Development of High Throughput One Dimensional Proteomics for the Analysis of Meat and Muscle

DISSERTATION

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By

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ABSTRACT

In this dissertation, some of the limitations and issues related to current popular proteomic approaches will be discussed and a case will be made for the benefits of using one dimensional electrophoresis based proteomics for agricultural study setups. One dimensional electrophoresis based proteomics have an important advantage over conventional two dimensional proteomic technologies because they are statistically robust and cost effective. The incorporation of greater numbers of samples and replicates can help alleviate the inherent variability of agricultural models that can adversely bias and influence the results. The goal of this dissertation is to develop proteomic applications which can be applied to agricultural experimental designs. Specifically, 3 one-dimensional electrophoresis (1DE) based proteomic applications for meat science, food science, and muscle biology interests are proposed. The rationale for this dissertation resides in the popularity of proteomic research over the past decade, where its application has extended over to multiple fields. The proteomic techniques used more often are two-dimensional electrophoresis (2DE) based. Proteomic studies that employ 2DE can deliver high resolution but suffer from replication issues which make them unsuitable for large population inference. The implementation of high throughput proteomics based on the high repeatability of 1DE can provide the robustness required for agricultural experimental designs. Each of the three studies presented explores a different aspect in which an experiment of agricultural interest is coupled to a 1DE based
proteomic method. The first study explores meat tenderness prediction after meat aging from the raw meat sampled just prior to fabrication. This was achieved by obtaining myofibrillar fraction proteomic patterns that were analyzed through a linear regression model to identify the electrophoretically separated bands that are associated with a tenderness estimator (Warner-Bratzler Shear force). The second study attempted a semi-quantitative abundance estimation of individual proteins identified through mass-spectrometry in cooked meat. The third study explored proteomic profiles through a multivariate approach. By the use of principal component analysis, the proteomic profiles of turkey breasts were segregated by genotype, nutritional status and developmental stage. The studies presented increase our knowledge about meat tenderization mechanisms; explore possible implications for the authentication of meat cut identification in cooked meat, which has never been done before; and, provide a proteomic tool that would allow animal scientists to design and evaluate the proteomic variability in their experimental designs.
DEDICATION

I would like to dedicate this accomplishment to my wife Ileana, my daughter Sofia and my son Dario. Also, I dedicate this dissertation to my brother Rodrigo, and my parents.
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CHAPTER 1

Literature Review

The development of newer analytical techniques with application in comparative studies can have an enormous impact on how science is currently conducted. One of those newer technologies being developed are proteomics. Proteomic techniques were developed to analyze proteome characteristics in a more comprehensive way, a way that can identify and evaluate proteome patterns as a whole and not just by adding up single protein comparisons. However each research field poses its own challenges and thus, the marvels of proteomic research still have to be adapted to overcome those challenges. In this dissertation, an effort is made to adapt proteomic techniques to agricultural research, specifically to meat and muscle research. A series of separate studies demonstrates the potential application of proteomic techniques for the prediction of meat quality, the characterization of muscle traits for authentication purposes and a proteomic profile evaluation tool for monitoring developmental patterns. The expectation of this dissertation is to deliver relatively low cost proteomic methodologies accessible to scientists suitable for common, animal science or any biological experimental designs where inference over large populations is required. The methodologies presented will provide an alternative for scientists when performing proteomic studies.
Advances in proteomic research

Genome/RNA based techniques have been increasing our knowledge of transcriptional events of growth and development in animals. However, it is ultimately the transcribed proteins, the proteome, that give rise to the phenotypes responsible for their variability. Over the past 15 years the study of the proteome has become a frontline tool to elucidate some of the vast amount of information gathered by functional genome characterization (Bendixen 2005; Han and Wang 2008). Although the genetic information, in terms of the sequence of the nucleotides, within an animal remains relatively unchanged throughout its life, the transcriptional patterns of genes into mRNAs, that are ultimately translated into proteins, are influenced by developmental, and environmental factors (Hollung, Veiseth et al. 2007). Absolute quantification of gene expression events, based on transcriptional events is affected by post-translational modifications of proteins (Gygi, Rochon et al. 1999). Proteomic methodologies attempt to translate this diverse and ambiguous genomic information into a tangible and quantifiable description of the protein biological systems that are carrying out physiological functions.

The most common proteomic technologies available are based on two dimensional electrophoresis (2DE) (Gorg, Postel et al. 1980) in which proteins are first resolved based on their isoelectric point then subsequently resolved based on their relative molecular size. This method has been further refined into a technique termed Differential Image Gel Electrophoresis (DIGE) that compares electrophoretically resolved spots (proteins) from two samples in which each of two samples has been
stained with different colored fluorescent dyes (Alban, David et al. 2003; Issaq and Veenstra 2007). Although this methodology can theoretically resolve more than a thousand proteins based on their individual physicochemical properties, the method is limited by issues of relatively high cost, spot recognition, difficulties in quantification, low throughput, and low reproducibility (Mann 1999; Kang, Techanukul et al. 2009).

Non-gel based methodologies such as Isotope-Coded Affinity Tags (ICAT) (Gygi, Rist et al. 1999) and isobaric Tag for Relative and Absolute Quantitation (iTRAQ) (Ross, Huang et al. 2004) are techniques used for quantitative proteomic analysis and are extremely efficient but are unsuitable for the large experimental setups, because of the vast amounts of information generated. Innovative combinations of proteomic methodologies are being developed constantly, mostly because of the necessity to adapt current technologies to specific research scenarios to bypass specific hurdles and to improve the efficiency of current methods. A recent innovative method combined iTRAQ labeling and 2DE to quantify an artificial mixture of various different proteins which demonstrates the potential applicability of this method for quantitative mass-spectrometry proteomics (Wiese, Reidegeld et al. 2007). Another study performed 2DE immuno-blotting of brain, myelin fraction and myelin-axolemmal proteins from biotinylated cerebrospinal fluid derived from multiple sclerosis patients. Proteins that reacted with the biotinylated sera were further analyzed by a Matrix Assisted Laser Desorption Ionization-Tandem Time of Flight-Time of Flight mass spectrometry (MALDI-TOF-TOF). This method allowed for the identification of potential biomarkers without the need to pool or
concentrate samples (Menon, Steer et al. 2011). The hurdles encountered in proteomic studies are often unique to the individual fields of research.

In agricultural research, variability is major concern. Agricultural studies are subject to a multitude of external factors that are hard to identify, isolate, and control. These factors include temporal and spatial events during growth and development, season, and geography. Environmental factors such as nutrition, pollution, husbandry practices (Stein 1998; Bannayan, Sadeghi Lotfabadi et al. 2011), and genetic variability are important factors that need to be taken into account in agricultural studies (Cartwright 1974; Haydar, Mandal et al. 2007; Sullivan 2007). In summary, in order to implement proteomic studies in agricultural studies it is important to develop proteomic tools that can accommodate large sample sizes and have high reproducibility. Although several studies have used 2D based proteomics this review will establish that the use of 1DE as an initial protein resolution step is capable of addressing a wider range of variables encountered in proteomic analyses of animals of agricultural interest and is thus a powerful tool for elucidating the proteins playing roles in the biological mechanisms of interest to agricultural scientists.

Proteomics in meat science

In the animal science field, proteomic studies have become more common in the past decade (Lippolis and Reinhardt 2008). The goals of the meat industry are to increase muscle growth and meat quality characteristics including tenderness in fresh meat and water holding and rheological characteristics of processed meats. In meat animal
agriculture ante mortem pressures, including genetics, nutrition and stress, combine with postmortem aging to influence the economic value of meat. Ante mortem mechanisms give rise to muscle growth and development which are important to increasing the amount of muscle and meat produced. Advances in proteomic technologies are helping researchers to identify the differential protein expression patterns and the proteins associated with the mechanisms of muscle growth, post mortem aging and meat processing characteristics.

Proteomic research in the meat science field is motivated by the revenue that can be generated by increasing meat production and the improvement in meat quality. Globally, meat consumption continues to increase steadily over the last 50 years where a 1.5 fold increase occurred from 1961 to 2003. In the United states meat consumptions has nearly doubled from 1909 to 2007 (Daniel, Cross et al. 2011). Because of the power of proteomics to enhance knowledge of the proteins playing roles in biological phenomenon, it is not hard to understand why proteomic research has been applied to such a wide variety of areas in meat science research (Bendixen 2005)such as growth and development in pigs (Lametsch, Kristensen et al. 2006), cattle (Bouley, Meunier et al. 2005), poultry (Doherty, McLean et al. 2004; Teltatham and Mekchay 2009) and fish (Reddish, St-Pierre et al. 2008), postmortem metabolism (Jia, Hollung et al. 2006), meat tenderization (Sawdy, Kaiser et al. 2004; Zapata, Zerby et al. 2009), water holding capacity (Updike, Zerby et al. 2005), meat processing and food authentication (Piñeiro, Barros-Velázquez et al. 2003).
Phenotypic variations within muscle tissue and between similar muscle tissues from animals with different genetic and environmental backgrounds are important in meat science because it is these variations that comprise the elements that will ultimately determine meat quality. Meat producers, processors, and customers have identified inconsistency in meat quality, as defined by a lack of uniformity/consistency in marbling and tenderness, as one of the most important economic factors negatively impacting the meat industry (Boleman, Boleman et al. 1997; McKenna, Roebert et al. 2002). Meat quality parameter variation has been successfully analyzed using high throughput 1DE based proteomics studies where meat tenderness was predicted from the band intensity of electrophoretic bands (Sawdy, Kaiser et al. 2004; Zapata, Zerby et al. 2009). In addition, 1DE based proteomics have also been used for describing the differences in thermally induced meat gels (Updike, Zerby et al. 2005). Other areas have also been explored such as the proteomic characterization of yellow perch in relation to fish weight and length (Reddish, St-Pierre et al. 2008).

The post-mortem mechanisms that occur during the “aging” process that gives rise to meat tenderization have remained unsolved (Ouali, Herrera-Mendez et al. 2006). To elucidate the mechanisms of meat tenderization a multitude of studies using both genomic and proteomic approaches have been performed. Investigators using a transcriptomics based study using RNA based microarray technology suggested that the expression of a heat shock protein, that had been previously described as having an anti-apoptotic role, accounted for 60% of the variability meat tenderness in the genetic line investigated (Bernard, Cassar-Malek et al. 2007). However, the expression of mRNA is
not always directly related to protein concentration (Gygi, Rochon et al. 1999), although, in average, the correlation between a population of genes and protein abundance are in substantial agreement (Greenbaum, Jansen et al. 2002). In a few instances, phenotype variation is related to the mutation of a single gene, like the structural differences in pigs muscle due to the RN allele (Alarcón-Rojo, Zapata et al. 2008), double muscling in cattle due to mutations in the myostatin gene (McPherron and Lee 1997), pale soft exudative syndrome in pigs due to a mutation in the ryanodine receptor (Otsu, Khanna et al. 1991), or the structural differences in Callipyge lamb muscle (White, Vuocolo et al. 2008).

Ultimately it is the proteins that underlie the function of the cell, tissue and organ. For example, it has been shown that RNA degradation is not extensive enough to be the limiting factor for postmortem protein synthesis (Fontanesi, Colombo et al. 2008). Regardless of RNA viability, energy availability required for protein synthesis plus the decrease in the functionality of protein synthesis machinery by the low pH abolishes the de novo protein synthesis (Pösö and Puolanne 2005). Thus, several investigators have suggested that proteomic pattern alteration, in postmortem skeletal muscle, is mostly the result of postmortem protein modification and proteolysis (Goll, Boehm et al. 1998; Geesink, Kuchay et al. 2006; Ouali, Herrera-Mendez et al. 2006).

Food authentication has become a major concern for consumers in the recent years. Consumers are concerned about labeling of products in terms of origin, meat substitution, meat processing treatment and non-meat ingredient addition. There are several DNA based protocols currently used to identify the species, sex or even the breed of animals present in meat products. There are also several technologies that can identify
country of origin, rearing and feed intake based on specific markers using ELISA, chromatography, DNA, microscopy and spectroscopy. However, there are currently no quantitative methodologies available for the identification of meat cuts (Ballin 2010). The value of fresh meat is predicated on the meat cut. Processor and retailers, including restaurants, charge based on the meat cut being supplied. Meat cut differences can be identified during a visual inspection by trained personnel and some general aspects of their differences can be spotted by consumers. Meat quality parameters have been characterized for individual muscles in the carcass of an animal. For instance, inherent differences in terms of meat quality parameters were associated to muscle types in lambs (Tschirhart-Hoelscher, Baird et al. 2006), in pigs (Hu, Wang et al. 2008) and beef (Cho, Kim et al. 2010). Meat cut differences are phenotypic, that is they are based on the inherent muscle structure within the cut of meat at both the macroscopic (or visual) and the cellular level. After water, muscle is composed predominantly of protein. Therefore, it is reasonable to hypothesize that proteomic technologies could be used to differentiate muscle groups or individual muscles based on protein abundance patterns unique to each of the muscle groups, and thus authenticate the cut of meat. In addition, the proteomic technologies would also be capable of differentiating meat cuts even after meat processing treatment including cooking.

The impact on nutrition to meat quality is an active area of research. Over the past decade, consumers have shown an increased interest for natural and organically grown meat. There is interest in the benefits of grass fed beef, which is viewed a more natural and healthy, versus grain fed beef, which is the most common meat industry feeding
practice (Xue, Mainville et al. 2010). The same phenomenon affects the preference of grass fed over grain fed lamb (Font i Furnols, Realini et al. 2011). Proteomic analysis was used to characterize the differences between grass fed and grain feed beef (Shibata, Matsumoto et al. 2009). These investigators concluded that a muscle fiber type conversion to oxidative (slow-twitch) from glycolytic (fast-twitch) was associated to the feeding regime. They suggested that the conversion was related to a change in the energy metabolic enzyme balance that is motivated by grazing in the latter fattening period.

Understanding the mechanisms of skeletal muscle growth and development has the potential of increasing the economic value meat animals and is therefore an active focus in meat science research. Growth and development studies have focused on the participation of anabolic steroids and growth factors. For example, IGF-1 has been shown to play a predominant role in promoting normal muscle growth by increasing protein synthesis and inhibiting protein degradation by the ubiquitin-proteasome system (Sjogren, Liu et al. 1999). The use of hormone implants to promote animal growth has been subject to extended research (Montgomery, Dew et al. 2001). For example, a study in steers showed the benefits of animal hormone implantation with trenbolone acetate and estradiol on feedlot performance, carcass characteristics and carcass composition (Johnson, Anderson et al. 1996). The role of muscle specific growth promoters has been widely studied. Myostatin, a TGF-β protein family member that inhibits muscle differentiation and growth has been intensively studied and its signaling pathways are well characterized in non-economically important species (Lee 2004). In addition, developmental gene pattern determination has been successfully characterized in
common animal models like the fruit fly (Tomancak, Beaton et al. 2002). Unfortunately, no developmental gene pattern studies exist for animals of agricultural importance. At this point, this suggests that the use of proteomic studies for agricultural research is necessary.

Understanding the growth and developmental patterns of animals of economic importance is valuable not only because it has the potential to improve production and quality parameters but because it will likely have broader impacts by generating insights into human muscle growth, development and disease. In complex systems involving animals it is important to develop technologies that permit animal scientists to accommodate factorial designs in their proteomic studies where the effects of several variables over multiple levels need to be evaluated.

**Current proteomic research hurdles and challenges for agricultural studies**

There is a need for improved or more robust approaches to experimental design and analyses. Proteomic studies, which are founded on data generated by mass spectrometry, are often hindered when researchers are faced with vast amounts of data, derived from downstream mass spectrometric methodologies, which need to be assembled into a coherent explanation of a biological mechanism responsible for the observed phenomenon. Specifically, putting mass spectrometry data into a coherent and relevant picture is daunting because of the difficulty of interpreting mass spectrometry results. The interpretation of mass-spectrometry results is confounded because the role of the individual proteins in the biological phenomenon is not straightforward. The
interpretation of the mass-spectrometry results has been facilitated, recently, by the implementation of protein sorting tools such as the KEGG pathway/module database (Kanehisa 1996; Kanehisa, Goto et al. 2004) which can segregate the proteins identified into the pathways in which they participate. However, this method limited reliability for proteins involved in major physiological pathways such energy metabolism. For example, creatine kinase, a critical energy metabolism enzyme in skeletal muscle, is also associated with a number of cellular functions, including calcium homeostasis regulation (Wallimann 1994; Wallimann, Dolder et al. 1998).

Protein abundance estimation in a comparative study can be erroneous because of variations in the efficiency of sequence-dependent peptide ionization, the suppression of neighboring signals because of dominant peptides and missing MS/MS observations of peptide peaks because of algorithm and threshold limit settings that limit the detection (Vogel and Marcotte 2009). This is a general limitation to proteomic studies. Some of these technical limitations have been improved with the development and integration of newer mass-spectrometry technologies including LTQ/Orbitrap, which is capable of higher mass resolution, mass accuracy, a wider mass/charge range and a wider dynamic range compared to the conventional LTQ ion-trap or TOF detectors (Hu, Noll et al. 2005).

Breakthroughs in mass-spectrometry have greatly surpassed many of these protein abundance estimation limitations by offering new evaluation tools and more accurate technologies. A recent study using Leptospira interrogans demonstrated a novel methodology that permitted the abundance estimation and fold difference comparison of
more than a thousand proteins (Malmstrom, Beck et al. 2009). This technology consists in a combination of an isotopic labeling of a selected number of proteins, an average estimation of the three best detected peptides for each protein and spectral counting based. However, this technology is limited for the study of eukaryotic cells where multiple protein isoforms are generated as a result of gene splicing. Also, even though the methodology could potentially be adjusted to any experimental design, as the authors assert, it is still unlikely that it would have routine application in agricultural studies due to the large number of samples generally used in animal studies. It is likely that the analyses of multiple samples by this technology would also pose funding limitations.

Another limitation of proteomic studies is the ability to segregate relevant proteins away from the irrelevant proteins. That is, electrophoretically resolved bands and spots potentially contain more than one protein/peptide species in which it is likely that only one or two of the protein/peptides is causal or integral in the biological difference observed and the other non-causal or integral protein/peptides are simply co-migrating because of limited resolving power. Unfortunately, most of the new evaluation tools need to be adapted to the specific needs of each research field, as sample characteristics can differ enormously from cattle, humans, mice, rats including the more rarely annotated species such as fish and insects; or across fields such as agriculture medicine and horticulture.

There are important economic considerations of proteomic analyses in animal agriculture. Novel technologies are being developed in the biomedical field where core grants are more likely to facilitate the use expensive technologies for core members. This
is especially true when by additional funding augments the acquisition of new cutting
gedge equipment and services to core biomedical research facilities which helps defray the
cost of expensive technologies. Agricultural research is normally funded at lower levels
and due to the number of samples and replications involved in animal agriculture studies;
researchers are not able of taking advantage of these cutting edge services and equipment.

Limitations of 2DE based proteomics that can be bypassed by 1DE based
proteomics

Biological replicate limitations

Most studies employing 2DE methods have a limitation in the number of replicates they analyze. As an example, a recent proteomic study performed on the livers of chemically induced fibrogenesis and sclerosing cholangitis mice only analyzed three individual mice as controls and five individual mice as treated (Henkel, Roderfeld et al. 2006).

Because of the statistical power, proteomic experiments using a large number of replicates can facilitate the identification of proteins that are present in different levels across treatments and not just random animal variation. Sample replication is a limitation of most 2DE based studies. A typical 2DE based study can cost hundreds of dollars per sample, thus limiting the study due to financial barriers. This limitation can be avoided and more robust information obtained with the use of multi-dimensional high throughput proteomics based on 1DE separation followed by further protein separation using reverse phase chromatography (Li, Wang et al. 2004; Reddish, St-Pierre et al. 2008; Zapata,

**Gel and mass spectrometry limitations**

It is widely perceived that a 2DE gel each spot corresponds to an individual protein and thus the number of spots identified in a gel is the same number of proteins that can be identified after mass spectrometry, but that is not the case. It is very common in studies using 2DE based technologies to report incomplete sequencing results because of spots that yielded no identifiable proteins after the mass-spec sequencing analysis. For example, a study published in 2010 investigating yeast meiosis, using DIGE, a 2DE based methodology, identified 590 spots in the matched gels from which 79 spots showed significant differences. From those 79 spots only 66 could be cut from the gel for downstream analysis. From those 66 only 48 spots gave protein fragment identifications (Scaife, Mowlds et al. 2010). These technical issues are present to some extent on most, if not all the proteomic technologies. An older comparative study published in 2009 comparing plasma from dengue fever patients with healthy subjects, using DIGE, identified 359 spots based on Cy-5 staining. However, only 336 were still detectable after colloidal Coomassie post-staining. From the initial 359 spots, 73 spots were found to be significantly different but only 65 could be visualized after colloidal Coomassie post-staining. All 65 spots were cut from gel but only 37 yielded positive identifications which corresponded to 14 differentially expressed proteins (Albuquerque, Trugilho et al. 2009). In another comparative study, published in 2004, using DIGE to investigate the effect of
retinoic acid-induced differentiation of human leukemia cells identified 32 significantly
different spots from an undisclosed number of spots. From those 32 spots, only 22
yielded positive protein identifications (Wang, Jensen et al. 2004). These studies indicate
that proteomic limitations have persisted for nearly a decade.

However, the use of high throughput 1DE based proteomics based on a large
number of replicates coupled with a robust statistical analysis is be able to reduce the
technical issues of protein identification associated with 2DE based systems. It is
important to note that 1DE based proteomics advantage over 2DE comes at the expense
of some resolution as bands from a 1DE gel suffer more from co-migration issues than
spots from a 2DE gel.

**Resolution limitations**

It is widely perceived that 2DE has a greater resolving power than 1DE and that
each spot in a 2DE gel corresponds to an individual protein, in contrast to 1DE which can
suffer from co-migrating protein/peptides. However, there are limitations associated with
the resolving dynamics of 2DE gels. Two dimensional gels are, generally, only capable of
resolving proteins/peptides within the molecular weight range of ~20 to 100 kDa and
isoelectric points within the pH range of 3 to 10. These ranges can be extended by using
specialized low or high pH range isoelectric focusing strips and specialized gel
compositions that allow the separation of proteins with low or high molecular weights. A
brief review of the current literature reveals that the use of these strips and specialized
gels in proteomic studies with a non-selective shotgun approach is not a common
practice. A recent proteomic study with the objective to identify tumor associated
proteins as biomarkers in human esophageal squamous cell carcinoma by a 2DE approach used isoelectric focusing in the pH range from 3 to 10 in the first step and a molecular weight range from ~14 to 97 kDa in the second step (Zhang, Wang et al. 2011). In another example, a study characterizing ovine corpus luteum protein abundance patterns in by a 2DE approach, performed isoelectric focusing in the pH range from 3 to 10 and a molecular weight range from ~5 to 95 kDa (Arianmanesh, McIntosh et al. 2011).

However, appropriate these tactics were for the biological systems being investigated by these researchers, they are not generally useful for the analyses of muscle tissue since the molecular weight and the isoelectric point ranges employed leave a large proportion of proteins unresolved and thus out of the analysis. As such, these tissues are good candidates for 1DE based proteomic analyses as one of the multiple resolution stages prior to mass spectrometry (Reddish, St-Pierre et al. 2008; Zapata, Zerby et al. 2009). In addition, a research report demonstrated by using liquid chromatography and 1DE versus 2DE in a shotgun characterization of human dorsolateral prefrontal cortex tissue that 23% of the proteins/peptides identified had isoelectric points outside of the 4 to 8 pH range used in this study and that 31% had molecular weights outside the 25 to 100 kDa range used in this study. Thus, these out of range proteins could not have been resolved in a 2DE gel based system (Martins-de-Souza, Guest et al. 2011).

One dimensional electrophoresis has a clear advantage over two dimensional systems in the dynamic range of its molecular weight resolution capability. Sample fractionation and wide dynamic range of the resolving power of 1D gel characteristics
can improve the detection of proteins outside of the analysis range of 2DE gels. For example, a study of plasma proteome analyzed the low molecular weight protein fraction obtained by centrifugal ultrafiltration, this fraction was followed by 1DE and LC-MS/MS (Simpson and Greening 2011). Proteins with molecular weights outside of the range of 2DE can be separated effectively by 1DE as demonstrated in a characterization study of wheat high molecular weight glutenin subunits that range from 70 to 140 kDa. These proteins were separated by 1DE and then analyzed by MALDI-TOF-MS (Gao, Ma et al. 2010). High molecular weight proteins can be separated sufficiently to allow isoform characterization as demonstrated in a study that used 4% glycerol added to acrylamide gels to separate myosin heavy chain isoforms. The method was able to resolve myosin heavy chain isoforms type 2A, 2X, 2B and 1 from each other and allowed the investigators to estimate isoform ratios (di Maso, Caiozzo et al. 2000).

Besides isoelectric focusing and molecular weight range limitations, 2DE systems are not exempt from co-migration issues. Co-migrating proteins can confound the detection of important causal proteins and the interpretation of results (Gygi, Corthals et al. 2000). In the development of a proteome map of *maize rachis* by 2DE and LC-MS/MS it was found that 416 spots represented 517 distinct proteins. From those 416 spots, 143 (34%) contained multiple proteins and that 103 proteins (19.9%) were present in multiple spots, likely due to isoform ambiguity and post-translational modifications (Pechanova, Pechan et al. 2010). It has been suggested that a combination of methods be used to validate the results of 2DE based proteomic analysis (Ou, Kesuma et al. 2006).

*Statistical methods to resolve co-migration.*
Despite the advantages of high throughput, high reproducibility and relative low cost, the major drawback of 1DE based proteomics is the confounding effect of the number of protein(s)/peptide(s) co-migrating in a given band (Sawdy, Kaiser et al. 2004; Zapata, Zerby et al. 2009). Protein ID interpretation is difficult because there is uncertainty resulting from the presence of a mixture of proteins that may participate in the biological mechanism studied and irrelevant proteins that likely make up the background (Reddish, St-Pierre et al. 2008; Zapata, Zerby et al. 2009).

The incorporation of protein abundance estimators has the potential of sorting out the co-migration issue associated to 1DE based proteomics. Protein abundance estimation methods like the exponentially modified protein abundance index (emPAI) (Ishihama, Oda et al. 2005), or the, absolute protein expression (APEX) based fragment count estimations from MS/MS data (Lu, Vogel et al. 2007), or the quantification of peptides of interest by comparison to a calibrated isotope labeled reference (Lange, Picotti et al. 2008), or by the use of a modified spectral count index (mSCI) derived from the incorporation of mRNA and protein expression data (Sun, Zhang et al. 2009), or even a combined method (Malmstrom, Beck et al. 2009) that uses multiple estimators can be incorporated into the analysis of 1DE bands. The incorporation of abundance estimators can provide precise results that can sort the relevant proteins out of a mixture of confounding background proteins.

The reliability of mass spectrometry data and the protein identifications derived from it is a generally accepted by the proteomics community. However, an additional issue associated with 2DE gel resolution resides in the ability of any software to
recognize a spot consistently across several gels. In theory 2DE has the ability to separate more than a thousand spots, but the reproducibility of that separation across technical and biological replicates is not consistent. Spot recognition and matching is performed with the aid of software which relies on complicated algorithms that are not perfect. A study compared the efficiency of three popular DIGE analysis software packages; DeCyder V6.5 (GE-Healthcare), Progenesis SameSpots V3.0 (Nonlinear Dynamics), and Dymension 3 (Syngene) on the analysis of two cancer cell lines. The study revealed differences in each of the software’s capability to detect spots. Fold changes were substantially different across the different software packages which indicates that spot quantification was software dependent despite the use of internal standards (Kang, Techanukul et al. 2009). Gel analysis in a 2DE experiment is very subjective and carries operator bias. Automation and standardization efforts to eliminate user bias are desired but are not always achieved (Franziska, Stefan et al. 2010). These investigators highlight the potential that a set of gels matched in one laboratory by a specific operator will not be matched the same way by a different laboratory with a different operator. It is likely that even within the same laboratory differences will exist. The low reproducibility inherent in 2DE based separations makes the matching required for the analysis of 2DE gels more complex than the matching required for the analysis of 1DE gels. In the study of differential muscle protein concentrations associated with and predictive of tenderness determined that there was no statistical difference between the multiple 1DE gel experiments performed over a period of several days (Sawdy et al., 2004). Thus, the
matching required for the analysis of 2DE gels is more complex and potentially less reliable than the matching required for the analysis of 1DE gels.

**Statistical analysis limitations**

Proteomic studies handle vast amounts of data, thus the likelihood of reporting false discoveries is large. In a mass spectrometry protein identification, fragment ion spectra are assigned to peptide sequences by using database search engines like Mascot, Sequest, or X!Tandem. Then protein identifications are inferred by assembling the identified peptide sequences into proteins (Reiter, Claassen et al. 2009). Neither database entries, nor sequencing results are perfect. Therefore, it is important to control the reliability of protein identifications. Proteomic studies expect to identify proteins that are truly differentially expressed due to environmental and or genetic influence, but the large number of comparisons encountered in a typical proteomic study makes statistical multiple testing issues a major concern (Franziska, Stefan et al. 2010). While performing a large number of independent statistical tests at a certain confidence level, the likelihood of observing false differences is proportional to the sum of the error for each test (Karp, McCormick et al. 2007). This means that in an experiment performing 500 independent tests at a 99% confidence level the experiment will likely contain 5 erroneous tests (0.01 times 500). If the confidence level is dropped to 95%, the experiment will likely contain 25 erroneous tests (0.05 times 500). The most popular method available for the control of false discovery is known as the false discovery rate correction. This method uses the outputs of MS/MS search engines to calculate $q$ values for each comparison made. The $q$ value is the expected proportion of false positives incurred when a significant difference
(Higgs, Knierman et al. 2007) is found through the use of a $p$ value. The $p$ value is a measure of significance in terms of the false positive rate of the test for an individual test. However, the $q$ value is a measure in terms of the false discovery rate which is dependent on the entire number of individual tests performed in the study. Thus, by using this method, to declare a significant different, the test must pass the $p$ value and the $q$ value criteria (Karp, McCormick et al. 2007). To illustrate this point, a study that evaluated the response to mobile phone radiation of two types of human primary endothelial cells by DIGE, which subsequently used a t-test approach with a 99.99% confidence level and a false discovery rate correction, found that from the 1746 spots analyzed in the gels 368 spots were found to differ between the two cell types, which was expected. However, when analyzing each of the cell types separately to evaluate the effects of mobile phone radiation researchers found, using independent t-tests at a 95% confidence level and no false discovery rate correction, that one cell type displayed 35 significantly different protein spots compared to its control while the other cell type displayed 2 significantly different protein spots compared to its control. When the false discovery rate was implemented, all the significantly different spots detected previously were recognized as false positives (Nylund, Kuster et al. 2010). In a separate study investigating plasma biomarkers in pediatric patients undergoing cardiopulmonary bypass by 2D-DIGE (Umstead, Lu et al. 2010), identified 556 protein spots in all gels. Out of those 556 spots 175 were significantly changing according to independent t-tests at a 95% confidence level. The results were grouped by protein name and a total of 25 proteins were identified. In a very interesting step, the results were reevaluated by applying a
Bonferroni correction for multiple comparisons and the protein list was reduced to 17 proteins identified after grouping. In an alternate approach, samples were subject to a multianalyte profile assay where the samples are challenged against 90 different human antigens related to tissue damage, inflammation and other pathologies. The multianalyte profile assay showed that 49 out of the 90 proteins assayed changed significantly at a 95% confidence level; however, after a Bonferroni adjustment the number of proteins assayed that were significant were now 21. Even when the authors of that study did not comment on the discrepancies as the study is merely designed as exploratory, it is evident to the reader that inference based on proteomic results without an experiment wise error correction can be risky, based on the observation that a large number of significant proteins are lost after an experiment wise correction. Conclusion discrepancies due to the use of an experiment wise error correction are rarely reported, thus its impact cannot be reliably estimated from a survey of the literature. Because of this, a formal proteomic study was performed in which the performance of the significance outcome obtained by independent uncorrected t-tests were compared to the significance outcome obtained using Bonferroni and False discovery rate adjustments (Ting, Cowley et al. 2009). In this study, 1DE fractionated microbial proteins extracts of cultures grown at 10 °C versus 30 °C. The 1DE fractions were further analyzed by LC-MS/MS which consistently found 954 proteins across the two treatments. Out of those 954 proteins an uncorrected t-test identified that 325 were differentially expressed while only 56 were identified by Bonferroni and 272 were identified by false discovery rate. All tests were performed at a 95% confidence. It was concluded that the Bonferroni method offered results that were
far too conservative for proteomic application. The false discovery rate method identified more than four times the number of proteins than Bonferroni but was more conservative and offers more confidence information than the uncorrected t-tests.

The use of methods that correct for experiment-wise error such as, Bonferroni’s adjustment or the false discovery rate method can improve the overall confidence of a study by offering results with less uncertainty. It is true that the use of experiment wise error correction can eliminate a large number of proteins that could be related significantly to the biological question, it is relevant to question if those proteins are really lost using these statistical analyses. The discovery of potential biological participants is ultimately related to chance since every discovery made has a potential of being true or false because its discovery is associated to the experiment characteristics and the statistical analysis used. Therefore, it is not possible to ascertain the validity of a discovery at a 100% certainty. However, the inference that can be made based on statistical methods that yield more confident results leads to more trustworthy conclusions.
CHAPTER 2

Study 1

Functional proteomic analysis predicts beef tenderness and the tenderness differential

Summary

Inconsistent tenderness is one of the most detrimental factor of meat quality.

Functional proteomics were used to associate electrophoretic bands from the myofibrillar muscle fraction to meat tenderness in an effort to gain understanding of the mechanisms controlling tenderness. The myofibrillar muscle fraction of the *Longissimus dorsi* from 22 Angus cross steers was analyzed by SDS-PAGE, and linearly regressed to Warner-Bratzler shear values. Six significant electrophoretic bands were characterized by electrophoretic and statistical analysis and sequenced by nano-LC/MS/MS. The protein(s)/peptide(s) identified in these bands encompass a wide array of cellular pathways related to structural, metabolic, chaperone and developmental functions. This study begins to assemble information that has been reported separately into a more complete picture that will lead to the establishment of a coherent mechanism accounting for meat tenderness.
Introduction

Tenderness is considered one of the most important of all the organoleptic characteristics contributing to meat quality (Koohmaraie 1992). Previous reports (Smith 1996; Brooks, Belew et al. 2000; Roeber 2001; McKenna, Robert et al. 2002) highlight the inability of the current USDA beef quality grading system to accurately segregate carcasses into tenderness categories, and these reports stress the importance of tenderness characteristics to overall beef quality. This quality inconsistency results in consumers being dissatisfied with the beef products currently available at the retail and food service level (Brooks, Belew et al. 2000), with consumers indicating that they would pay a premium for beef of known tenderness (Boleman, Boleman et al. 1997).

The mechanisms controlling meat tenderness involve a multitude of cellular functions, which have proven difficult to develop into a coherent model. Variations in genetics (Dikeman, Pollak et al. 2005), final pH (Shackelford, Koohmaraie et al. 1994), the cathepsins, the calpain/calpastatin system, the proteasome (Ouali, Herrera-Mendez et al. 2006), collagen content, collagen cross-linking, myofibrillar degradation (Sifre, Berge et al. 2005), and, more recently, heat shock proteins (Bernard, Cassar-Malek et al. 2007; Morzel, Terlouw et al. 2008) are thought to play roles in the mechanisms controlling tenderness. It is likely that all of these processes participate, to some extent, in the mechanisms resulting in beef carcass tenderness.

A novel approach to gain understanding of the mechanisms controlling tenderness and to predict tenderness is by elucidating the state of the muscle cell through the use of functional proteomics, which is a combination of electrophoretic, image, statistical and
protein sequencing technologies that identifies the protein(s)/peptide(s) associated with Warner Bratzler Shear (WBS), a common method of assessing beef tenderness (Sawdy, Kaiser et al. 2004; Reddish, St-Pierre et al. 2008).

Therefore, the objective of the present study was to perform a functional proteomic analysis to associate electrophoretic bands from the myofibrillar fraction of meat samples at 36 h postmortem that are statistically significant with meat tenderness at 72 h and 14 d of aging and/or the tenderness differential and determine the sequence of the protein(s)/peptide(s) in those bands.
Materials and Methods

Experimental Design

The selection of the two time points for WBS was designed to capture two different scenarios of postmortem aging, which are the non-aged meat phase, represented by the 72 h time point; and the aged meat phase, represented by the 14 d time point. Samples for proteomic analysis were taken at 36 h.

Animals and Sample Collection

Twenty two Angus crossbred steers with no Brahman influence, of approximately 18 mo of age, were harvested in the abattoir at The Ohio State University Meat Laboratory following USDA accepted welfare practices. No electrical stimulation was performed. All muscle samples were taken from the 8th rib region of the *Longissimus dorsi* muscle. Samples for proteomic analysis were taken from one side of the carcass at 36 h and steak samples for tenderness value determination by WBS were taken at 72 h and 14 d postmortem (during cold room aging) were taken from the other. Samples destined for proteomic analysis were flash frozen in liquid nitrogen and stored at –20 °C.

Warner-Bratzler Shear Force Analysis

Steaks aged for 72 h or 14 d were cooked on a cooking grill (George Foreman® Next Grilleration, Salton, Inc.; Miramar FL) set to 190.5 °C and cooked to an internal temperature of 71.1 °C monitored with a scanning thermocouple thermometer (Digi-Sense 12-Channel Benchtop Thermocouple Scanner, Barnant Co.; Barrington, IL). The cooked steaks were then cooled to room temperature and six cores, 1.27 cm in diameter, were sampled parallel to the muscle fiber orientation. Shear force values of the cores
were obtained using a TA-XT Plus Texturometer (Stable Micro Systems Ltd.; Godalming, UK). The average WBS value of the six cores was calculated and used as the shear force value for the samples.

**Proteomic Analysis**

A myofibrillar fraction of each muscle sample was prepared by homogenizing 250 mg of muscle for 1 min with 3 ml of a low salt buffer (50 mM NaCl, 0.1% NaN3 0.4 mM Pefabloc SC Plus®, pH 7.2 (Boehringer Mannheim Corp. Indianapolis, IN) on ice. The homogenate was centrifuged at 10,000 × g for 10 min. The supernatant was discarded and the resulting myofibril pellet was suspended in 2 ml of low salt buffer and centrifuged at 10,000 × g for 10 min. This step was repeated three times. The supernatant was discarded and the 50 mg of the pellet (myofibrillar fraction) was dissolved in sample buffer (8 M urea/ 2 M thiourea, 75 mM DTT, 50 mM Tris, 3% SDS and 0.004% bromophenol blue, pH 6.8) at a ratio of 1 mg of sample per 30 μl of sample buffer. The samples were then incubated on ice for 30 min. Samples were centrifuged at 10,000 × g for 10 min prior to loading onto a 1 mm × 12 cm × 14 cm polyacrylamide slab gel consisting of 10% to 20% gradient resolving gel (30:0.8, acrylamide: N, N’- bis-methylene acrylamide) and a 3% stacking gel containing 1% SDS. Electrophoretic separation was carried out at a constant voltage of 10 Vcm⁻¹. After electrophoretic separation, gels were placed in fixing solution (50% methanol, 7% acetic acid) for 12 h and then stained with SYPRO Ruby™ protein gel stain (Bio-Rad Laboratories, Inc. Hercules, CA) for 24 h protected from light. The gels were then placed in destain solution (10% methanol, 7% acetic acid) for 30 min and rinsed with deionized water prior to imaging. The gels were scanned using a Typhoon®
9400 laser scanner (GE Healthcare; Chalfont St. Giles, UK) using the SYPRO Ruby™ setting, but with the 457 nm blue laser. Digital images were analyzed using the Total Lab TL120® (Nonlinear Dynamics Inc.; Newcastle upon Tyne, UK) software. The bands were identified and then analyzed to determine the percentage contribution of each band in relation to the total band volume in the lane.

**Statistical Analysis**

A multiple linear regression model previously developed and published (Reddish, St-Pierre et al. 2008) was fitted using the dependent variables. The percent contribution of each band, obtained from Total Lab TL120®, in each lane was imported into SAS v.9.1.3 (SAS Institute Inc., Cary, NC) for subsequent multiple linear regression analysis. This model consists of a stepwise multiple linear regression within the REG procedure of SAS. In a stepwise model, bands are removed or added iteratively from the model in each step of the procedure according to their significance ($P = 0.05$ was used in this study) starting with a model that includes no bands and ending with a model that includes all bands that meet the significance criteria. The purpose of the model is to determine which bands from the proteomic analysis were contributing to the variation of the dependent variable as described below:

$$Y_i = \beta_0 + \beta_j X_{ij} + \varepsilon_{ij},$$

where $Y_i$ is the dependent variable measured of the $i$th sample ($i = 1, ..., n$), $\beta_0$ is the intercept, $\beta_j$ is the regression parameter associated with the $j$th band ($j = 1, ..., 30$), $X_{ij}$ is the percent contribution for the $j$th band of the $i$th sample, and $\varepsilon_{ij}$ is the random error inherent to each sample, which is assumed to be independent and normally distributed.
**Band Sequencing**

The bands that were determined by the multiple linear regression model as being predictive of the dependent variable were submitted for characterization by mass spectrometry as previously described (Reddish, St-Pierre et al. 2008) with the following modifications. The associated bands from three animals that showed the highest values of the dependent variable were pooled together and were analyzed as one; the same procedure was performed with the associated bands from three animals that showed the lowest values of the dependent variable. All mass spectrometry was performed at the Campus Chemical Instrument Center of The Ohio State University using established methods. Briefly, selected bands were excised from the gel, washed in 50% methanol/5% acetic acid solution, and dehydrated in acetonitrile. Gel bands were rehydrated and incubated with 5 mg/ml of DTT in 100 mM ammonium bicarbonate solution prior to the addition of 15 mg/ml Iodoacetamide in 100 mM ammonium bicarbonate solution. Gel bands were washed with acetonitrile and ammonium bicarbonate (100mM) and were dried in speed vac, the protease is driven into the gel pieces by rehydrating them in 50 μl of sequencing grade modified trypsin (Promega, Madison, WI) and the mixture was incubated at 25°C for overnight. Peptides were extracted from the poly-acrylamide with a 50% acetonitrile/5% formic acid solution. The extracted pools were concentrated in a speed vac to ~30 μl. Pools were analyzed by capillary-liquid chromatography-nanospray tandem mass spectrometry (Nano-LC/MS/MS). Nano-LC/MS/MS was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray source operated in positive ion mode. The LC system was an UltiMate™ Plus system (Dionex Co.,
Sunnyvale, CA) with a Famos autosampler and Switchos column switcher. The solvent A was water containing 50 mM acetic acid and the solvent B was acetonitrile. Five microliters of each sample was eluted into a 5 cm 75 μm ID ProteoPep II C18 column (New Objective, Inc. Woburn, MA) packed directly in the nanospray tip. Peptides were eluted directly off the column into the LTQ system using a gradient of 2-80% solvent B over 50 minutes, with a flow rate of 300 nl/min. The scan sequence of the mass spectrometer was based on the TopTen™ method; the analysis was programmed for a full scan recorded between 350 through 2000 Da, and a MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive instrument scans of the ten most abundant peaks in the spectrum. Sequence information from the MS/MS data were searched using Mascot Daemon v.2.2.1 (Matrix Science, Boston, MA) and the database searched against the full SwissProt database v.55.3 (366,226 sequences; 132,054,191 residues). The mass accuracy of the precursor ions were set to 2.0 Da given that the data was acquired on an ion trap mass analyzer and the fragment mass accuracy was set to 0.5 Da. Modifications considered were methionine oxidation and carbamidomethyl cysteine. Two missed cleavages for the enzyme were permitted. Protein identifications were checked manually.

**Sequencing Result Interpretation**

The two main confounders that complicate the interpretation of mass spectrometry data are that the identified sequences can be found on multiple sequences found in the database and proteins/peptides can co-migrate in bands resolved by one dimensional SDS-PAGE. Because of this, the list of protein sequences found in each band sequenced
had to be trimmed to facilitate our interpretation. The criteria employed to trim the list was as follows:

1. Remove all identified sequences from trypsin (the protease used to digest the bands) and keratin (a common contaminant of samples that comes from the contact of skin with the device or its components).

2. Remove all sequences identified from non bovine sources.

3. Remove all sequences when significance was based on only one fragment significantly identified. This is a common practice, because single fragment identifications can lead to misinterpretations (Harry, Wilkins et al. 2000).
Results and Discussion

It has been previously shown that electrophoretic analyses at 36 h postmortem can be used to predict tenderness at 7 d (Sawdy, Kaiser et al. 2004). It is likely that this is true for 72 h tenderness and 14 d tenderness, as well. The descriptive statistics of the WBS values, he change from 72 h WBS to 14 d WBS (Δ WBS) are presented in Table 1. Δ WBS, was used to estimate the tenderness differential between those two time points. As expected, WBS force values declined from 72 h to 14 d postmortem, which can also be observed in the mean Δ WBS, which has a negative value. One-way ANOVA supported the difference in WBS force values between the two time points (P < 0.001).

Table 1. Descriptive statistics for Warner-Bratzler Shear force (WBS).

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>n</th>
<th>Mean (N)</th>
<th>Std. Dev. (±)</th>
<th>Minimum (N)</th>
<th>Maximum (N)</th>
<th>Range (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 h WBS</td>
<td>22</td>
<td>53.37</td>
<td>12.58</td>
<td>37.06</td>
<td>75.82</td>
<td>38.76</td>
</tr>
<tr>
<td>14 d WBS</td>
<td>22</td>
<td>36.69</td>
<td>6.38</td>
<td>26.98</td>
<td>51.85</td>
<td>24.87</td>
</tr>
<tr>
<td>Δ WBS</td>
<td>22</td>
<td>-16.68</td>
<td>12.37</td>
<td>-40.99</td>
<td>4.45</td>
<td>45.44</td>
</tr>
</tbody>
</table>

Δ refers to the dynamic change over time (Δ WBS = 14 d WBS – 72 h WBS).

Functional Proteomic Analysis of Myofibrils

Functional proteomics relies on statistically analyzing the variation of the staining intensities of bands in an electrophoretic profile. This allows for identification of bands that are significantly associated with a dependent variable. In this study, that variable was WBS or tenderness. The protein(s)/peptide(s) present in the bands were sequenced by tandem mass spectrometry.

Thirty protein/peptide bands across all samples were electrophoretically resolved and matched across all samples by Total Lab TL120®. A representative gel, comparing an animal that observed the maximum value of the dependent variable to animal that
observed the minimum value is shown in Figure 1. In some of the cases the differences can be spotted by the naked eye however it is enhanced by the software. The synthetic lane is shown in Figure 2 along with the estimated molecular weights. The synthetic lane

![Image](image-url)

**Figure 1.** Comparative 10 to 20% gradient SDS-PAGE gel showing the samples that observed the minimum (Min) and the maximum (Max) value for the respective dependent variable (72 h WBS, 14 d WBS and $\Delta$ WBS). The arrows show the location of the bands found to be significantly associated to the dependent variable. BR is the Broad Range molecular weight marker.

is an artificial reference lane that contains all of the bands separated through all samples
and allows band matching across samples. To avoid the confounding factor of uneven protein loading, the percentage that each band contributed to the total staining of all bands within the lane was estimated by the software. This value was determined from the integration of the intensity of each peak in the electropherogram after background removal.

**Figure 2.** Representation of the synthetic lane used to align and compare the SDS-PAGE profiles. The 30 resolved bands are displayed. Estimated molecular weights (MW) were calculated using the Broad Range standard. The bands found to be associated are circled. * = Band associated to 72 h WBS; ** = Band associated with 14 d WBS; *** = Band associated with Δ WBS.

Intact actin and myosin band images were removed from further analyses following the recommendation of previous literature (Sawdy, Kaiser et al. 2004). In order to generate quantifiable bands of the proteins/peptides present in muscle, other than actin and myosin, it was necessary to load sufficient sample to maximize the number of resolvable bands as determined by the image analysis software; this resulted in overloading of actin and myosin. Thus, this overloading of actin and myosin meant that the linear increases in the amount of sample loaded did not result in a linear increase in the band intensities of actin or myosin. This discrepancy would prevent quantifying the
loss of these proteins during the aging process. Linear losses of these two proteins during aging would be observed in other quantifiable bands.

The multiple linear regression models were fitted using the dependent variables previously discussed (72 h WBS, 14 d WBS and Δ WBS) and the bands that were found to be significant for each multiple linear regression were identified. Three separate multiple linear regressions were fitted and the equations obtained were:

\[
72 \text{ h } WBS = 57.044 + 2.652 (\text{Band 2}) - 9.678 (\text{Band 25}); \\
(R^2 = 0.508; P < 0.001; \text{SEM} = 9.276 \text{ N})
\]

\[
14 \text{ d } WBS = 19.738 + 1.105 (\text{Band 12}) + 4.134 (\text{Band 24}) - 2.902 (\text{Band 30}); \\
(R^2 = 0.674; P < 0.001; \text{SEM} = 3.934 \text{ N})
\]

\[
\Delta \text{ WBS} = -23.074 - 18.455 (\text{Band 9}) + 10.768 (\text{Band 25}); \\
(R^2 = 0.576; P < 0.001; \text{SEM} = 8.472 \text{ N})
\]

One band (band 25) was found to be significantly predictive for two of the dependent variables. No band was found to be predictive for all three variables. A total of six bands were identified in this study.

It should be noted that in the case of 72 h WBS and 14 d WBS, a positive regression coefficient in a specific band means that, as the band intensity increases, the WBS value increases, which translates to less tender meat. Thus, a negative regression coefficient will describe the opposite phenomenon; if the band intensity increases, the WBS value decreases, which results in more tender meat. In the case of Δ WBS, the interpretation is different; if the regression coefficient is positive, it indicates a smaller WBS differential, which translates to a lesser range of tenderization during aging. As well, a negative
regression coefficient indicates larger WBS differential, which indicates a greater range of tenderization during aging.

_Table 2._ Comparison of individual samples to be pooled together for band sequencing.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Level</th>
<th>Individual WBS (N)</th>
<th>Pooled WBS (Mean) (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 h WBS</td>
<td>H</td>
<td>74.32, 75.46, 75.82</td>
<td>75.20</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>37.06, 40.33, 40.53</td>
<td>39.31</td>
</tr>
<tr>
<td>14 d WBS</td>
<td>H</td>
<td>45.51, 49.66, 51.85</td>
<td>49.01</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>26.98, 30.84, 30.97</td>
<td>29.60</td>
</tr>
<tr>
<td>Δ WBS</td>
<td>H</td>
<td>-5.22, -3.20, 4.45</td>
<td>-1.32</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>-40.99, -34.78, -34.70</td>
<td>-36.82</td>
</tr>
</tbody>
</table>

_The three samples that showed the highest (H) and the three lowest (L) values for each dependent variable were combined for sequencing._

**Band Sequencing and Protein Identification**

The highest three and the lowest three most extreme samples, for each of the dependent variables, were selected for sequencing. This was done to normalize the animal to animal variation, thus avoiding the observation of results from a single animal. The details of the selected samples are displayed in _Table 2_. The six bands found to be predictive of and associated with WBS force values were cut from the gel to be sequenced. The Mascot Daemon software sequencing identification process blasts the peptide sequences found in the band against the Swiss-Prot protein database for matching against known protein sequences. The software sorts the results based on the statistical significance of the identification. Only results found to be significant (_P_ ≤ 0.05) are considered. The significance is determined by the summation of the probability of each fragment identified and matched to that specific sequence, which is expressed as a score value (MOWSE Score).
### Table 3. Sequencing data of bands associated with 72 h WBS.

<table>
<thead>
<tr>
<th>Band No. (MW&lt;sub&gt;e&lt;/sub&gt;&lt;sup&gt;4&lt;/sup&gt; (kD))</th>
<th>Association Type&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Identification</th>
<th>Swiss-Prot Accession No.</th>
<th>MOWSE Scores&lt;sup&gt;2&lt;/sup&gt; (H/L)</th>
<th>Fragments Identified&lt;sup&gt;3&lt;/sup&gt; (H/L)</th>
<th>MW&lt;sub&gt;t&lt;/sub&gt;&lt;sup&gt;5&lt;/sup&gt; (kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (240.4)</td>
<td>+</td>
<td>Myosin heavy chain 1</td>
<td>MYH1_BOVIN</td>
<td>5672/6451</td>
<td>272/341</td>
<td>223.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myosin heavy chain 2</td>
<td>MYH2_BOVIN</td>
<td>5647/6100</td>
<td>257/328</td>
<td>224.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myosin heavy chain 7</td>
<td>MYH7_BOVIN</td>
<td>3552/4095</td>
<td>165/196</td>
<td>223.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sarcoplasmic reticulum calcium ATPase 1</td>
<td>AT2A1_BOVIN</td>
<td>229/284</td>
<td>5/7</td>
<td>110.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myosin light chain 2, skeletal</td>
<td>MLRS_BOVIN</td>
<td>0/76</td>
<td>0/3</td>
<td>19.11</td>
</tr>
<tr>
<td>25 (23.6)</td>
<td>–</td>
<td>Myosin light chain 2, skeletal</td>
<td>MLRS_BOVIN</td>
<td>929/895</td>
<td>43/37</td>
<td>19.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myosin light chain 2, ventricular</td>
<td>MLRV_BOVIN</td>
<td>580/406</td>
<td>29/16</td>
<td>18.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heat shock protein beta 6</td>
<td>HSPB6_BOVIN</td>
<td>144/146</td>
<td>5/5</td>
<td>17.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Creatine kinase M type cardiac</td>
<td>KCRM_BOVIN</td>
<td>130/105</td>
<td>5/2</td>
<td>43.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Troponin C, slow skeletal &amp; cardiac</td>
<td>TNNC1_BOVIN</td>
<td>120/0</td>
<td>3/0</td>
<td>18.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crystallin alpha B chain</td>
<td>CRYAB_BOVIN</td>
<td>47/0</td>
<td>2/0</td>
<td>20.02</td>
</tr>
</tbody>
</table>

The protein sequences identified using these criteria are presented in Tables 3, 4 and 5, based on the dependent variable analyzed. Thirty six individual proteins/peptides were identified, though many of the same proteins/peptides were identified in more than one band. This may be due to proteolytic fragments of the intact protein co-migrating in a band of lower molecular weight than that of the intact protein. It was also observed, in Table 3, that in band 2 myosin heavy chain co-migrated along with myosin light chain 2 at an estimated molecular weight of ~240 kDa even though myosin light chain 2 has a molecular weight of ~19 kDa. It is generally accepted that both myosin light and heavy chains are associated via non-covalent bonding. In some cases, proteins co-migrated in

---

1. Type of association with the dependent variable;
2. The MOWSE score is a numeric descriptor of the likelihood that the identification is correct;
3. Number of fragments sequenced and matched to that particular protein;
4. MW<sub>e</sub> is the experimental molecular weight;
5. MW<sub>t</sub> is the theoretical molecular weight.
bands with larger molecular weights. This phenomenon has been shown to occur with myosin heavy chain due to the covalent crosslinking activity of transglutaminase in the muscle (Eligula, Chuang et al. 1998). The same phenomenon has also been observed in

Table 4. Sequencing data of bands associated with 14 d WBS.

<table>
<thead>
<tr>
<th>Band No. (MW\text{e}\textsuperscript{4} (kD))</th>
<th>Association Type\textsuperscript{1}</th>
<th>Identification</th>
<th>Swiss-Prot Accession No.</th>
<th>MOWSE Scores\textsuperscript{2} (H/L)</th>
<th>Fragments Identified\textsuperscript{3} (H/L)</th>
<th>MW\text{t}\textsuperscript{5} (kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 (41.6) +</td>
<td>Tropomyosin beta chain</td>
<td>TPM2_BOVIN</td>
<td>1006/1286</td>
<td>46/49</td>
<td>32.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Creatine kinase M type</td>
<td>KCRM_BOVIN</td>
<td>784/886</td>
<td>42/43</td>
<td>43.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Actin alpha, skeletal</td>
<td>ACTS_BOVIN</td>
<td>724/724</td>
<td>44/42</td>
<td>42.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tropomyosin alpha 3 chain</td>
<td>TPM3_BOVIN</td>
<td>546/459</td>
<td>22/19</td>
<td>32.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde 3 phosphate</td>
<td>G3P_BOVIN</td>
<td>210/226</td>
<td>5/7</td>
<td>36.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dehydrogenase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aspartate aminotransferase,</td>
<td>AATM_BOVIN</td>
<td>133/135</td>
<td>4/2</td>
<td>47.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mitochondrial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Creatine kinase, sarcomeric</td>
<td>KCRS_BOVIN</td>
<td>116/0</td>
<td>3/0</td>
<td>47.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde 3 phosphate</td>
<td>G3PT_BOVIN</td>
<td>0/94</td>
<td>0/2</td>
<td>43.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dehydrogenase, testis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crystallin alpha B chain</td>
<td>CRYAB_BOVIN</td>
<td>488/347</td>
<td>18/11</td>
<td>20.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Myosin light chain 2, skeletal</td>
<td>MLRS_BOVIN</td>
<td>221/154</td>
<td>8/8</td>
<td>19.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Creatine kinase M type</td>
<td>KCRM_BOVIN</td>
<td>215/0</td>
<td>6/0</td>
<td>43.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Actin alpha, skeletal</td>
<td>ACTS_BOVIN</td>
<td>94/0</td>
<td>4/0</td>
<td>42.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cysteine and glycine-rich</td>
<td>CSRP3_BOVIN</td>
<td>62/0</td>
<td>2/0</td>
<td>21.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>protein 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Myosin light chain 1, skeletal</td>
<td>MLE1_BOVIN</td>
<td>568/604</td>
<td>24/30</td>
<td>21.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobin subunit beta</td>
<td>HBB_BOVIN</td>
<td>442/487</td>
<td>14/15</td>
<td>16.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Histone H4</td>
<td>H4_BOVIN</td>
<td>239/337</td>
<td>9/13</td>
<td>11.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome C oxidase subunit 5A,</td>
<td>COX5A_BOVIN</td>
<td>185/245</td>
<td>6/8</td>
<td>16.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mitochondrial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Myosin light chain 2, skeletal</td>
<td>MLRS_BOVIN</td>
<td>96/0</td>
<td>4/0</td>
<td>19.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome B-C1 complex subunit 7</td>
<td>QCR7_BOVIN</td>
<td>77/0</td>
<td>3/0</td>
<td>13.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Galectin 1</td>
<td>LEG1_BOVIN</td>
<td>0/229</td>
<td>0/7</td>
<td>15.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP synthase subunit E,</td>
<td>ATP5I_BOVIN</td>
<td>0/121</td>
<td>0/3</td>
<td>8.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mitochondrial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobin subunit alpha 1/2</td>
<td>HBA_BOVIN</td>
<td>0/88</td>
<td>0/5</td>
<td>15.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP synthase subunit F,</td>
<td>ATPK_BOVIN</td>
<td>0/59</td>
<td>0/2</td>
<td>10.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mitochondrial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde 3 phosphate</td>
<td>G3PT_BOVIN</td>
<td>0/54</td>
<td>0/2</td>
<td>43.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dehydrogenase, testis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The comparison between the pooled samples of animals with high 14 d WBS values (H) versus the pooled sample of animals with low 14 d WBS (L) values is displayed.

\textsuperscript{1} Type of association with the dependent variable;

\textsuperscript{2} The MOWSE score is a numeric descriptor of the likelihood that the identification is correct;

\textsuperscript{3} Number of fragments sequenced and matched to that particular protein;

\textsuperscript{4} MW\text{e} is the experimental molecular weight;

\textsuperscript{5} MW\text{t} is the theoretical molecular weight.
vitro with troponin T (Bergamini, Signorini et al. 1995). Our observation suggests that a covalent association between the two molecules is formed postmortem.

**Table 5. Sequencing data of bands associated with the change from 72 h WBS to 14 d WBS (Δ WBS).**

<table>
<thead>
<tr>
<th>Band No. (MWe&lt;sup&gt;4&lt;/sup&gt; (kD))</th>
<th>Association Type&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Identification</th>
<th>Swiss-Prot Accession No.</th>
<th>MOWSE Scores&lt;sup&gt;2&lt;/sup&gt; (H/L)</th>
<th>Fragments Identified&lt;sup&gt;3&lt;/sup&gt; (H/L)</th>
<th>MWt&lt;sup&gt;5&lt;/sup&gt; (kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 (59.5)</td>
<td>–</td>
<td>Myosin heavy chain 2</td>
<td>MYH2_BOVIN</td>
<td>2031/812</td>
<td>82/26</td>
<td>224.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myosin heavy chain 1</td>
<td>MYH1_BOVIN</td>
<td>1919/753</td>
<td>77/27</td>
<td>223.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Desmin</td>
<td>DESM_BOVIN</td>
<td>1447/634</td>
<td>62/22</td>
<td>53.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATP synthase subunit alpha, heart</td>
<td>ATPA1_BOVIN</td>
<td>1160/1358</td>
<td>42/49</td>
<td>59.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myosin heavy chain 7</td>
<td>MYH7_BOVIN</td>
<td>712/292</td>
<td>23/13</td>
<td>223.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tubulin beta 2C chain</td>
<td>TBB2C_BOVIN</td>
<td>290/342</td>
<td>12/11</td>
<td>50.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tubulin beta 3 chain</td>
<td>TBB3_BOVIN</td>
<td>228/0</td>
<td>11/0</td>
<td>50.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Actin alpha, skeletal</td>
<td>ACTS_BOVIN</td>
<td>224/456</td>
<td>7/17</td>
<td>42.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATP synthase subunit beta, mitochondrial</td>
<td>ATPB_BOVIN</td>
<td>131/372</td>
<td>4/11</td>
<td>52.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Creatine kinase M type</td>
<td>KCRM_BOVIN</td>
<td>62/0</td>
<td>3/0</td>
<td>43.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tubulin alpha 3 chain</td>
<td>TBA3_BOVIN</td>
<td>54/0</td>
<td>2/0</td>
<td>50.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tubulin alpha 4A chain</td>
<td>TBA4A_BOVIN</td>
<td>0/91</td>
<td>0/2</td>
<td>50.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial</td>
<td>ODO2_BOVIN</td>
<td>0/60</td>
<td>0/3</td>
<td>49.28</td>
</tr>
<tr>
<td>25 (23.6)</td>
<td>+</td>
<td>Myosin light chain 2, ventricular</td>
<td>MLRV_BOVIN</td>
<td>762/618</td>
<td>40/33</td>
<td>18.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myosin light chain 2, skeletal</td>
<td>MLRS_BOVIN</td>
<td>666/854</td>
<td>27/42</td>
<td>19.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Troponin C, slow skeletal &amp; cardiac</td>
<td>TNNC1_BOVIN</td>
<td>245/226</td>
<td>8/6</td>
<td>18.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heat shock protein beta 6</td>
<td>HSPB6_BOVIN</td>
<td>230/48</td>
<td>10/2</td>
<td>20.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crystallin alpha B chain</td>
<td>CRYAB_BOVIN</td>
<td>215/162</td>
<td>7/5</td>
<td>20.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cysteine and glycine-rich protein 3</td>
<td>CSRP3_BOVIN</td>
<td>155/0</td>
<td>5/0</td>
<td>21.85</td>
</tr>
</tbody>
</table>

The comparison between the pooled sample of animals with high Δ WBS values (H) versus the pooled sample of animals with low Δ WBS (L) values is displayed.

<sup>1</sup> Type of association with the dependent variable;

<sup>2</sup> The MOWSE score is a numeric descriptor of the likelihood that the identification is correct;

<sup>3</sup> Number of fragments sequenced and matched to that particular protein;

<sup>4</sup> MWe is the experimental molecular weight;

In this study, we identified protein(s)/peptide(s) that have structural, metabolic, chaperone and/or developmental functions that appear to be participating, in unison, in
the mechanism of meat tenderness. The limitation of our study resides in the co-migration of protein(s)/peptide(s) within a given band that confound the ability to definitively identify which protein(s)/peptide(s) are involved with a biological mechanism or pathway. Therefore, we were unable to definitively identify which protein(s)/peptide(s) were playing a direct role in the mechanisms underlying postmortem tenderness, but several trends are apparent.

One trend was the presence of structural proteins specific to the sarcomere. Both intact and proteolytic fragments of myosin heavy chains, in bands 2 and 9, and myosin light chains, in bands 2, 24, 25 and 30, tended to be found in bands associated with all WBS variables. Myosin heavy chains and myosin light chains are crucial to muscle function in terms of contraction velocity and power that is associated with fiber type composition. Fiber type composition is based on the myosin heavy chain and myosin light chain isoform composition which, ultimately, determines the main source of energy for the muscle (Bottinelli 2001). This observation supports the notion that the muscle fiber type composition is related, in some way, to the postmortem proteolytic events that may lead to meat tenderization. These results are also consistent with recent reports on meat tenderness. Muscle fiber type is associated with tenderness in Angus and Brahman crossbreeds (Stolowski, Baird et al. 2006). Post mortem concentrations of both myosin heavy chain fragments and myosin light chain 1 have been associated with pork quality and tenderness (Lametsch, Karlsson et al. 2003; Lametsch, Roepstorff et al. 2004) and beef tenderness (Sawdy, Kaiser et al. 2004). In addition, recent reports in cattle have shown that bovine myosin light chain 1 is fragmented soon after harvest and that the
fragment increases 11 fold by 24 h post harvest (Jia, Hollung et al. 2006). Additionally, both 1D and 2D electrophoresis experiments have shown that myosin heavy chain is proteolyzed by μ-calpain (Lametsch, Roepstorff et al. 2004).

Another trend was the presence of the structural protein actin in a band with a molecular weight higher than 40 kDa in band 9 that was found to be negatively associated with Δ WBS. Thus, the presence of actin migrating at a higher molecular weight than the full molecular weight suggests it is covalently bound to a myosin fragment migrating at a lower molecular weight than its reported value of 223 kDa. It is interesting to speculate that this phenomenon is fiber type specific. Myosin binds to actin to perform its biological function of muscle contraction. Muscle contraction can be observed postmortem during rigor onset. The actomyosin bond requires the binding of ATP in order to return the structure to the relaxed state. Post mortem actomyosin release is limited by ATP availability. This availability has been reported to be dependent of fiber type composition (Bowker, Botrel et al. 2004). It can also be due to transglutaminase activity as described before.

Research has shown that degradation of the structural proteins desmin and troponin T is also correlated with increased tenderness in beef (Ho, Stromer et al. 1997) and lamb (Wheeler and Koohmaraie 1999) during aging. In the present study, desmin was identified in band 9 migrating at an estimated molecular weight of 59 kDa, which is close to its intact molecular weight of 52 kDa. Even though troponin T was not identified in any of the bands in this study, another member of the troponin complex, troponin C, was identified in band 25 at~22 kDa and was negatively associated with 72 h WBS and
positively associated with Δ WBS; the protein migrated close to its reported full length molecular weight of ~19 kDa.

Another trend was the observation in all six statistically significant bands of at least one enzyme involved in energy metabolism. Overall, the metabolic enzymes identified were: several ATP synthase subunits, a pair of creatine kinase isoforms, a mitochondrial aspartate aminotransferase isoform, two different glyceraldehyde 3 phosphate dehydrogenase isoforms, one cytochrome BC1 subunit, one cytochrome C oxidase subunit and a oxoglutarate dehydrogenase component. An important point to note is the presence of these proteins in the myofibrillar fraction. Creatine kinase and glyceraldehyde 3 phosphate dehydrogenase are soluble proteins that are normally found in the low salt soluble sarcoplasmic fraction of muscle tissue. Precipitation of these proteins in the myofibrillar fraction may be the result of higher temperatures and lower pH conditions present in postmortem tissue, as suggested by research performed on pork (Fischer and Hamm 1979; Joo, Kauffman et al. 1999). We do not have an explanation for this phenomenon. It is possible that these peptides were trapped interstitially during the extraction process or that these proteins were precipitated by isoelectric precipitation (Joo, Kauffman et al. 1999). The presence of fragments of these sarcoplasmic proteins is likely due to proteolytic events occurring during the aging process since we are not aware of pH being capable of fragmenting of proteins in postmortem conditions.

Previous research found that increased metabolism immediately postmortem resulted in higher WBS values, less tender meat, and that inhibiting glycolysis resulted in increased tenderness, lower WBS values (Jerez, Calkins et al. 2003). In that study, the
addition of glycolytic inhibitors to enhance tenderness was studied. Muscles that were treated with glycolytic inhibitors were more tender, lower WBS, than muscles that received a control treatment. Additionally, it has been shown that tenderness is enhanced with decreased glycolytic activity due to the activity of the calpain system, which has been shown to be more active at a pH closer to physiological levels (Beltrán, Jaime et al. 1997). However, reduced glycolytic potential naturally within muscle is generally accepted to not lead to increased tenderness. Rather, the opposite has been shown to occur. In one study, muscle with decreased glycolytic potential was more likely to exhibit a dark, firm and dry (DFD) condition. The DFD beef was less tender and had more off-flavors than beef from muscles with increased glycolytic potential (Wulf, Emnett et al. 2002). It is likely that postmortem energy metabolism plays an undefined, yet critical, role in determining beef tenderness.

One more trend was the presence of participation of the heat shock protein or chaperone beta 6 (hspb6) in band 25. Heat shock proteins have been included in recent studies involving meat tenderness. One of those studies, in beef, found a negative relationship between tenderness and heat shock protein 40 (hsp40), a protein that helps to retard cell death in muscle tissue (Bernard, Cassar-Malek et al. 2007). In another study, it was reported that heat shock protein 27 (hsp27) was down-regulated in animals that gave lower tenderness values (Morzel, Terlouw et al. 2008). Results of our study associated hspb6 with tenderness variability. Hspb6 is alternatively known as heat shock protein 20 (hsp20) (Kappé, Franck et al. 2003) and has been described as having a strong affinity to actin thin filaments in muscle tissues and to be highly homologous to hsp27 (Kato, Goto
Hsp20 has been related to muscle relaxation during high calcium concentration events during situations of acute stress (Rembold, Foster et al. 2000). In our study, hspb6 was shown to be negatively associated with 72 h WBS and positively associated with ΔWBS, which is consistent with the literature (Morzel, Terlouw et al. 2008). It seems reasonable to assume that the function of hsp20 is comparable to the function of hsp27, which is intimately related to the stability of the actin thin filament based on the fact that the sequence elements responsible for their activity are highly conserved between the two (Guay, Lambert et al. 1997; Rembold, Foster et al. 2000). Thus, it seems reasonable to postulate that alterations in the regulation of hspb6 may lead to decreased stability of the actin filament, which may be associated with increased tenderness (Morzel, Terlouw et al. 2008).

The presence of hspb6 in the myofibrillar fraction and has to do with the conditions of the extraction process. We used a low salt solution adjusted to a pH of 7.2. A relevant difference from other small heat shock proteins is that hspb6 is not soluble in a pH ranging from 6 to 8 (this range includes the pH of our solution). Its difference in solubility from other similar HSPs is suspected to be related to sub-cellular specialization (Quraishe, Asuni et al. 2008). Hspb6’s singular solubility explains its presence in our myofibrillar preparation.

A trend, unique to our study, related tenderness to muscle differentiation. The cysteine and glycine-rich protein 3 (found in band 24), also known as muscle LIM, was found to be positively associated with 14 d WBS. The muscle LIM protein contains the
LIM- domain which is cysteine-histidine rich, zinc-coordinating domain, consisting of two tandemly repeated zinc fingers. The proteins called LIM contain the LIM domain. The LIM domain takes its name from Lin11, Isl-1 and Mec-3 proteins in which the domain was first discovered (Bach 2000). This protein functions as a positive developmental regulator that works as a cofactor of MyoD, MRF4, and myogenin to promote myoblast terminal differentiation (Kong, Flick et al. 1997). The RNA expression of this protein has been observed to be increased in muscle tissue of beef cattle undergoing nutritional stress (Lehnert, Byrne et al. 2006), but this is the first report of its link to tenderness. The presence of this protein indicates that the muscle is undergoing hyperplastic growth, which leads to an increased cell number that requires additional structural scaffolding leading to perimysium accumulation. LIM was also positively associated with Δ WBS (band 25). The additional structural scaffolding required for supporting the increased cell numbers may be sufficient to offset the proteolytic breakdown that occurs postmortem, which is reflected by a smaller WBS differential.

This idea is further supported by the presence of alpha and beta tubulin isoforms in band 9, which are specific to microtubules. Microtubules provide structural support that helps maintain the shape of cells. Recently, microtubules have been reported to be apoptotic targets (Adrain, Duriez et al. 2006; Moss, Betin et al. 2006) of caspases and granzyme B. The participation of apoptotic pathways in muscular postmortem metabolism related to meat tenderization has been previously suggested (Ouali, Herrera-Mendez et al. 2006).

Finally, both alpha and beta hemoglobin subunits were identified in band 30 which
was negatively associated with Δ WBS. This observation is interesting, because a correlation of hemoglobin concentration in meat and tenderness variation has not been previously reported. In regards to meat quality, pre-slaughter hemoglobin concentration in blood has been studied for prediction of carcass color (Wilson, Egan et al. 1995; Klont, Barnier et al. 2000). In the same manner, there were also other protein(s)/peptide(s) identified in the bands; however, their relation with meat tenderness has never been reported or suggested. As mentioned before, the electrophoretic co-migration of protein(s)/peptide(s) currently confounds our ability to quantify the protein(s)/peptide(s) that identify the direct participants involved in the mechanism of meat tenderization.
CHAPTER 3

Study 2

A semi-quantitative comparative proteomic approach can be used to define
postmortem aging time and muscle type in cooked meat

Summary

A simple semi-quantitative method, which can be applied to comparative
proteomic studies, was developed. The objective of this study was to present a proteomic
methodology that would allow the comparison of proteomic patterns of a complex,
processed meat sample (cooked meat). The methodology presented was used to explore
the possibility of meat cut authentication which currently cannot be assessed using a
formal analytical methodology. Three bands were statistically identified to contain the
most relevant proteins that define the factors studied. First, a 99.5 kDa band was
associated primarily to postmortem aging time. Second, a 31.1 kDa band was associated
primarily to muscle type; and third, a 17.1 kDa band was associated to the interaction of
both factors. The outcome of this study demonstrates the potential application of
proteomic studies in the development of meat cut authentication tools.
Introduction

Although proteomic techniques were developed over thirty years ago, proteomic analyses have become more common in the meat science field only during the past decade. Proteomics has the ability to enhance and clarify the vast amount of information that has been gathered by functional genome characterization (Bendixen 2005; Han and Wang 2008). Although the genetic information within an animal remains relatively unchanged throughout its life, the expression patterns of genes into mRNAs, that are ultimately translated into proteins, is altered by developmental, and environmental factors (Hollung, Veiseth et al. 2007). In addition to such factors, posttranslational modifications of proteins can also prevent absolute quantification of protein abundance based on mRNA expression (Gygi, Rochon et al. 1999). Proteomic studies attempt to translate the often ambiguous genomic information into tangible descriptions of the protein biological systems that are responsible for carrying out physiological functions. Physiological functions determine the state of the living tissue in which they are being developed. The state of a living tissue varies according to genetics of the organism and environmental factors affecting the organism. Phenotypic variation within muscle tissue is important in the meat science field because it is this variation that comprises the elements that are considered to determine meat quality. Meat science research has a focus on meat quality prediction, which is motivated by the revenue that can be generated by meat quality improvement. Previous studies have found relationships between the proteome of uncooked meat and meat quality (Joo, Kauffman et al. 1999; Ouali, Herrera-Mendez et al. 2006).
Recently, authentication of meat products has gained major interest from consumers due to the prevalence of meat fraud because of incorrect labeling because it is difficult to measure. There are several analytical tools available that can be used to authenticate meat products. Meat characteristics such as sex, breed, feed, age, wild vs. farmed, organic vs. conventional and even geographic origin can be authenticated. However, no analytical tools have been developed to authenticate meat cuts (Ballin 2010).

An interesting question can thus be posed; can a proteomic analysis be used to identify meat cuts at the time point and under similar conditions at which it is typically enjoyed by the consumer based on the differential abundances of specific proteins/peptides? It becomes relevant to note that the organoleptic, and physicochemical evaluations of meat are performed on cooked meat (Safari, Fogarty et al. 2001; Hoffman, Muller et al. 2003) and cooked meat is what the consumer enjoys.

Elucidating the relevant proteins underlying the variability in meat quality using proteomic methodologies based on electrophoretic protein separation is often confounded by the presence of irrelevant proteins that co-migrate with the relevant proteins (Sawdy, Kaiser et al. 2004; Zapata, Zerby et al. 2009). The inability to identify individual protein(s) within an electrophoretically separated band or spot limits its interpretation. Such limitation is in terms of which protein(s) are playing a role in the biological mechanisms under study (Sawdy, Kaiser et al. 2004).

While interpreting mass-spectrometry protein identifications, researchers are faced with vast amounts of data that need to be assembled into a coherent explanation of
a biological mechanism. Putting the mass spectrometry data into a coherent and relevant picture is a difficult challenge because of the difficulty of presented by co-migrating proteins and peptides. Recent breakthroughs in mass-spectrometry (Vogel and Marcotte 2009) have greatly surpassed many of these limitations by offering new evaluation tools to objectively segregate relevant proteins from irrelevant proteins which likely are just part of the protein background carried in the band being analyzed. No studies, to date, have semi-quantified the individual proteins/peptides within a one dimensional, electrophoretically resolved band that contains several co-migrating proteins/peptides.

Therefore, the objective of this study was to present a highly repeatable proteomic methodology that would allow the comparison of proteomic patterns of a complex sample while accounting for biological replicate variability by using a highly repeatable technique. To demonstrate, the application of this proteomic methodology, the characterization of meat cuts, based on specific protein abundance differences, was used to demonstrate the application of this novel proteomic methodology. It is important to note that the objective of this study was not to characterize the differences between cooked and raw meat.

The variables investigated in this study were muscle type and postmortem aging time before cooking. Variables were statistically analyzed to determine an association with the electrophoretically resolved bands from cooked meat samples. Significant bands were sequenced by nano-LC-MS/MS to obtain protein IDs and their emPAI abundance estimations (Ishihama, Oda et al. 2005). The proteomic analysis techniques performed in this study were selected for being accessible, simple to replicate, and relatively
inexpensive. Therefore, this proteomic technique can be a viable alternative for researchers interested in proteomics.
Materials and Methods

Experimental Design Overview

The present study used paired lamb chops obtained from *Longissimus*, the *Psoas major* and the *Semitendinosus* muscles of commercial grade lambs. One of the chops was aged for 72 h and then cooked, while the other paired chop was aged for 7 d and then cooked. Samples from six biological replicates were used. One sample was obtained from the internal part of each cooked chop. A myofibrillar fraction for SDS-PAGE was prepared and separated to generate one dimensional proteomic profiles. The proteomic profiles were annotated by image analysis software. Band percentage contribution to the total volume of the lane was calculated and was statistically analyzed by a mixed model. The mixed model consisted of muscle type × postmortem aging time × number of identified bands in the proteomic profile factorial design. The model also considered the covariance of the repeated measurements and the random effect of the biological replicates. Proteins contained within the bands found to significantly vary across the factors were sequenced by capillary-liquid chromatography-nano spray tandem mass spectrometry. The comparisons of the sequencing results across treatment groups were manipulated and normalized by incorporating electrophoretic data to permit fair comparisons of individual protein participants likely to be responsible for the variation among the treatment groups.

Animals and Sampling

Six lambs from the mating of Suffolk × Texel rams with Rambouillet ewes were used for this experiment. Animals weighing 62.9 ± 9.5 kg of weight and 220 ± 23.3 d of
age were harvested at The Ohio State University Meat Laboratory according to accepted animal welfare practices. Postmortem time was measured from the time of exsanguination. After harvest, carcasses were placed in a cold room at 4 °C for 36 h prior to chop fabrication. To fabricate the chops the whole muscles were dissected from the carcass and then chops were cut to 2.5 cm thickness. Paired chops were matched from each side of the carcass so the assessment was performed on the same location in the muscle. The chops were selected from the Longissimus between the 12th and 13th rib, and the chops selected from the P. major were cut between the 15th and 16th rib. Chops selected from the Semitendinosus were cut at the midpoint of the muscle. All chops were vacuum packaged (Prime Source Vacuum Pouches, KOCH Supplies Inc., Kansas City, MO) and aged at 4 °C. From the paired chops, one chop was aged for 72 h postmortem while the paired chop was aged for 7 d postmortem.

**Cooking Process**

Chops aged for 72 h or 7 days were cooked on a cooking grill (George Foreman Next Grilleration; Salton, Inc., Miramar, FL) set to 190.5 °C and cooked to an internal temperature of 71.1 °C monitored with a scanning thermocouple thermometer (DigiSense 12-Channel Benchtop Thermocouple Scanner; Barnant Co., Barrington, IL). The cooked chops were then cooled to room temperature and then sliced with a knife into one centimeter cubes. Samples were taken from the center of the cube by removing the browned meat that was in contact with the cooking surface. Only the lean tissue free of evident fat deposits and connective tissue was sampled. Samples were flash frozen in liquid nitrogen and then stored at -80 °C until they were prepared for SDS-PAGE.
SDS-PAGE and Image Analysis

All samples from the selected muscles and selected time points of aging were evaluated by SDS-PAGE. Myofibrillar fractions of each meat sample were prepared as previously described (Zapata, Zerby et al. 2009). The myofibrillar fraction was dissolved in sample buffer (8 M urea/2 M thiourea, 75 mM DTT, 50 mM Tris, 3% SDS, and 0.004% bromophenol blue, pH 6.8). After the last centrifugation step the pellets where weighed and only 75 mg of the pellet were kept, the rest of the pellet was discarded. The 75 mg of pellet were dissolved with 1.5 ml of sample buffer. Muscle samples were then incubated on ice for 30 min. Samples were centrifuged at 10000g for 10 min prior to loading onto a 1 mm × 12 cm × 14 cm polyacrylamide slab gel consisting of 10-20% gradient resolving gel [30:0.8, acrylamide/N, N’-bis (methylene acrylamide)] and a 3% stacking gel containing 1% SDS. Electrophoretic separation was carried out at a constant voltage of 10 V/cm.

After electrophoretic separation, gels were placed in fixing solution (50% methanol, 7% acetic acid) for 12 h and subsequently stained with SYPRO Ruby® stain (Bio-Rad Laboratories, Inc., Hercules, CA) for 24 h. The gels were then placed in destain solution (10% methanol, 7% acetic acid) for 30 min and then rinsed with deionized water prior to imaging. Gels were scanned using a Typhoon 9400 laser scanner (GE Healthcare, Chalfont St. Giles, U.K.) using the SYPRO Ruby® setting, but with the 457 nm blue laser to obtain digital images. Images were then analyzed using the Total Lab TL120 (Nonlinear Dynamics Inc., Newcastle upon Tyne, U.K.) software. Band boundary limits were automatically set by the software without manual intervention of the user.
Individual bands were identified and analyzed to determine the percent contribution of each band to the total band volume of the individual sample lane.

**Statistical Analysis**

All statistical analyses were carried out using the statistical package SAS v.9.1.3 (SAS Institute Inc., Cary, NC). The experiment was carried out at a 95% confidence level. To analyze proteomic variation in samples as measured by band percentage, a mixed model was used to estimate the mean band percentage values and mean differences across postmortem aging times and muscle types. The effect of postmortem aging times, the effect of muscle types and their interaction were treated as fixed effects in the statistical analysis. Treatment groups were defined by the muscle type by postmortem aging time interaction resulting in a total of six groups. The effect of the individual animal was treated as a random effect. In addition, the experiment contained repeated measurements from the same animal. All estimates were obtained per band, which implies that all the previous variables had the band interaction effect included. The model is described as follows:

\[
\text{Band percentage}_{ijkl} = \text{Band}_i \mid \text{Aging time}_j \mid \text{Muscle type}_k + \text{animal}_l + \varepsilon_{ijkl}
\]

where \(\text{Band percentage}_{ijkl}\) is the dependent variable measured on the \(i^{th}\) band of the \(j^{th}\) aging time of the \(k^{th}\) muscle type from the \(l^{th}\) animal; \(\text{Band}_i\) is the effect of the \(i^{th}\) electrophoretically resolved band matched across lanes and gels in the image analysis software \((i = 1, \ldots, 32)\); \(\text{Aging time}_j\) is the effect of the \(j^{th}\) postmortem aging time \((j = 72 \text{ h or } 7 \text{ d})\); \(\text{Muscle type}_k\) is the effect of the \(k^{th}\) muscle type \((k = \text{Longissimus, P. major, Semitendinosus})\); \(\text{animal}_l\) is the random effect of the \(l^{th}\) individual animal \((l = 1, \ldots, n)\);
and \( \varepsilon_{ijkl} \) is the random error inherent to each measurement, which is assumed to be independent of other observations and normally distributed with mean zero and standard deviation of \( \sigma^2 \). Mean differences were only estimated within the same band using a Bonferroni adjusted alpha to control the error in an experiment-wise manner. Slice effects (Winer 1971) were estimated to simplify the data interpretation.

**Protein Sequencing**

For protein sequencing from the selected bands, three random samples from each treatment group were pooled together in equal proportions as previously described (Zapata, Zerby et al. 2009). All mass spectrometry was performed at the Mass Spectrometry & Proteomics Facility of the Campus Chemical Instrument Center of The Ohio State University using established methods.

Briefly, bands were excised from the SDS-PAGE gel and washed in 50% methanol/5% acetic acid and further dehydrated in acetonitrile. Gel bands were rehydrated and incubated with DTT solution (5mg/ml in 100 mM ammonium bicarbonate) prior to the addition of 15mg/ml iodoacetamide in 100 mM ammonium bicarbonate solution. Gel bands were washed again with cycles of acetonitrile and ammonium bicarbonate (100 mM) and dried in a speed-vac. The protease is driven into the gel pieces by rehydrating them in 50 \( \mu \)L of sequencing grade modified trypsin (Promega, Madison WI) at 20 \( \mu \)g/mL in 50 mM ammonium bicarbonate and incubated at room temperature overnight. Peptides were extracted from the polyacrylamide with 50% acetonitrile and 5% formic acid several times and pooled together. Extracted pools were concentrated in a SpeedVac (Thermo Fisher Scientific, Waltham, MA) to ~30 uL.
Capillary-liquid chromatography-nanospray tandem mass spectrometry (nano-LC/MS/MS) was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray source operated in positive ion mode. The LC system was an UltiMate™ 3000 (Dionex, Sunnyvale, CA). Solvent A was water containing 50mM acetic acid and solvent B was acetonitrile. Five microliters of each sample were injected and washed with 50 mM acetic acid and then eluted onto a 5 cm 75 μm ID ProteoPep II C18 column (New Objective Inc., Woburn, MA) for chromatographic separation. Peptides were eluted off the column into the LTQ system using a gradient of 2 to 80% solvent B with a flow rate of 300 nl/min. The total run time was 65 minutes. The nanospray source was operated with a spray voltage of 3 KV and a capillary temperature of 200 °C. The scan sequence of the mass spectrometer was based on the TopTen method. The analysis was programmed for a full scan recorded between 350 to 2,000 Da, and a MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive instrument scans of the ten most abundant peak in the spectrum. The CID fragmentation energy was set to 35%. Dynamic exclusion was enabled with a repeat count of 30 s, exclusion duration of 350 s and a low mass width of 0.5 Da and high mass width of 1.50 Da. Sequence information from the MS/MS data was processed by converting the raw data files into a merged file (.mgf). The resulting files were searched using Mascot Daemon v.2.2.1 (Matrix Science, Boston, MA) and the searched against the NCBInr database v.2009-07-10 (9,283,978 sequences; 3,180,197,137 residues) across all Metazoa sequences. The mass accuracy of the precursor ions were set to 2.0 Da given that the data was acquired on an ion trap mass analyzer and fragment mass accuracy was
set to 0.5 Da. Variable modifications considered were methionine oxidation and carbamidomethyl cysteine. Two missed cleavages for the enzyme were permitted. Protein identifications were checked manually and proteins with a MOWSE score of 50 or higher with a minimum of two unique peptides from one protein having a -b or -y ion sequence tag of five residues or better were accepted.

**Sequencing Data Mining**

Mascot sequencing data across the individual bands were merged across all six groups into single datasets, one per individual band. The data was mined following the algorithm described in Figure 3.

**Validation**

A validation experiment was performed by spiking random samples with known amounts of a protein not present in muscle tissue. This separate experiment was performed in order to verify the validity of the estimates obtained through the normalization made on the emPAI values and the adjustment to band percentages. Random samples were spiked with known amounts of beta-galactosidase (Thermo Fisher Scientific, Waltham, MA) in a 1:2:4 ratio, eg. 0.01125 mg/ml, 0.0225 mg/ml, and 0.045 mg/ml.

Three random samples were selected from the main experiment. Samples were electrophoresed, imaged, LC-Ms/MS sequenced and data mined using the same methodology described previously. The difference from the experimental method used in this validation experiment was that bands corresponding to the beta-galactosidase protein
were not selected by a statistical model; instead, they were visually selected based on relative 1:2:4 band intensity and molecular weight using the same image analysis performed to obtain the band percent contribution to the total of the lane that would correspond to the band containing beta-galactosidase. One graph was constructed for each of the separate samples selected from the main experiment. Normalized abundance

**Figure 3.** Data manipulation algorithm followed for semi-quantitative determination of protein ratios from mass spectrometry data. The algorithm describes how to manipulate Mascot search results to obtain protein ratios present within a given band. This method allows for valid comparisons across independent mass spectrometer runs by relying on the emPAI as a protein abundance estimator.
mean differences were estimated by ANOVA using the normalized abundance as the
independent variable and the ratio (1:2:4) as the dependent variable.
Results

Proteomic Profiles of Cooked Meat

Thirty two bands were matched across all samples by using Total Lab TL120. Bands were numerically labeled from the top of the gel to the bottom of the gel for matching across samples and the band percentages from the matched bands were statistically analyzed. From the 32 bands, statistical analysis of main effects and interactions revealed 3 bands that varied significantly across the main effects of muscle type and postmortem aging time and across the interaction effect of muscle type by postmortem aging.

Figure 4. One dimensional electrophoretic profiles of cooked lamb chops of Longissimus, Psoas major and Semitendinosus muscles that were aged for 72 h or 7 d postmortem before cooking. BR = Broad Range molecular weight marker. The asterisk highlights the band number of the resolved bands that were found to be significantly different across muscles and postmortem aging by statistical analysis.
postmortem aging time (Figure 4). Significant bands were band 3, band 22 and band 30. The estimated molecular weights of the significant bands estimated by the image analysis software were 99.5 kDa for band 3, 31.1 kDa for band 22, and 17.1 kDa for band 30.

Table 6. Source of statistical differences across treatment groups.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Band</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F value</th>
<th>P &gt; Fa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band*Aging Timeb</td>
<td>b3</td>
<td>1</td>
<td>186</td>
<td>14.61</td>
<td>0.0002</td>
</tr>
<tr>
<td>Band*Muscle Typec</td>
<td>b3</td>
<td>2</td>
<td>186</td>
<td>4.39</td>
<td>0.0137</td>
</tr>
<tr>
<td>Band<em>Aging Time</em>Muscle Typec</td>
<td>b3</td>
<td>5</td>
<td>186</td>
<td>8.37</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Band*Aging Timeb</td>
<td>b22</td>
<td>1</td>
<td>186</td>
<td>0.01</td>
<td>0.9043</td>
</tr>
<tr>
<td>Band*Muscle Typec</td>
<td>b22</td>
<td>2</td>
<td>186</td>
<td>15.59</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Band<em>Aging Time</em>Muscle Typec</td>
<td>b22</td>
<td>5</td>
<td>186</td>
<td>7.41</td>
<td>&lt;.0001</td>
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<tr>
<td>Band*Aging Timeb</td>
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<td>0.1816</td>
</tr>
<tr>
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</tr>
<tr>
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<td>b30</td>
<td>5</td>
<td>186</td>
<td>6.24</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

The source of the effects was obtained by estimating the slice effect across bands from the main model.

a Significances are declared when the P > F values are less than the Bonferroni adjusted alpha.
b For the Band*Aging Time slice effect the Bonferroni adjusted alpha is 0.05/64.
c For the Band*Muscle Type slice effect the Bonferroni adjusted alpha is 0.05/96.
d For the Band*Aging Time*Muscle Type slice effect the Bonferroni adjusted alpha is 0.05/192.

To identify the source of variation, slice effects of the main factors were estimated and are displayed in Table 6. The significance of band 3 was mainly due to the main effect of postmortem aging time (P = 0.0002) but also the main effect of muscle type was close to being significant (P = 0.0137). A statistical trend that related the effect of muscle type in band 3 can be derived from this observation. It is important to note that these comparisons utilize the Bonferroni cut-off value of 0.05/64, where 64 (32 bands × 2 postmortem aging times) is equal to the total number of pair-wise comparisons within individual bands over the entire dataset. In the case of band 22, the significance was due
to the main effect of muscle type (P < 0.0001) rather than to the main effect of postmortem aging time (P = 0.9043). In the same manner as in band 3, the Bonferroni cut-off value was used. However, in this case the Bonferroni cut-off value was 0.05/96, where 96 (32 bands × 3 muscle types) is equal to the total number of pair-wise comparisons within individual bands over the entire dataset. Finally, for band 30, the significance did not reside in any of the main effects (P = 0.0028 for the main effect of muscle type and P = 0.1816 for the main effect of postmortem aging time). Significance of this band resided solely on the interaction effect of muscle type by postmortem aging time (P < 0.0001). The corresponding Bonferroni cut-off value was 0.05/192, where 192 (32 bands × 2 postmortem aging times × 3 muscle types) is equal to the total number of pair-wise comparisons within individual bands over the entire dataset.

Separately defining the main effects or interaction effects as the main source of the significance within a given band allows for data grouping which facilitates the interpretation of the statistical analysis. In Figure 5, the estimated mean band percentages are displayed by treatment group and then grouped by the main source of significant variation for each specific case. The values reported for the grouped variables are the least square means estimated by the model without considering the interaction effect. The difference between the largest mean band percentage and the smallest mean band percentage across all six treatment groups was calculated to illustrate the range of variation. These differences were 5.14 for band 3, 5.06 for band 22, and 4.90 for band 30. In Figure 5, the ungrouped data means as well as the grouped data mean are displayed. For band 3 the mean band percentage of the 7 d group was significantly greater (P =
For band 22, the mean band percentage of the *P. major* was significantly greater (\(P < 0.0001; \alpha = 0.05/96\)) than the mean band percentage of the *Semitendinosus* but was not different than the mean band percentage of the *Longissimus* (\(P = 0.0011; \alpha = 0.05/96\)). The *Semitendinosus* was not significantly different from the *Longissimus* (\(P = 0.0889; \alpha = 0.05/96\)). For band 30, 0.0002; \(\alpha = 0.05/64\)) than the mean band percentage of the 72 h group. For band 22 the mean band percentage of the *P. major* was significantly greater (\(P < 0.0001; \alpha = 0.05/96\)) than the mean band percentage of the *Semitendinosus* but was not different than the mean band percentage of the *Longissimus* (\(P = 0.0011; \alpha = 0.05/96\)). The *Semitendinosus* was not significantly different from the *Longissimus* (\(P = 0.0889; \alpha = 0.05/96\)). For band 30,
within the 72 h group, the Longissimus mean band percentage was significantly greater than the mean band percentage from both the P. major and the Semitendinosus (P = 0.0001 and P < 0.0001; α = 0.05/192). However, within the 7 d group no significant differences were detected. Additionally, the band percentage of the Longissimus at 72 h was greater than the band percentage of the Semitendinosus at 7 d (P < 0.0001; α = 0.05/192).

**Protein Sequencing and Data Mining**

Each treatment group had three bands submitted for protein sequencing by nano-LC/MS/MS (band 3, 22 and 30). In the initial Mascot search results dataset for band 3 an average of 42 protein IDs were identified, for band 22 an average of 38 protein IDs were identified, and for band 30 an average of 24 protein IDs were identified. The data were mined by removing incomplete entries and low score protein IDs (Figure 3, step 1). The average number of protein IDs remaining in the analysis was 22 for band 3, 38 for band 22, and 16 for band 30. After sorting by simplified protein names, only 16 unique proteins were kept for band 3, 29 for band 22, and 14 for band 30. Grouped data are displayed by band in **Appendix Data Tables 1, 2, and 3**. Some protein IDs were consistently found across all treatment groups. These protein IDs are listed in these supplementary data tables.

**Beta-galactosidase validation experiment**

One random sample from each muscle of cooked meat was selected for this part of the study. The samples correspond to Semitendinosus aged for 72 h, Psoas major aged for 7 d and Longissimus aged for 7 d. Samples were electrophoresed, imaged and
sequences determined as described previously. Protein IDs were semiquantified by following the process described in Figure 3. Protein ID comparisons from the beta-galactosidase spiked samples are displayed in Figure 6.

Figure 6. Beta-galactosidase experiment normalized abundance of the proteins identified. Samples spiked with 1:2:4 ratio of a 0.01125 mg/ml of purified beta-galactosidase. Only the protein IDs that contributed a mean of at least 5% of the total were included. A) Semitendinosus aged for 72 h sample used as background. B) Psoas major aged for 7 d sample used as background. C) Longissimus aged for 7 d sample used as background.

To simplify the display of results for this experiment, only the protein IDs with a
mean contribution of at least 5% of the ratio are displayed. Graphs show an evident increase that resembles the proportions (1:2:4 ratio) of beta-galactosidase added. Mean and standard deviation in arbitrary units of the three separate runs were of 1.13 ± 0.69 for the 1X load, 2.40 ± 0.59 for the 2X load and 6.11 ± 0.18 for the 4X load. All three comparisons were significantly different from each other by ANOVA; 1X vs. 2X (P = 0.0270), 1X vs. 4X (P < 0.0001) and 2X vs. 4X (P = 0.0001) (SEM = 0.5324).

**Comparison of Protein Ratios**

The protein ID comparisons obtained after the process described in Figure 3 are presented in Figure 7 panel A for band 3, panel B for band 22 and panel C for band 30. The normalized ration between treatment groups are displayed in each figure. Ratio differences are subjective; however relative amounts can be considered a reasonable approximation as observed in the beta-galactosidase validation experiment. Confidence intervals cannot be calculated due to the experiment setup. For band 3 (Figure 7 panel A), the proteins that had the greatest abundance difference across the treatment groups were myoglobin, ATPase, glycogen phosphorylase and actinin. The rest of the proteins did not show large abundance differences. Myoglobin, ATPase and actinin were more abundant in the 7 d samples than in the 72 h samples. Glycogen phosphorylase was more abundant in the 72 h samples compared to the 7 d samples. For band 22 (Figure 7 panel B), the proteins that had the greatest abundance difference were carbonic anhydrase, followed by ADP/ATP translocase, myosin light chain, troponin, prohibitin, triosephosphate isomerase, phosphoglycerate mutase, cytochrome C, creatine kinase and tyrosine 3-monoxigenase. In the *P. major* samples, carbonic anhydrase, ADP/ATP
translocase, myosin light chain, troponin, phohibitin, cytochrome C, creatine kinase and tyrosine 3-monoxigenase had greater abundance compared to either, *Longissimus* and *Semitendinosus* samples. However, in the *P. major* samples, triosephosphate isomerase,

**Figure 7.** Identified proteins and their abundance within individual bands based on normalized emPAI abundance ratios by treatment group. All identified proteins are included. A) Proteins identified in band 3. B) Proteins identified in band 22. C) Proteins identified in band 30.
and phosphoglycerate mutase had lower abundance values as compared to either the Longissimus or the Semitendinosus samples. Carbonic anhydrase, thriosephosphate isomerase, and phosphoglycerate mutase were expressed at higher levels in the Longissimus, compared to Semitendinosus. For Band 30 (Figure 7 panel C), the proteins that had the greatest abundance differences were myoglobin, myosin light chain, troponin, cytochrome C oxidase and the NADH-dehydrogenase. The rest of the proteins did not show any evident abundance differences. In this case, the 72 h Longissimus had higher levels of myoglobin abundance compared to the other five treatment groups. Myosin light chain was not found in the Semitendinosus at 7 d. Other protein ratio differences were not described because of the presence of 6 treatment groups; however, the abundance differences were not as great as the difference observed for myoglobin.
Discussion

One Dimensional Proteomic Profiles of Cooked Meat

Proteomic analysis based on one dimensional electrophoresis is not a common practice because of the availability of more sophisticated methodologies that generate larger amounts of information. The major drawback of one dimensional electrophoresis based proteomic experiments is the confounding effect of the number of protein(s)/peptide(s) co-migrating in a given band. Protein ID interpretation is difficult because there is uncertainty resulting from the presence of a mixture of proteins that may participate in the biological mechanism studied and irrelevant proteins that likely make up the background (Reddish, St-Pierre et al. 2008; Zapata, Zerby et al. 2009).

The inability to objectively separate the relevant proteins from the irrelevant proteins limits the application of one dimensional electrophoresis in modern proteomic analysis. This limitation obscures some of the advantages of one dimensional electrophoresis of high reproducibility, the high throughput ability of running several replicates on a single gel, technical simplicity, and low cost of equipment and reagents (Issaq 2001). Other technologies available used for proteomic analyses are based on two dimensional electrophoresis (Gorg, Postel et al. 1980) which has been further refined into DIGE (Alban, David et al. 2003; Issaq and Veenstra 2007); however these methodologies are still limited by issues of spot recognition and quantification, and low throughput and reproducibility (Mann 1999; Kang, Techanukul et al. 2009). There are also tag based methodologies like ICAT (Gygi, Rist et al. 1999) and iTRAQ (Ross, Huang et al. 2004) which are popular choices for quantitative proteomic analysis.
In addition, it was established by several studies (Parsons and Lawrie 1972; Armstrong, Richert et al. 1982) that the analysis of cooked meat proteins by SDS-PAGE was a viable method because even meat samples heated with a high-temperature (cooked) can be dissolved under the denaturing conditions of 2% sodium dodecyl sulphate. It was demonstrated that protein recovery using when using 3% of sodium dodecyl sulfate was not different between cooked and raw meat (Lee, Rickansrud et al. 1975).

**Experimental Design and Procedure Overview**

Our experimental approach is similar to two-dimensional electrophoresis, where the first dimension consists of sorting the protein(s)/peptide(s) by their respective isoelectric point followed by the second dimension which separates the protein(s)/peptide(s) by their respective molecular weights (Gorg, Postel et al. 1980). The methodology reported in this study is similar to 2DE in that it is based on a two dimensional system. The first dimension consists of electrophoretic separation by one dimensional electrophoresis where the sample is separated into bands based on molecular weights. The bands from the one dimensional electrophoresis can be matched across samples and gels similar to spots are matched in a two dimensional gel but in a much simpler way. In the system reported in this study, the data set obtained from the image analysis can be handled by any statistical package. SAS was used in this study; statistical packages are widely available and are more intuitive compared to specialized software packages where the parameter settings tend to be not clearly defined (Shen, Li et al. 2009). The second dimension separation is carried out by the mass spectrometer itself and is only performed on the bands that are considered to be relevant to the experimental
design from the statistical model. The manner in which we evaluated the sequence data by coupling mean band percentages with emPAI establishes an objective method to differentiate biologically relevant proteins from irrelevant background proteins. This is because emPAI calculates protein/peptide abundance in a given band based on the number of enzymatic fragments identified compared to the number of potential enzymatic fragments for a particular protein/peptide in the band of interest.

The advantages of the methodology presented in this study are: First, the high reproducibility of the one dimensional electrophoretic separation system. Second, the simplicity of the band matching across multiple samples (in our system, each lane was loaded with a biological replicate) as compared to matching two dimensional gels. Third, the transparency of the statistical analysis. Finally, the ability to segregate proteins which are related to the biological question posed from those that are not related. Although it was not performed in this study, the methodology presented can be further refined by performing independent nano-LC-MS/MS runs on biological replicates. Another point to be highlighted about the statistical modeling employed in this study is the estimation of slice effects (Winer 1971). The benefits and practical application of slice effect estimates have been previously described and reviewed (Schabenberger, Gregoire et al. 2000). Briefly, the estimation of slice effects is a statistical tool that aids researchers on the interpretation of factorial models. Slice effects represent the orthogonal contrasts that account for comparisons of levels of a given factor at a particular level of another factor. In the present study, the factors (referred to as treatment groups) investigated were muscle type and the postmortem aging time within a given band. Only the comparisons
across muscle type and postmortem aging time within a given band made biological sense, thus the estimation of slice effects within bands was justified.

Incorporating the band percentage solves the problem that arises from the fact that the raw emPAI value calculation does not incorporate any information that reflects the abundance of the sequenced sample within the tissue. Normalized protein ratios only indicate the proportions in which the proteins are found in the trypsin digested sample. However, the proportional variation compared to another sample can be made which produces semi-quantitative results. Unlike the simple comparisons of a single treatment versus a control conducted using 2DE based proteomics, the methodology developed in the present study generates pair-wise comparisons across multiple treatments. Thus, the abundance difference of individual proteins from one treatment group is relative to what treatment group is being compared.

Some potential pitfalls that must be acknowledged using our methodology include the necessity to fractionate samples prior to one dimensional electrophoresis which can potentially omit unfractionated proteins due to recovery losses. In this experiment, a myofibrillar fraction was analyzed. However, fractionation of complex samples is commonly suggested by experts (Gygi, Corthals et al. 2000; Gorg, Weiss et al. 2004), and is routinely performed. Another potential pitfall is due to the low band resolution. Band resolution can be improved to a large extent by the use of sensitive fluorescent stains (Cong, Hwang et al. 2008) and the use of gradient gels matrices (Margolis and Kenrick 1967) as used in this study. A major limitation to many 1DE or 2DE based proteomic studies is the reliability of the protein IDs abundance estimations. This was
solved in this study by the development of normalized emPAI values. In this experiment the emPAI values (Ishihama, Oda et al. 2005) were used as abundance estimators. EmPAI values were selected because they are provided by default on the Mascot search result summary. Although more efficient methods to estimate protein abundance from a proteomic experiment are available, discussed below, emPAI values were selected because they could be obtained without complex experimental designs or additional computations. The normalization performed in the present study allowed fair comparisons across treatment groups.

Protein abundance estimation methods like the absolute quantification based on the three most intense tryptic fragments in a MS/MS run (Silva, Gorenstein et al. 2006), absolute protein expression (APEX) based fragment count estimations from MS/MS data (Lu, Vogel et al. 2007), quantification of peptides of interest by comparison to a calibrated isotope labeled reference (Lange, Picotti et al. 2008) a combined method encompassing the three previous methods mentioned (Malmstrom, Beck et al. 2009), or by the use of a modified spectral count index (mSCI) derived from the incorporation of mRNA and protein expression data (Sun, Zhang et al. 2009) will increase the complexity of the analysis. In theory, the more advanced abundance calculation methods can provide more precise results than those obtained by utilizing the emPAI values; however the incorporation of such abundance calculation methods into a routine analysis is more complex to perform which can hinder their application. The downstream process of the methodology reported in this study can be easily adapted to incorporate the more complex estimators.
The present study was performed on lamb which is a species whose genomic or expressed sequence tag data is not as complete or as well characterized as human or mouse data; at the time of manuscript preparation the NCBI protein database contained 8,117 sequences designated as lamb “Ovis aries” compared to 556,706 designated as Homo sapiens or 257,356 designated as Mus musculus. Protein identification is based on peptide sequence matching to protein sequence databases. In the case of organisms with poorly characterized proteins, the matching is affected by the evolutionary divergence between the species. In addition, the large amount of sequences of protein fragments can also affect the data because the calculation of the emPAI relies on the sequence coverage (Ishihama, Oda et al. 2005).

**Beta-galactosidase validation experiment.**

Results from this experiment showed that it is possible to determine a difference of the abundance of a protein by as little as one fold. This notion helps base our rationale of using emPAI values in combination with band percentage contribution of the band to the total of the lane as estimators of protein abundance. In further experiments, it may be relevant to assess the impact of band cutting prior to nano-LC-MS/MS by performing technical replicates of the same sample. Even when the normalized abundance estimates are not exact as they carry some error, the method provides good resolution that can find differences among treatments and spot candidate proteins that can be further analyzed in a more directed approach. It is important to mention that this methodology does not replace other popular methods for protein abundance comparison as two dimensional electrophoresis based methods but it can aid on directing them.
Identified Proteins Significantly Associated to Postmortem Aging Time and Muscle Type

This experiment determined three bands which were each uniquely significant to a single factor in the statistical model as defined by the slice effects. Band 3 was mainly related to postmortem aging time, band 22 to muscle type, and band 30 to the postmortem aging time by muscle type interaction. Significances were observed even when, the confidence of the analysis of this experiment was very tight. Such tightness was controlled by using the Bonferroni adjustment, which decreases the chance of committing a Type I error but increases the chance of committing a Type II error. In other words, the likelihood of having the significant bands found to be associated when, in reality, they are not is very small but the chance of not finding other significant bands that are also associated is large (Gordi and Khamis 2004). Therefore, some relevant information was inherently sacrificed because of the use of the Bonferroni adjustment. Even when data was sacrificed, I decided to use the Bonferroni approach because higher confidence in the band/protein identifications was preferred rather than a larger number of band/protein identifications with lower confidence.

To be able to discuss the association of the identified proteins with the variables included in the statistical model, it is necessary to understand some generalities about postmortem metabolism in skeletal muscle. Although it has been shown that RNA degradation is not extensive enough to be the limiting factor for postmortem protein synthesis (Fontanesi, Colombo et al. 2008), the energy availability required for protein synthesis plus the decrease in the functionality of protein synthesis machinery by the low
pH resulting from the production of lactic acid, the end product of anaerobic metabolism (Pösö and Puolanne 2005) abolishes the de novo protein synthesis. This notion suggests that proteomic pattern alteration, in postmortem skeletal muscle, is mostly the result of postmortem protein modification, proteolysis and protein denaturation (Ouali, Herrera-Mendez et al. 2006). Protein denaturation, which entrapped the sarcoplasmic proteins during the cooking process, is the likely explanation for the observation of soluble proteins (sarcoplasmic) in the insoluble fraction (myofibrillar), used in this study (Joo, Kauffman et al. 1999). Transglutaminase catalyzed protein covalent cross-linking has been reported to occur in postmortem muscle which could result in the observation, in this study, of proteins migrating at a higher molecular weight (Eligula, Chuang et al. 1998). This study is novel since classical studies of postmortem events in muscle that lead to meat is usually conducted on raw muscle tissue prior to cooking.

Due to the large number of protein IDs, the discussion was reduced only to the protein IDs that contributed at least an average of 5% of the total load among the six treatment groups. The resulting protein IDs are displayed in Figure 8 panel A, for band 3, Figure 9 panel A, for band 22 and Figure 10 for band 30. As mentioned in the Results section, main effects were identified for band 3 and band 22 to be determinant of their variation; this was intended to facilitate the interpretation of the results. Figure 8 panel B shows the protein ID abundances by postmortem aging time while Figure 9 panel B shows the protein ID abundances by muscle type. The normalized protein ratios obtained in this experiment showed some interesting patterns. The most obvious pattern was the relation of the band percentage pattern across treatment groups (Figure 5). In band 30,
Figure 8. Trimmed list of identified proteins and abundance found in band 3. Only the protein IDs that contributed a mean of at least 5% of the total were included. 

A) Most abundant identified proteins by treatment group. 

B) Most abundant identified proteins averaged by postmortem aging time, postmortem aging time was found via the statistical analysis to be the most determinant factor for the abundance variation of this band.

The protein that had the greatest abundance difference across treatment groups was myoglobin. The normalized ratio pattern of myoglobin across treatment groups (Figure 10) was almost identical to the band percentages for that same band (Figure 5, panel C). Previous studies have shown that the amount of myoglobin is muscle type and
Figure 9. Trimmed list of identified proteins and abundance found in band 22. Only the protein IDs that contributed a mean of at least 5% of the total were included. A) Most abundant identified proteins by treatment group. B) Most abundant identified proteins averaged by muscle type, muscle type was found via the statistical analysis to be the most determinant factor for the abundance variation of this band.

developmental stage dependent in lambs (Gardner, Hopkins et al. 2007). In addition, myoglobin was found at a higher level or concentration in band 3 in the meat aged for 7 d as compared to the meat aged for 72 h. A similar trend was observed across aging time in band 30, but in this case, the relation was the opposite. Because band 3 has an estimated molecular weight of 99.5 kDa and full length myoglobin is ~17kDa, it can be assumed that myoglobin was covalently associated with another protein. This
Figure 10. Trimmed list of identified proteins and abundance found in band 30. Only the protein IDs that contributed a mean of at least 5% of the total were included. Note that for this band the determinant factor for the abundance variation was the interaction between postmortem aging time by muscle type.

association is formed during the aging period between 72 h and 7 d by an unknown mechanism. As discussed earlier, transglutaminase catalyzed protein covalent cross-linking has been reported to occur in postmortem muscle which could result in the phenomenon (Eligula, Chuang et al. 1998). In addition, transglutaminase cross-linking has been reported with proteins of the globin family (Manjunath, Chung et al. 1984). Other protein abundance ratio differences found in band 3 may be related to the statistical trend observed which involves the muscle type effect. Differences in the abundance ratio of actinin and glycogen phosphorylase and ATPase have been previously reported to be associated to fiber type (Mills, Yang et al. 2001; Pösö and Puolanne 2005; Quinlan, Seto et al. 2010).

A trend was observed in the band percentage of band 22 (Figure 5, panel B) and
on the normalized protein ratios for band 22 (Figure 9). Psoas major samples had protein abundance patterns which were different from Longissimus and Semitendinosus samples. This difference could be attributed to fiber type composition (Kolczak, Pospiech et al. 2003; Melody, Lonergan et al. 2004) which have differences in their fundamental energy metabolism (Bottinelli 2001). The greatest abundance difference was observed in carbonic anhydrase, which has been reported to be related to fiber type composition (Henry, Bilger et al. 2001). In addition, ADP/ATP translocase is related to mitochondrial activity which is indicative of oxidative metabolism pathways (McMillin and Pauly 1988). Higher abundance of oxidative pathway related enzymes in the P. major suggests its greater content of slow muscle fibers as compared to the Longissimus and Semitendinosus. Enzymes associated with the glycolytic pathway are also represented in the band. Triosephosphate isomerase and phosphoglycerate mutase, two enzymes that that participate in the glycolytic pathway (Scrutton and Utter 1968), were found at higher concentrations in the Longissimus and Semitendinosus muscles as compared to the P. major. Thus, such finding reflects the predominance of fast Type II muscle fibers in the Longissimus and Semitendinosus muscles compared to the P. major. The presence of myosin light chain in band 22 and band 30, which were significantly associated to muscle type, is also related to fiber type composition (Kolczak, Pospiech et al. 2003; Brotto, Biesiadecki et al. 2006).

The association of muscle type related proteins and meat quality has been demonstrated previously in pig (Nam, Choi et al. 2009) and in beef (Renand, Picard et al. 2001) where higher meat quality ratings were associated to greater amounts of slow
muscle (Type I) fiber content. In the present study, greater abundance of oxidative enzymes were found in the *P. major* which has a higher concentration of slow Type I fibers than either the *Longissimus* or *Semitendinosus* muscles. In cattle, the *P. major* muscle is commonly marketed as a steak derived from the tenderloin which is the most valued cut of the carcass (USDA 2010).

An interesting finding was the abundance variability across treatment groups of tyrosine 3-monooxygenase. Tyrosine 3-monooxygenase was expressed in higher concentrations in the *P. major* muscle followed by the *Longissimus* muscle and finally by the *Semitendinosus* muscle. This enzyme plays a role in the biosynthetic pathway of catecholamines (Fitzpatrick 1999). This protein was found migrating at a lower molecular weight (31.1 kDa) than of the full length protein (~ 58 kDa) (Ikeda, Okuno et al. 1991) which suggest that the protein was likely proteolyzed during postmortem aging. A previous study in beef reported an association of catecholamides in urine to meat quality (Lowe, Devine et al. 2004). These investigators report that higher concentrations of catecholamides, which lead to more rapid glycogen depletion, has an impact on ultimate pH which is related to several meat quality variables such as tenderness, color and flavor.

The present study does not yet provide a definitive methodology that will permit the authentication of meat cuts. There was no specific protein/peptide that was found significant in only one type of meat cut.
CHAPTER 4

Study 3

Multivariate evaluation of one dimensional sarcoplasmic protein profile patterns of breast muscle during early post hatch development

Summary

Proteins are the main participants in metabolic pathways. However, the analysis of protein abundance patterns associated with those pathways is complicated by the large number of proteins involved. In this study, the objective was to present the application of principal component analysis (PCA) to permit the visualization of developmental proteomic patterns of breast muscle sarcoplasmic proteins. Different turkey genotypes and nutritional regimens were used to potentially increase the variability within the sarcoplasmic protein profile. Sarcoplasmic protein fractions from turkey breast muscle samples were collected at 6 ages from 7 d to 24 d. Breast muscle samples were collected from two distinctly different turkey lines. The poult within each line were either ad libitum or restrict fed. Proteomic PCA plots displayed a visual developmental pattern from 7 d until 17 d. Multivariate analysis of variance (MANOVA highlighted the effect of the time point and the feeding regime among profile patterns. The use of different genotypes and feeding regimens did influence variability which was measured by their mean Euclidean distances and the ellipses of the PCA plots. These treatment effects,
however, did not mask the developmental patterns. After 17 d, the proteomic patterns converged, suggesting that a level of biological stability was achieved regardless of the genotype or treatment. The developmental pattern obtained by the PCA methodology can aid in the planning of more efficient experimental designs so the developmental stage of individuals can be more accurately assessed.
Introduction

Sarcomeric proteins are the primary components of metabolic pathways, thus proteomic profiles may provide some insight into the metabolic status of an organism. However, proteome analysis and proteomic data interpretation is complicated due to the large number of proteins found within the cell(s). It is often necessary to utilize discriminative statistical methods to identify which proteins or protein clusters are associated with a specific trait or metabolic phenomenon. Protein pattern recognition is also complicated due to the complex interactions that invariably rig biological processes (Barrett, Brophy et al. 2005). The incorporation of statistical tools into proteome analysis has allowed for the development of structured methods that facilitate proteomic data interpretation. It has been reported that one-dimensional electrophoresis coupled with principal component analysis (PCA) is an effective method for proteomic pattern recognition (Supek, Peharec et al. 2008). For example, PCA has been used to analyze the associations of numerous variables in a multivariate approach to describe sensory analysis of deboned chicken breast (Liu, Lyon et al. 2004) and the evaluation of microbial community structures within poultry litter (Lovanh, Cook et al. 2007; Cressman, Yu et al. 2010).

Breast meat production is a primary economic driver for the turkey industry and the tandem effects of genetic selection and nutrition on body weight gain and breast muscle development have been extensively studied (Bayyari, Huff et al. 1997; Christensen, Donaldson et al. 1999). Turkey breast muscle was used in the current experiment to illustrate the functionality of the proteomic methodology developed. A
study (Havenstein, Ferket et al. 2007) was specifically designed to address the interactive effects of diet and genotype on the growth and development of a slow growing, random bred turkey line representative of commercial turkeys available in the mid 1960’s (RBC2) and a sub-line of the RBC2 selected for body weight alone at 16 wk (F line; (Nestor, Anderson et al. 2008). Selection of the F line was initiated in 1969 and there have been numerous studies conducted over the last 30 years comparing the RBC2 and F line for various aspects of growth, reproductive fitness, and disease resistance (Nestor, Lilburn et al. 1999; Nestor, Saif et al. 1999). The F line weighs approximately twice as much as the RBC2 at selection age (16 wk) but there are no correlated increases in the proportional weight of the breast muscles. There are, however, line differences in the expression of key proteins associated with breast muscle growth and development (Updike, Zerby et al. 2005). One critical protein that is relevant to muscle development is myosin. Myosin has been reported to go through developmental isoform transitions during early growth in chickens (Tidyman, Moore et al. 1997) and turkeys (Maruyama and Kanemaki 1991) and the temporal expression of these isoforms can be influenced by both genetics and nutritional regimen (Graham 2004) Huffman, 2010; unpublished data).

Based on the previous reports of distinct myosin isoform transition patterns, it was hypothesized that the expression of breast muscle sarcomeric proteins would likely go through similar temporal transitions. These transitions would be reflected in differences in protein abundance that could be observed by one dimensional electrophoresis.

In order to utilize proteomic data analysis for the purpose of improving animal
production, it is necessary to develop tools that allow for the visualization of the proteomic status of animals from different genotypes and nutritional environments. With this rationale in mind, a methodology for the visual analysis of proteomic data during turkey muscle growth in pouls was developed. Thus, the objective of this study was to present a statistical application that would permit the visualization of proteomic developmental patterns of breast muscle sarcoplasmic proteins during the first 24 days of age. Different genotypes and nutritional regimens were included in the study to potentially increase the developmental variability in the sarcoplasmic protein profile.
Materials and Methods

Animals, Diets and Experimental Design

Turkey poults from the RCB2 and F line were used in this experiment. The poults from each line were reared in heated Petersime battery brooders. All birds were fed the Ohio State University turkey starter diet which met or exceeded the NRC nutrient recommendations for turkeys (NRC 1994). Poults were allowed either *ad libitum* access to feed or given access to feed for 30 min per day beginning at 4 days of age (Restrict Fed). At the beginning of the experiment, 60 RBC2 and 60 F line poults were randomly assigned to 20 pens (10 pens for RBC2 and 10 pens for F line). Of the 10 pens assigned to each turkey line, the poults in 5 pens had *ad libitum* access to feed while the other 5 pens were restrict fed. To accommodate for replication and avoid having each pen represent a single experimental unit, one bird per pen was randomly selected from each of the pens at each sample age for the respective line and treatment combination. During the course of the experiment some animals died, so the samples collected taken at later ages represented a reduced number of poults. At the end of the experiment, samples from 56 RBC2 poults and 56 F line poults were used for the analysis.

At 7, 11, 14, 17, 21, or 24 days of age, poults from each genetic line and feed treatment were individually weighed and euthanized with CO₂ (FASS 1998). Muscle samples were removed from the *Pectoralis major* breast muscle, flash frozen in liquid nitrogen and subsequently transferred to a -80 °C freezer until further analysis. In summary, the experimental design was a 2 (genotype) × 2 (diet) × 6 (age) factorial arrangement of treatments with four to five biological replicates within each treatment
All animals were handled in compliance with Institutional Animal Care and Use Committee (IACUC) policies and guidelines at The Ohio State University.

### One Dimensional Proteomic Profiling

The sarcoplasmic fraction of muscle tissue was prepared by homogenizing 250 mg of frozen tissue in 2 ml of rigor buffer [10 mM Trismaleate, 60 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.4 mM Pefabloc SC Plus, (Boehringer Mannheim Corp., Indianapolis, IN), pH 6.8] (Ishiwata and Funatsu 1985) on ice. The homogenate was centrifuged at 10,000 ×g for 5 min. The supernatant was saved and re-centrifuged under the same speed and time conditions. The supernatant obtained after the second centrifugation step was designated as the sarcoplasmic fraction. Five hundred microliters of the sarcoplasmic fraction were combined with 1 ml of sample buffer (8 M urea / 2 M thiourea, 75 mM DTT, 50 mM Tris, 3% SDS, 0.004% bromophenol blue, pH 6.8) and kept in ice for 30 min.

For the one dimensional electrophoretic separation, samples were centrifuged again at 10,000 ×g for 10 min before loading onto a 1 mm × 12 cm ×14 cm discontinuous polyacrylamide slab gel consisting of a 10% resolving gel [30:0.8, acrylamide/N,N-bis(methylene acrylamide)] and a 3% stacking gel. Electrophoretic separation was carried out at a constant voltage of 10Vcm⁻¹. After electrophoresis, the gels were stained with SYPRO® Ruby protein gel stain (Bio-Rad Laboratories, Inc., Hercules, CA) for 24 h protected from light. The gels were subsequently destained in 10% methanol, 7% acetic acid for 30 min and rinsed with deionized water prior to imaging according to the manufacturer’s recommendation. Imaging was performed as previously described.
(Zapata, Zerby et al. 2009) on a Typhoon 9410 laser scanner (GE Healthcare, Chalfont St. Giles, U.K.). Digital images were analyzed using the Total Lab TL120 software (Nonlinear Dynamics Inc., Newcastle upon Tyne, U.K.). Bands were matched across samples and gels and assigned a band index number starting from the top of the gel. Band intensity was measured and expressed as a band percentage of the total of the lane to normalize for uneven sample loading.

**Statistical Analysis**

**Body weight differences.** Body weight data were imported into SAS v9.2 (SAS Institute Inc., Cary, NC) and analyzed by a mixed model using the MIXED procedure. Significant differences were declared at a 95% confidence level. Briefly, body weight was estimated by including the fixed effects of turkey line, dietary treatment, age and all interactions. In addition, the experiment included a repeated measurement term to identify the birds that were raised together in the same battery pen. Only the three-way interaction terms were estimated. The model is described as follows:

\[
\text{Body weight}_{ijkl} = \text{Line}_i \mid \text{Treatment}_j \mid \text{Day}_k + \epsilon_{ijkl}
\]

where \(\text{Body weight}_{ijkl}\) is the dependent variable measured on the \(i^{th}\) Line of the \(j^{th}\) Treatment of the \(k^{th}\) Day from the \(l^{th}\) bird; \(\text{Line}_i\) is the effect of the \(i^{th}\) turkey line (\(i = \text{RBC2 or F}\)); \(\text{Treatment}_j\) is the effect of the \(j^{th}\) treatment (\(j = \text{full feed or restricted feed}\)); \(\text{Day}_k\) is the effect of the \(k^{th}\) timepoint (\(k = 7, 11, 14, 17, 21, 24\) d); and \(\epsilon_{ijkl}\) is the random error inherent to each measurement, which is assumed to be independent of other observations and normally distributed with mean zero and standard deviation of \(\sigma^2\).

**One dimensional proteomic profile analysis.** Data were exported from the
image analysis software into a statistical analysis software package SAS v.9.2 (SAS Institute Inc., Cary, NC). PCA was performed using the correlation matrix of the band percentages of the total of the lane as the variables to be analyzed by the PRINCOMP procedure. From the data obtained from the PCA, a component loading plot of the variables (bands) and score plots of the observations (individual samples) were generated by the GPLOT procedure. A further decomposition of the score plots and the addition of 95% confidence ellipses were obtained through the SGPLOT procedure.

**Principal component score pattern analysis.** To analyze the variation among the principal component score clusters obtained from the analysis of the proteomic data a second multivariate approach was used by using multivariate analysis of variance (MANOVA). Component scores from the entire data were used as a secondary dataset. Scores were set as dependent variables while the coded variables of turkey line, dietary treatment, age and all their interactions were set as independent variables. MANOVA analyses were performed in SAS using the GLM procedure. Two separate analyses were performed; the first one used the first two principal component scores as depicted in the plots generated and a second one with the first five principal component scores. To identify differences among treatment combinations within each time point (line*treatment), independent MANOVAs were performed using a subset of the data for each time point. A set of orthogonal contrasts was used to test each pair-wise comparison within a time point. Significant differences were declared at a 95% confidence level.

To evaluate the spread of the score clusters formed from the initial principal component analysis the mean Euclidean distances for each observation’s set of scores to
the centriole of the cluster was calculated. This value represents how spread out the observations are within each cluster. To obtain these values, a subset of the principal component score data for each cluster was analyzed using cluster analysis using Minitab v.15.1 (Minitab Inc., State College, PA) The mean Euclidean distance was calculated separately to the first two principal component scores and to the first five principal component scores within a cluster.
Results

Weight Gain

Mean body weights of the RBC2 and F line poults from each feeding treatment are presented in Figure 11. The ad libitum fed F line poults weighed significantly more than the RBC2 poults at all ages (P < 0.05). The line differences observed are consistent with previous reports for these two lines (Nestor 1984; Nestor, Anderson et al. 2000; Nestor, Anderson et al. 2008). Body weight in both lines was significantly reduced by feed restriction (P < 0.05) but within the feed restriction treatment, there were no between-line differences in body weight.

Figure 11. Mean body weights measured before harvest. F corresponds to the fast grow commercial line while RBC2 corresponds to the random bred control line. Error bars represent the standard deviation of the sample. Each point represents the mean of 4-5 biological replicates.

Principal Component Analysis of the One Dimensional Proteomic Profiles

After image analysis of the one dimensional electrophoresis, 42 bands were
consistently resolved in samples collected from the total population of 112 birds. A gel image of two representative samples is displayed in Figure 12, Panel A. Band percentages were calculated and a correlation matrix was constructed. Principal component analysis was performed and only the first two component loadings and the first two component scores are reported and displayed in Figure 12, Panels B and C, respectively. The first principal component explained 21.2% of the variation while the

**Figure 12.** A) Representative SDS-PAGE lanes of the sarcoplasmic fraction of two RBC2 turkeys one sampled at 7d post hatch and the second sampled at 24d post hatch. B) Principal component analysis score plots of observations (samples). C) Principal component analysis component loadings of variables (bands). Arrows in B indicate samples shown in A. In B and C only the first two scores/components are displayed.
second principal component added 12.6% for a total of 33.8% of the variation explained by the two principal components. For the extended dataset analysis, the first five principal components were included. These five principal components accounted for 54.2% of the variation.

Principal component score loadings represent individual samples and show the spread of the samples in relation to the principal component loadings. Each individual animal sampled is represented by a score in the plot. Samples where scores are clustered together have more in common than samples that are further apart. The spread observed in the sample scores is related to the amount of variation that exists among them. The relationship of the individual scores with the loadings is a reflection of which variables are different among samples. Loadings that appear in the center of the plot have less variation than those that are located closer to the edges. Based on this interpretation, abundance of band 42 appears to be relatively homogeneous across all samples (Figure 12, Panel C), while abundance of band 13 appears to be more heterogeneous across all samples. In Figure 12, Panel B, the sample score labeled as “7 d” has a greater abundance of bands 13, 6, 1, 34, 14, and a lesser abundance of bands 17, 21, 26, 27. In contrast, the sample score labeled as “24 d” exhibits the opposite scenario, bands 17, 21, 26, 27 are in greater abundance than bands 13, 6, 1, 34, 14.

Score plots were decomposed to help the visualization of developmental patterns related to line and feeding regime. Table 7 displays the outcome of the MANOVA analyses on the principal component score data. Significances are consistent between the two separate analysis performed on the first two and first five principal component scores.
Table 7. P values of main and interaction effects of component scores by MANOVA

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<th>Effect</th>
<th>First 2 component scores</th>
<th>First 5 component scores</th>
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<td>line</td>
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</tr>
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<td>treatment</td>
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<td>&lt;0.0001</td>
</tr>
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<td>line*treatment</td>
<td>0.0433</td>
<td>0.0026</td>
</tr>
<tr>
<td>day</td>
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<td>&lt;0.0001</td>
</tr>
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<td>line*day</td>
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<td>0.0991</td>
</tr>
<tr>
<td>treatment *day</td>
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<tr>
<td>line* treatment *day</td>
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<td>0.0005</td>
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Independent analyses were performed on the first 2 and the first 5 principal component scores. P-values were obtained through Hotelling’s-Lawley $T^2$ tests.

except on the interaction effects of treatment*day and line*treatment*day. Table 8 displays the mean Euclidean distance of component scores to the cluster centriole. The mean Euclidean distance to cluster centriole measures the spread of the observations within each cluster. The spread observed in a cluster represents the variability among the observations just as in the principal component loadings explained in the previous paragraph. In addition to the mean Euclidean distances in Table 8, the outcome of the independent MANOVAs pair wise comparisons are displayed.

The decomposed score plots are displayed in Figure 13. At 7 d (Figure 13, Panel A), the cluster of observation scores is located on the right side of the plot, all the observations at 7 d have positive scores for the first principal component with some observations having scores above 10. Restrict fed poults, regardless of line; exhibit a greater degree of variability than the ad libitum fed poults as observed in the values in Table 8. In addition, the RBC2 poults show a greater degree of variability than the F line, as observed in the values in Table 8. At 11 d (Figure 13, Panel B), the cluster of observation scores have moved left, closer to the center of the plot as now 11 of the observations have negative scores. This is because variations in protein abundances are
Table 8. Mean Euclidean distance of component scores to cluster centriole.

<table>
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<tr>
<th>Treatment</th>
<th>First 2 component scores</th>
<th>First 5 component scores</th>
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<tr>
<td><strong>7 days</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-line, full fed</td>
<td>1.565 ab</td>
<td>2.219 a</td>
</tr>
<tr>
<td>F-line, restricted fed</td>
<td>3.160 ab</td>
<td>3.817 b</td>
</tr>
<tr>
<td>RBC2 line, full fed</td>
<td>2.417 a</td>
<td>3.643 a</td>
</tr>
<tr>
<td>RBC2 line, restricted fed</td>
<td>4.392 b</td>
<td>5.886 a</td>
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<tr>
<td><strong>11 days</strong></td>
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<td></td>
</tr>
<tr>
<td>F-line, full fed</td>
<td>2.221</td>
<td>2.816</td>
</tr>
<tr>
<td>F-line, restricted fed</td>
<td>2.691</td>
<td>3.197</td>
</tr>
<tr>
<td>RBC2 line, full fed</td>
<td>1.732</td>
<td>2.793</td>
</tr>
<tr>
<td>RBC2 line, restricted fed</td>
<td>2.712</td>
<td>3.286</td>
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<tr>
<td><strong>14 days</strong></td>
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<td></td>
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<tr>
<td>F-line, full fed</td>
<td>1.903</td>
<td>2.445 a</td>
</tr>
<tr>
<td>F-line, restricted fed</td>
<td>2.008</td>
<td>2.355 b</td>
</tr>
<tr>
<td>RBC2 line, full fed</td>
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<td>2.448 a</td>
</tr>
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<td>RBC2 line, restricted fed</td>
<td>2.208</td>
<td>2.735 b</td>
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<tr>
<td><strong>17 days</strong></td>
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<tr>
<td>F-line, full fed</td>
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<td>3.727 ab</td>
</tr>
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<td>4.548 ab</td>
<td>6.187 b</td>
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<td>1.732 ab</td>
<td>2.510 a</td>
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<tr>
<td><strong>21 days</strong></td>
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<td>F-line, full fed</td>
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<td>F-line, restricted fed</td>
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<td>RBC2 line, full fed</td>
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<td>RBC2 line, restricted fed</td>
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<td>1.529</td>
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</table>

The mean Euclidean distance to cluster centriole measures spread of the observations within each cluster. Each cluster corresponds to the scores that correspond to observations within the same treatment. Significant differences are declared only in the time points where they are found and are represented by different literals. Significances are declared at a 95% confidence level. Independent analyses were performed on the first 2 and the first 5 principal component scores.

reduced at 11 d compared to 7 d while the greatest degree of variability is still observed in restrict fed pouls, similar to what was observed at 7d (Table 8). At 14d (Figure 13, Panel C), the cluster of observation scores has continued to move slightly to the left of the plot as now only 5 observation have positive scores. The restrict fed pouls
Figure 13. Decompositions of the principal component analysis score plots of samples. Each panel shows only the samples from a single time point. Each plot is coded by line (F line = squares; RBC2 line = circles) and by diet (Full feed = clear; Restricted feed = filled). 95% Confidence ellipses are displayed for the mean of each line and diet combination.

continue to show greater variation compared with the ad libitum fed poults (Table
8), similar to what was observed at 7 d and 11 d. At 17 d (Figure 13, Panel D), the cluster of observation scores showed little change from what was observed at 14 d as now only 3 observations have positive values. At this age, however, the cluster of observation scores increased over the vertical axis. This is evident in the F line restrict fed poults and the RBC2 ad libitum fed poults. The cluster of observation scores at 17 d, however, continues the trend of moving to the left of the plot. At 21 d (Figure 13, Panel E), the movement of the cluster of observation scores to the left has stopped as only one observation has a positive score. The amount of variation at this point is at its smallest and there is no apparent difference in variation among the four treatment groups (Table 8). The increase in spread over the vertical axis observed at 17 d is no longer present. At 24 d (Figure 13, Panel F), the cluster of observation scores is similar to what was observed at 21 d and only two observation scores have positive values. At this time point the ellipses are wider than at 21 d but this feature is influenced because the sample size for the calculation of the ellipses is smaller since some of the samples were lost during the experiment, this notion is supported by comparing the mean Euclidean distances which is very similar to the values at 24 d. Based on the ellipses, it is not possible to establish if the increase in variation observed at 24 d reflects a biological phenomenon. This notion is unlikely given that the spread of the cluster of observation scores is similar to what was observed at 21 d and the mean Euclidean distances are just slightly larger (Table 8).

The spread over the vertical axis appears to be homogeneously distributed around 0 at each age and decreases slightly with age with the exception of 17 d. This
phenomenon is largely due to the variation observed in the RBC2 full fed group at day 17 where the mean Euclidean distance for that group (Table 8) is oddly larger than the rest of the groups and is also even larger than values from the previous time point. The variation is increased at 17 d along the vertical axis and at 21 d returns to the progression observed from 7 d to 24 d.
Discussion

Principal Component Analysis of the One Dimensional Proteomic Profiles

Figure 3 panels A through C shows that restrict fed poults, regardless of line, display greater variation, represented by their mean Euclidean distances, than the *ad libitum* fed poults. The increased variation observed in restrict fed RBC2 poults, suggests that they are using a larger cadre of proteins to adapt to the restricted feeding regimen when compared with restrict fed F line poults. This may be due to the ability of the RBC2 animal to express a greater number of genes in response to environmental challenges compared to the F line. The variation difference observed between groups, subject to different feeding regimen, indicates that there is a greater effect of feed restriction on sarcomeric protein abundance at early developmental stages.

The spread over the vertical axis represents the second principal component scores. The reason for the variation at the particular age is unknown but it is connected mostly to a single treatment. The increased variation observed at this time point may be due to an unknown physiological adjustment or to an external factor that was not taken into consideration and does not correspond to genotype or nutritional regimen main effects.

The movement of the cluster of score patterns from right to left across the horizontal axis, corresponding to the first principal component, reflects the effect of age/developmental stage on the sarcoplasmic fraction proteomic profiles. The cluster of observation scores at 7 d is located on the right side of the plot and moves progressively from right to left and stops at approximately 17 days. This movement across the
horizontal axis suggests a metabolic adaptation process that starts at hatch and is nearly complete at approximately two weeks. The vertical axis, which corresponds to the second principal component, reflects a source of variation that is not accounted for by our experimental design. The clusters of observation scores along the vertical axis are not related to the effect of genotype or nutritional regimen.

Mean Euclidean distances and the shape of the clouds are indicative of the variation in the abundance of the variables or bands. The larger the mean Euclidean distance and the cloud area are indicative of increased variation that may be associated with nutritional and or genetic influences. In our study, the first two principal components were able to differentiate treatment groups within a time point (Table 8); however, this differentiation is more evident and more precise in the analysis performed using the first five principal component scores where smaller P-values are observed. Some features observed in the plots and in the mean Euclidean distances are indicative of the genotype and nutritional regimen participation in the biological. For instance, at 7 and 11 days the mean Euclidean distances and the size of the cloud were larger for restrict fed poults than ad libitum fed poults indicating that nutrition was delaying the convergence of protein abundances as animals accommodated the limited nutritional plane.

It is likely that while the type and abundance of sarcoplasmic proteins present in the breast muscle of developing turkeys undergoes an adaptation process. However, in this study the protein abundance of individual proteins was not determined and is being actively pursued with further proteomic analysis by LC/MS/MS. As the poults age the variability in the abundance of sarcoplasmic proteins appears to not only change but
become more stable and shows less variability. This suggests that there are fewer different types of individual proteins being expressed and results in a more biologically efficient metabolism. The shift toward a more stable state is affected by both genotype and nutritional regimen but these treatment effects do not alter or delay the shift, at least not during the first 24 days of age and thus appear to be temporally driven, similar to what has been reported for myosin.

The developmental patterns reflected in the sarcoplasmic protein profiles evaluated in this study illustrate two points. The first point is that animals converge to a common biological status that is independent of genetic differences or nutritional regimen although this convergence can be delayed. In the course of evolution, natural selection has facilitated the ability of animals to optimize many biological functions. These functions have become highly conserved throughout the phylogenetic tree. The expression of some of the genes controlling biological functions may be temporally influenced by genotype or nutritional regimen, but the ultimate expression of the trait remains the same. This is best illustrated in the current study by the homogeneity of the component scores among lines and treatment groups at 21 d. The second point is that there are different paths that animals can take prior to convergence to a similar biological status. This is suggested by the increased variability due to genotype and nutritional regimen at younger ages. This was particularly true with respect to differences in how each genetic line adapted to the restricted feeding treatment.
CHAPTER 5

Epilogue

In the first study, WBS values were observed to be associated with the structural proteins, myosin heavy chains, myosin light chains, actin, desmin and tubulin or their fragments. Results are consistent with previous reports showing that degradation of thick filament components at 36 h is predictive of future tenderness in beef (Sawdy, Kaiser et al. 2004). Finding structural proteins that were predictive of tenderness in this study supports a hypothesis previously suggested (Goll, Geesink et al. 1995; Goll, Boehm et al. 1998) that the architecture within the myofibril in postmortem muscle may be an important contributor to the eventual tenderness after aging due to the weakening of the actomyosin interaction during postmortem proteolysis.

Besides the participation of structural proteins in meat tenderization processes, proteins with metabolic, developmental and chaperone functions have also been identified. The participation of metabolic enzymes in meat tenderization can be easily related to muscle fiber type composition; however, proteins with developmental or chaperone functions are harder to relate. By identifying the mechanisms through which tenderness is mediated, it will be possible to develop more precise breeding strategies to produce cattle with greater and more consistent tenderness. This study identified multiple proteins that have been previously reported in separate studies that are related to meat...
tenderization. The methodology used in this study has the potential to capture the participants of multiple processes in a single experiment. The results of this study begin, for the first time, to assemble those proteins into participants in a coherent mechanism underlying the tenderization process.

The second study presents researchers with an accessible approach to perform a semi-quantitative proteomic analysis. Its accessibility resides in the use of common techniques including one dimensional electrophoresis, a comprehensive statistical analysis and mass-spectrometry protein sequencing. Although mass-spectrometry protein sequencing technology is not commonly available in every research lab, central facilities that perform such services for a fee put this technology within the economic reach of an average researcher.

This study also demonstrates that the use of proteomic techniques can be employed on experimental setups that can otherwise be unsuitable because of the possibility of damage to sample integrity by the cooking process. Specifically in the food science field, much information can be obtained from the analysis of processed food products. The methodology presented displayed potential proteins/peptides that were associated with specific muscle types. Such proteins/peptides can be used as indicators in further studies to develop more refined and precise methods for meat cut authentication.

In the third study, principal component analysis successfully displayed differences in the genotype and nutritional variables at the different ages post-hatch. The method successfully illustrated the extent of participation of a one variable compared to another over time. The comparative protein profiles of sarcoplasmic proteomic fractions from
turkey breast muscles were more variable at time points before 17 d. This information will be advantageous in future studies where the objective is to characterize differences that are most likely to have the greatest impact on the growth and development of poultry muscle.

The application of 1DE does not replace more refined technologies, because the resolution offered by 2DE methodologies. However, a low cost high throughput proteomic methods can provide an opportunity to research fields where the search for biomarkers and limited funding are common through the use of large number of samples. One dimensional electrophoresis has the advantages of being high throughput, highly reproducible and low cost. Thus, 1DE based proteomics can be very appropriate in low cost exploratory studies that can be used to guide studies that employ less reproducible, lower throughput but higher resolution methodologies.
REFERENCES


### APPENDIX

Cooked meat sequencing data

**Appendix Data Table 1.** Sequencing data of Band 3, band associated with the effect of postmortem aging time.

<table>
<thead>
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<th>Accession number</th>
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<td>gi</td>
<td>387090</td>
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*L = Longissimus, P = Psoas major, S = Semitendinosus*

72h = aged for 72 h postmortem, 7d = aged for 7 d postmortem.

ID Rank is the order in which the sequence appears on the Mascot result dataset.

MWt is the theoretical molecular weight.

The MOWSE score is a numeric descriptor of the likelihood that the protein identification is correct.

Number of queries sequenced and matched to that particular protein.
Appendix Data Table 2. Sequencing data of Band 22, band associated with the effect of muscle type.

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*L = Longissimus, P = Psoas major, S = Semitendinosus*

*b 72h = aged for 72 h postmortem, 7d = aged for 7 d postmortem.

c ID Rank is the order in which the sequence appears on the Mascot result dataset.

d MWt is the theoretical molecular weight.

e The MOWSE score is a numeric descriptor of the likelihood that the protein identification is correct.

f Number of queries sequenced and matched to that particular protein.
Appendix Data Table 3. Sequencing data of Band 30, band associated with the interaction effect of postmortem aging time and muscle type.

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*a* L = Longissimus, P = Psoas major, S = Semitendinosus

*b* 72h = aged for 72 h postmortem, 7d = aged for 7 d postmortem.

*c* ID Rank is the order in which the sequence appears on the Mascot result dataset.

*d* MWt is the theoretical molecular weight.

*e* The MOWSE score is a numeric descriptor of the likelihood that the protein identification is correct.

*f* Number of queries sequenced and matched to that particular protein.