase subunit alpha, was 25% more abundant (Figure 4.2, Table 4.3; \( P=0.06 \)) in the HSP samples. Isocitrate dehydrogenase catalyzes the oxidative decarboxylation of isocitrate to \( \alpha \)-ketoglutarate in the citric acid cycle (Tymoczko et al. 2013). This is the rate-limiting step of the TCA cycle (Tymoczko et al. 2013). Greater abundance of this enzyme could indicate the need for greater ATP production through the allosteric stimulation of ADP. This energy need could be a result of stress on the cell ultimately impacting the development of tenderness in the HSP samples. Greater abundance of these metabolic proteins demonstrates the greater need for and utilization of ATP the HSP samples may have had due to stress in postmortem muscle.

**Other Proteins**

Two other proteins were identified in the corresponding 3-10 pH range, mitochondrial aldehyde dehydrogenase (Spot 246) and adenylate kinase isoenzyme 1 (Spot 244). Other 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% great abundance (Figure 4.1 & 4.2, Table 4.2 & 4.3; $P=0.14$ 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). Additionally, 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 was needed in the HSP group.

Annexin A7 (Spot 272; $P=0.15$) and A5 (Figure 4.2, Table 4.3; Spot 371; $P=0.11$) were found in the 4-7 pH range to be 33% and 20% more abundant in the HSP group. Annexins function as calcium-dependent phospholipid binding proteins. It has been hypothesized that this family of proteins associate with the sarcoplasmic reticulum, possibly increasing the release of intracellular calcium (Gerke and Moss 2002). Annexin A7 is a
calcium and phospholipid-binding protein that assists with promotion of the fusion of membranes, exocytosis, and possible calcium regulation (Gerke and Moss 2002). Di Luca et al. (2013) reported that annexin A7 was less abundant in the sarcoplasmic fraction of pork LM of pork with greater drips loss compared with pork with intermediate drip loss (Di Luca et al. 2013). Annexin A5 potentially participates as molecular switch for calcium release under pH-dependent conditions (Sopkova-De Oliveira Santos et al. 2001). Another isoform of this family of proteins, Annexin A6, has been suggested to be related to calcium release and tenderness (Bjarnadóttir et al. 2012). It is feasible that the annexins may play a role in the postmortem metabolism that influences the development of fresh pork quality. Greater annexin isoform abundance may be related to calcium regulation impacting rigor development, rate of glycolysis, and autolysis of the calpains ultimately impacting the development of meat tenderness. Greater abundance of different annexin isoforms may impact the rate of rigor development, restricting rigor development and proteolysis due to lower free calcium concentrations in the HSP group.

In the pH range of 4-7, mitochondrial dihydrolipoyl dehydrogenase was 19% more abundant (Spot 244; Figure 4.2, Table 4.3; \(P=0.05\)) in the HSP group. Dihydrolipoyl dehydrogenase is a flavoprotein enzyme which oxidizes dihydrolipoamide to lipoamide and is also an enzyme required for several enzyme complexes related to energy production (Babady et al. 2007). This enzyme has also been suggested to be a protease of serine residues and was found to be consistently inhibited by serine protease inhibitors but has not shown consistent results from other inhibitors (Babady et al. 2007).

Protein disulfide isomerase was more abundant (Spot 256; Figure 4.2, Table 4.3; \(P=0.04\)) in the HSP group. This 55-kDa protein assists with protein disulfide bond formation
while also catalyzing the correction of disulfide bonds which were incorrectly formed (Wilkinson and Gilbert 2004). It has been demonstrated that protein disulfide isomerase plays a chaperone activity during the assembly of procollagen indicating a possible impact on connective tissue within muscle (Wilson et al. 1998). Protein disulfide isomerase is also known to impact oxidative stress through its chaperone function (Ali Khan and Mutus 2014). This could indicate that the HSP group had greater oxidative stress levels and subsequent need for greater protein disulfide isomerase abundance.

**Stress Response Proteins**

In the refined pH 4-7 range, 3 proteins were identified related to stress response; Peroxiredoxin-2 (Fragment; Spot 409; Figure 4.2, Table 4.3), 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. Peroxiredoxin-2 serves as an antioxidant protein to protect cells and mitigate damage caused by oxidative stress through the reduction of reactive oxygen species to less reactive molecules (Chae et al. 1993; Zhao and Wang 2012). Peroxiredoxin-2 also functions as a peroxidase, molecular chaperone, and signaler for oxidative stress (Oláhová et al. 2008). Peroxiredoxin-2 abundance in the sarcoplasmic fraction increased in beef longissimus thoracis from 1 to 24 h postmortem (Jia et al. 2007). Peroxiredoxin-2 content was also positively correlated (0.67) with Warner-Bratzler shear force tenderness value in pork longissimus muscle (Hwang et al. 2005). Carlson et. al. (2017) quantified peroxiredoxin-2 abundance in aged pork loins and confirmed significantly greater abundance in HSP samples suggesting the impact of oxidative stress that may have occurred early postmortem (Carlson et al. 2017b). The current results combined
with previous research demonstrates the need to identify further the role of peroxiredoxin-2 as a potential biomarker for tenderness.

Heat shock cognate 71 kDa protein (Spot 161; \( P=0.09 \)) and Hsc 70-interacting (Spot 273; Figure 4.2, Table 4.3; \( P=0.20 \)) 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 (Ohtsuka and Suzuki 2000). 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). Lower abundance of heat shock cognate 71 was exhibited in samples in the high drip loss group compared to the intermediate drip loss group (Di Luca et al. 2013). The results of the current experiment are consistent with previous findings showing less abundance of stress related proteins in the HSP group which demonstrated significantly greater purge at 1 d postmortem. Understanding the role of this protein in relation to water holding capacity would help to understand why this difference was seen between samples.

**Regulatory Proteins**

The only regulatory protein identified was myosin regulatory light chain 2, which was 46% more abundant (Spot 462; Figure 4.2, Table 4.3; \( P=0.05 \)) in the HSP group. Myosin light chain plays a regulatory role as part of myosin. Myosin is large protein (520-kDa) which has two heavy chains (approximately 220-kDa) and 4 light chains (approximately 20-kDa) (Lowey et al. 1969; Weeds and Lowey 1971; Clark et al. 2002). 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). Of the four light chains, two are
regulatory chains (20-kDa) while the others are essential light chains (17-27-kDa) (Weeds and Lowey 1971; Clark et al. 2002; Au 2004). Myosin light chain 2 specifically assists with fine tuning the myosin molecular motor (Clark et al. 2002). 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). These combined results demonstrate that dynamic changes in myosin light chain 2 may be associated the development of tenderness early postmortem.

**Conclusions**

Pork tenderness is impacted by a large variety of factors; 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 (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.
Proteins identified as being different between experimental groups included triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, mitochondrial aldehyde dehydrogenase, mitochondrial isocitrate dehydrogenase, and myosin regulatory light chain 2. The sarcoplastic proteome analysis results suggest that there is a difference in the potential for glycolytic metabolism between star probe groups 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 sarcoplastic proteome may suggest release of by-products of the mitochondrial electron transport chain, possibly due to rupture of mitochondria, causing subsequent increased use of stress related proteins or need for antioxidant proteins, such as the peroxiredoxins, to regulate these by-products, adversely impacting calpain activity and development of meat tenderness. These proteins were found in the sarcoplastic protein fraction which would allow for rapid detection without further extraction for identification. Once the role of these proteins in the development of pork tenderness is defined, robust protein biomarkers can identify products of differing quality.

Acknowledgements

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Seidler NW (2013) GAPDH: Biological Properties and Diversity


Figure 4.1 Representative 2D-DIGE gel from the sarcoplasmic fraction of aged pork *Longissimus dorsi* showing identified proteins. Immobilized pH gradient strips (11cm, pH 3-10) were loaded with 45 μg of CyDye labeled protein (15 μg each of CyDye 2, 3, and 5) and strips were run on 12.5% SDS-PAGE gel. Proteins labeled with CyDye2 are shown.
Figure 4.2 Representative 2D-DIGE gel from the sarcoplasmic fraction of aged pork Longissimus dorsi showing identified proteins. Immobilized pH gradient strips (11cm, pH 4-7) were loaded with 45 μg of CyDye labeled protein (15 μg each of CyDye 2, 3, and 5) and strips were run on 12.5% SDS-PAGE gel. Proteins labeled with CyDye2 shown.
Figure 4.3 Average star probe value (kg) of low star probe (n=6) and high star probe (n=6) groups from pork *Longissimus dorsi* chops across aging periods.
Figure 4.4 Representative Western blot of intact and degraded desmin in pork *Longissimus dorsi* (LM) whole muscle samples aged over time. Intact bands (55-kDa) and degradation bands (38-kDa) were compared to corresponding bands of a 0-d aged pork LM sample (Ref). Star probe values (kg) are provided for the samples and samples are labeled high/low depending on star probe force.
Figure 4.5 Representative Western blot of sarcoplasmic calpain-1 autolysis in 1 d aged *Longissimus dorsi* (LM) muscle pork samples. Samples in low and high star probe groups were compared to a day 0/7 mixed LM whole muscle sample (Ref) to identify the presence of intact calpain-1 (80-kDa), and autolyzed (78-kDa and 76-kDa) protein bands.
Table 4.1 Summary of fresh pork loin quality attributes, proteolysis of whole muscle protein fraction desmin and sarcoplasmic protein fraction calpain-1 autolysis in pork Longissimus dorsi (LM) of selected star probe groups.

<table>
<thead>
<tr>
<th>Item</th>
<th>Low Star Probe Group (n=6)</th>
<th>High Star Probe Group (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days Aged</td>
<td>1     8   14   21</td>
<td>1     8   14   21</td>
</tr>
<tr>
<td>Purge (%)^1</td>
<td>0.14  1.32  1.83^a  2.41^a</td>
<td>0.16  1.64  3.27^b  4.54^b</td>
</tr>
<tr>
<td>pH^2</td>
<td>5.82^ab 5.79^bc 5.86^a 5.86^a</td>
<td>5.76^c  5.73^c  5.78^bc  5.76^bc</td>
</tr>
<tr>
<td>Fabrication pH^3</td>
<td>5.94</td>
<td>5.96</td>
</tr>
<tr>
<td>Color Score^4</td>
<td>3.1   2.8   2.5   2.5   0.2</td>
<td>3.1   2.7   2.6   2.6   0.2</td>
</tr>
<tr>
<td>Marbling Score^5</td>
<td>2.0^a  2.3^a  2.0^a  2.5^a  0.2</td>
<td>1.3^b   1.5^b  1.4^b   1.7^b  0.2</td>
</tr>
<tr>
<td>Cook Loss (%)^6</td>
<td>22.40 17.32^a 20.47 18.71^a 1.00</td>
<td>19.71  20.49^b 22.49 22.22^b 1.00</td>
</tr>
<tr>
<td>Hunter L value^7</td>
<td>44.96 49.79 49.96 50.55 0.76</td>
<td>44.34  49.23 48.44 50.25 0.76</td>
</tr>
<tr>
<td>Hunter a value^7</td>
<td>11.81 13.91 13.87 13.79 0.26</td>
<td>11.97  13.19 13.93 13.58 0.26</td>
</tr>
<tr>
<td>Hunter b value^7</td>
<td>2.86^b 4.24^a 4.23^a 3.92^a 0.19</td>
<td>2.71^b  3.89^a 3.82  3.77  0.20</td>
</tr>
<tr>
<td>Intact Desmin^8</td>
<td>1.16^ab 0.58^de 0.47^e 0.38^e 0.12</td>
<td>1.32^a  0.81^cd 0.81^cd 0.93^bc 0.13</td>
</tr>
<tr>
<td>Autolyzed Calpain-1 (%)^9</td>
<td>53.67  -    -    -    9.14</td>
<td>57.83  -    -    -    9.14</td>
</tr>
</tbody>
</table>

1. Percent chop purge = (weight of package with purge - weight of package without purge/chop weight) x 100.
2. pH measurements were taken at the center of each chop.
3. pH measurements taken at the commercial processing plant at approximately 20 hours postmortem.
4. National Pork Board standards, 6-point scale (1 = pale pinkish gray/white; 6 = dark purplish red)
5. National Pork Board standards, 10-point scale (1 = 1% intramuscular fat; 10 = 10% intramuscular fat)
6. 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.
7. Hunter L, a, and b determined with Minolta Chroma Meter with D65 light source, 50 mm aperture, and 0° observer.
8. Ratio indicates abundance of intact desmin within samples at each day of aging compared to intact desmin from a 0 d aged reference sample (myofibrillar and sarcoplasmic proteins) present on each gel.
9. Percentage indicates the percent of autolyzed calpain-1 as a total of calpain-1 in each sample.
10. a, b Means within rows and day of aging with different superscripts are significantly different within classification (P<0.05).
Table 4.2 Identified proteins of 2D-DIGE experiment 11 cm immobilized pH gradient strip, pH 3-10.

<table>
<thead>
<tr>
<th>Spot Number</th>
<th>Protein</th>
<th>Ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>246</td>
<td>Mitochondrial Aldehyde Dehydrogenase</td>
<td>-2.14</td>
<td>0.14</td>
</tr>
<tr>
<td>248</td>
<td>Pyruvate Kinase</td>
<td>-1.84</td>
<td>0.16</td>
</tr>
<tr>
<td>272</td>
<td>Creatine Kinase M-Type</td>
<td>-1.64</td>
<td>0.12</td>
</tr>
<tr>
<td>305</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>-2.37</td>
<td>0.11</td>
</tr>
<tr>
<td>314</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>-1.59</td>
<td>0.15</td>
</tr>
<tr>
<td>353</td>
<td>Triosephosphate Isomerase</td>
<td>-1.54</td>
<td>0.13</td>
</tr>
<tr>
<td>355</td>
<td>Triosephosphate Isomerase</td>
<td>-1.52</td>
<td>0.13</td>
</tr>
<tr>
<td>444</td>
<td>Adenylate Kinase Isoenzyme 1</td>
<td>-2.08</td>
<td>0.08</td>
</tr>
</tbody>
</table>

1Ratio 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.
Table 4.3 Identified proteins of 2D-DIGE experiment 11 cm immobilized pH gradient strip, pH 4-7.

<table>
<thead>
<tr>
<th>Spot Number</th>
<th>Protein</th>
<th>Ratio¹</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>161</td>
<td>Heat Shock Cognate 71 kDa protein</td>
<td>1.14</td>
<td>0.09</td>
</tr>
<tr>
<td>214</td>
<td>Myc box-dependent-interacting protein 1</td>
<td>-1.23</td>
<td>0.07</td>
</tr>
<tr>
<td>244</td>
<td>Mitochondrial Dihydrolipoyl Dehydrogenase</td>
<td>-1.19</td>
<td>0.05</td>
</tr>
<tr>
<td>256</td>
<td>Protein Disulfide-Isomerase</td>
<td>-1.41</td>
<td>0.04</td>
</tr>
<tr>
<td>264</td>
<td>Mitochondrial Aldehyde Dehydrogenase</td>
<td>-1.32</td>
<td>0.02</td>
</tr>
<tr>
<td>271</td>
<td>Mitochondrial ATP Synthase Subunit Beta</td>
<td>-1.71</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>272</td>
<td>Annexin A7</td>
<td>-1.33</td>
<td>0.15</td>
</tr>
<tr>
<td>273</td>
<td>Hsc 70-Interacting Protein</td>
<td>1.14</td>
<td>0.20</td>
</tr>
<tr>
<td>310</td>
<td>Mitochondrial Isocitrate dehydrogenase subunit alpha</td>
<td>-1.25</td>
<td>0.06</td>
</tr>
<tr>
<td>332</td>
<td>Phosphoglycerate Kinase 1</td>
<td>-1.36</td>
<td>0.04</td>
</tr>
<tr>
<td>371</td>
<td>Annexin A5</td>
<td>-1.20</td>
<td>0.11</td>
</tr>
<tr>
<td>409</td>
<td>Peroxiredoxin-2 (Fragment)</td>
<td>-1.25</td>
<td>0.20</td>
</tr>
<tr>
<td>462</td>
<td>Myosin Regulatory Light Chain 2 Isoform</td>
<td>-1.46</td>
<td>0.05</td>
</tr>
</tbody>
</table>

¹Ratio 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.
CHAPTER 5. GENERAL CONCLUSION

Pork quality is a multifactorial issue that varies greatly in the current marketplace (Bachmeier et al. 2016). Being able to predict pork quality is difficult due to the diverse array of factors that impact its development. One fundamental part of pork quality is tenderness (Moeller et al. 2010). Pork tenderness is impacted by a variety of factors including rate and extent of pH decline (Melody et al. 2004; Boler et al. 2010), lipid content (Lonergan et al. 2007; Wilson et al. 2017), collagen content (Nishimura et al. 2009), and postmortem protein changes (Carlin et al. 2006; Liu et al. 2016; Carlson et al. 2017a). Postmortem protein variations mainly involve modifications to the structure and function of these proteins. Proteolysis is the primary contributor to these changes which may be affected by the rate and extent of pH decline, oxidation, protease activity, protease inhibitor activity, and post translation modifications. Emphasis on these proteins is essential to understanding how pork tenderness development occurs and can be predicted.

The rate of pH decline was not collected with this sample set. The rate of pH decline would have been beneficial in understanding how this decline may have impacted proteolysis, quality attributes and abundance of proteins in sarcoplasmic proteome. However, the fabrication pH was measured at approximately 24 hours postmortem and was not significantly different demonstrating similar pH values at that timepoint. Identifying muscle fiber type would also assist in determining if the observed differences in abundance of glycolytic proteins were due to fiber type differences between experimental groups. Additionally, examining posttranslational modifications and oxidation states of proteins would help to understand changes to proteins. Collection of these data would assist in further defining differences between groups classified by high and low star probe value.
Freezing is the most common method of extending shelf life and product safety (Leygonie et al. 2012). However, the process of freezing and thawing can negatively impact pork quality. Identifying ways to maintain pork quality through freezing and thawing will assist in maintaining pork quality. Implementation of post-aging freezing is proposed to minimize the negative impacts of freezing and thawing (Kim et al. 2011; Coombs et al. 2017). Even with the implementation of post-aging freezing, pork quality factors such as water holding capacity and color can still be significantly impacted by the freezing and thawing of pork. Freezing pork at 1 d postmortem will not allow for pork quality attributes to improve. A better understanding of the mechanisms that impact water holding capacity and color during post-aging freezing pork products will help to identify methodologies to maintain pork quality through freezing and thawing of pork.

Star probe and Warner-Bratzler shear are highly correlated instrumental tenderness measurements. The aging response of pork longissimus muscle over 21 d aging demonstrated star probe and Warner-Bratzler shear force values did not improve after 8 d aging. Based on our results, pork products need to be aged at least 8 d postmortem for proper tenderness development. It would be of interest to examine the aging response of pork over individual days between 1 and 8 d aging to identify if tenderness development continues to improve out to 8 d aging or if instrumental tenderness measurements cease to decline prior to 8 d aging. This would assist the industry in identifying the most suitable aging period for pork products.

Carlson et al. (2017b) demonstrated that pork quality attributes, proteolysis, and sarcoplasmic proteome differences exist in aged (11-16 d) pork loin classified by high and low star probe values. The current experiments identify consistent results except identified difference in early postmortem (1 d aged) pork loin chops. Pork loin chops from the LSP group had greater
marbling scores and pH values at each day of aging but were not different at the commercial processing facility. The LSP group also had greater protein degradation at 14 and 21 d aging compared with the HSP group. The HSP group had a greater abundance of some isoforms glycolytic proteins compared with the LSP group. These combined results demonstrate that differences in pork quality is multifactorial and cannot be determined based on a single factor. Investigating the role of these glycolytic proteins is essential to further understand how these proteins impact tenderness development. To understand further these protein’s roles in the development of pork quality, it would be beneficial to analyze posttranslational modifications such as phosphorylation and oxidation of these proteins to identify if their activity is enhanced or inhibited in either experimental group influencing the observed differences in SP value. Additionally, identifying the predominant fiber type and myosin light chain isoform would help to explain why the isoform identified in this experiment was greater in the HSP group (Bjarnadóttir et al. 2012). Also, determining the phosphorylation state of this isoform may indicate differences in the regulation of myosin and rate at which rigor development occurred during the conversion of muscle to meat, impacting the rate of pH decline. Investigating the presence of the considerable number of proteins originating from the mitochondria is of interest. If mitochondrial electron transport chain by-products such as reactive oxygen species are lost into the sarcoplasm due to possible bursting of the mitochondria membrane, this may result in greater activation of heat shock proteins or greater need for antioxidant proteins such as those in the peroxiredoxin family. This could explain their abundance differences in the sarcoplasmic proteome of the star probe groups. Identifying why these proteins are present in the sarcoplasmic proteome and how they may have influenced the development of tenderness would help understand the role of these mitochondrial proteins.
The continued use of modern technologies like proteomics, genomics, transcriptomics, metabolomics, and lipomics are essential to identifying robust biomarkers for understanding differences in pork quality. The use of the sarcoplasmic proteome to identify these biomarkers is also essential to the pork industry due to no further extraction of proteins being required. Rapid identification technologies must follow their identification to differentiate between high and low pork quality. Being able to quickly identify these differences will allow for implementation of a pork grading system to provide consumers with a consistently tender pork product.

**Literature Cited**


Table A.1 Proteins identified from 1 d aged pork *Longissimus dorsi* (LM) muscles (11 cm, pH 3-10 Immobilized pH Gradient strips).

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|    |               |               |        |      |    |       | MQHLIAR  
|    |               |               |        |      |    |       | GIFPVVCK | 14491
<p>| 272 | Creatine Kinase M-Type | Sus Scrofa | Q5XLD3 | 7.09 | 43.0 | 70.08 | HNNHMAK | LSVEALNLSTGEFK | AGHPFMWNEHLGYVLTCPSNLGTGLR | AGHPFMWNEHLGYVLTCPSNLGTGLR | SFLVWVNEEDHLR | LSVEALNLSTGEFKGK | DLFDPQIQDR | LGSSEVEQVQLVVDGVK | GTGGVDTAAGVSNDVSAD | RGTGGVDTAAGVSNDVSAD | SMTEQEQQQLIDDHFLFDKPVSPLLASGMAR | SMTEQEQQQLIDDHFLFDKPVSPLLASGMAR | SMTEQEQQQLIDDHFLFDKPVSPLLASGMAR | SMTEQEQQQLIDDHFLFDKPVSPLLASGMAR | TDLNHENLKGDLDLPNYVLSSR | AEEEPDLSK | GQSIDDMIPAQK | GQSIDDMIPAQK | GYTLPPHCSR | TDLNHENLKGDDLDLPNYVLSSR | TDLNHENLKGDDLDLPNYVLSSR | TDLNHENLKGDDLDLPNYVLSSR | GWHNDNK | LVMEMEK | LVMEMEK | LVMEMEK | HKTDLNHENLKGDDLDLPNYVLSSR | HKTDLNHENLKGDDLDLPNYVLSSR | HKTDLNHENLKGDDLDLPNYVLSSR | LVMEMEKK | PFGNTHNK | HGGYKPTDK | IEEIFKK | ALTLEIYK | 107860 |</p>
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1. Isoelectric point (pI) and molecular weight (MW) are theoretical.
2. Percentage of the MASCOT protein sequence covered by identified matching peptides from trypsin digest.
3. MOWSE – Molecular weight search, score used to calculate the similarity in molecular weight of the peptides from trypsin digest and the proteins from the MASCOT database.
Table A.2 Proteins identified from 1 d aged pork *Longissimus dorsi* (LM) muscles (11 cm, pH 4-7 Immobilized pH Gradient strips).

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|    | Mitochondrial ATP Synthase Subunit Beta | Homo Sapiens | P06576 | 5.40 | 56.5  | 42.00 | LVLEVAQHLGESTVR
|    |                                            |              |        |      |       |       | VLDSDAPIK
|    |                                            |              |        |      |       |       | VLDSDAPIKIPVGPETLGR
|    |                                            |              |        |      |       |       | TVIIMELINVAK
|    |                                            |              |        |      |       |       | TVIIMELINVAK
|    |                                            |              |        |      |       |       | VALTGLTVAEYFR
|    |                                            |              |        |      |       |       | VALVYGQMNEPPGAR
|    |                                            |              |        |      |       |       | VALVYGQMNEPPGAR
|    |                                            |              |        |      |       |       | VVDLLAPYAK
|    |                                            |              |        |      |       |       | TREGNDLYHEMIESGVINLK
|    |                                            |              |        |      |       |       | TIAMDGTEGLVR
|    |                                            |              |        |      |       |       | TIAMDGTEGLVR
|    |                                            |              |        |      |       |       | EGNLHHEMIESGVINLK
|    |                                            |              |        |      |       |       | EGNLHHEMIESGVINLK
|    |                                            |              |        |      |       |       | FTQAGSEVSALLGR
|    |                                            |              |        |      |       |       | AIAELGIYPAVDPLDSTSR
|    |                                            |              |        |      |       |       | AHGGYSVFAGVGER
|    |                                            |              |        |      |       |       | IGLFGGAGVVK
|    |                                            |              |        |      |       |       | IPSAVGYQPTLATDMGTMQER
|    |                                            |              |        |      |       |       | IPVGPETLGR
|    |                                            |              |        |      |       |       | IPSAVGYQPTLATDMGTMQER
|    |                                            |              |        |      |       |       | IPSAVGYQPTLATDMGTMQER
|    |                                            |              |        |      |       |       | IMDPNIVGSEHYDVAR
|    |                                            |              |        |      |       |       | IMDPNIVGSEHYDVAR
|    |                                            |              |        |      |       |       | IMNVIGEPIDER
|    |                                            |              |        |      |       |       | IMNVIGEPIDER
<p>|    |                                            |              |        |      |       |       | 8454  |</p>
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<td>Homo sapiens</td>
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<td>6.92</td>
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<td>23.00</td>
<td>MSDGLFLQK NVTAIQGPGGK SNVTAVHK TPYTDVNIVTIR TPIAAGHPSMNLLL R APIQWEER CSDFTEEICR IAEFAFEYAR</td>
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<td>Auto-PI</td>
<td>MW (%)</td>
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<td>LYGAYELK</td>
<td>GTVDHFPGFDER</td>
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1. Isoelectric point (pI) and molecular weight (MW) are theoretical.
2. Percentage of the MASCOT protein sequence covered by identified matching peptides from trypsin digest.
3. MOWSE – Molecular weight search, score used to calculate the similarity in molecular weight of the peptides from trypsin digest and the proteins from the MASCOT database.