Biochemical Basis of Fresh Ham Color Development

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In
Animal and Poultry Sciences

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July 21st, 2015
Blacksburg, VA

Keywords: pork quality, ham quality, muscle metabolism, meat color

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ABSTRACT

Commercial hams display variation in color uniformity across the cut surface, especially the *semimembranosus (SM)* muscle. This variation in fresh ham color, or two-toning, persists through further processing and contributes to production of a less desirable end product. In an attempt to understand the underlying source of this color variation, we evaluated the differences in muscle fiber-type composition and glycolytic metabolism in the SM muscle of fresh hams differing in color uniformity. Fifty-eight fresh SM muscles, ranging in color, were selected at 24 h postmortem and each partitioned into four distinct regions and three color classes based on color uniformity in the caudal region. The L* (lightness) values were greatest and a* (redness) values lowest in the most caudal portions of the muscle. The caudal portion also exhibited the lowest ultimate pH ($P < 0.0001$), lowest myoglobin ($P < 0.05$), greatest glycolytic potential (GP) ($P < 0.0001$) and the lowest myosin heavy chain type I isoform ($P < 0.0001$) abundance of all regions in "normal" colored hams. After segregating based on L* values, the caudal region had identical pH, GP, LDH, and MyHC-I, despite significant differences in L* ($P < 0.0001$). These data show the most caudal aspects of the SM are indeed more prone to adverse postmortem metabolism and suggest that inherent differences in muscles of the ham may make some areas of the ham more vulnerable to temperature abuse during harvesting.

DEDICATION

       I would like to dedicate this thesis to my friends and family who put many things on hold while I pursued this degree. Your kindness and patience did not go unnoticed, and for both I am eternally grateful. It was an experience in which I learned far more things about myself than I thought, in addition to the knowledge I gained about the meat industry. Things that have made me a better person, and more able to give back to our Lord, Jesus Christ, to make his world a better place. Also, to the many farmers, ranchers, 4-H leaders, and teachers in Pennsylvania and Virginia, who molded my passion for this industry, I hope to one day repay the very agriculture community that built me.

ACKNOWLEDGEMENTS

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Postmortem meat color development in hams
<u>Overview</u>

Ham quality, especially lean color, remains a problem for the meat industry. According to Troy and Kerry (2010), meat color is the most important quality attribute because it is the first quality characteristic perceived by the average consumer and either encourages or discourages a sale. According to the Pork Chain Quality Audit performed by Cannon et al. (1996), pork manufacturing companies identified muscle color as one of the top five concerns for the pork industry. Taken with other consumer concerns for quality, it has been estimated that 10% of the live animal's value is lost due to substandard quality characteristics. When studying fresh pork color, pork carcass quality is typically based on quality parameters evaluated on the cross-section of the *longissimus* muscle. While useful, the relationship between fresh quality parameters of the *longissimus* and ham are not readily apparent. Consequently, selection programs to improve fresh pork quality have focused on the loin alone.

Over 55% of hams sold in the United States are cured (NPB, 2014). During the ham curing process, nitrite is added to meat to create nitric oxide that binds to myoglobin producing the cured pink color known as nitrosylhemochromogen. While effective in stabilizing a particular pigment, color anomalies in fresh ham quality are only exacerbated after the curing process (McDonagh, Troy, Kerry, & Mullen, 2005). Therefore, understanding processed meat quality must start with the fresh ham quality.

Fresh meat color is largely predicated on myoglobin content and its functionality (Seideman, Cross, Smith, & Durland, 1984). A number of factors can impact myoglobin content in the muscle, mainly through changes in muscle fiber type composition. Factors such as age, exercise, nutrition, genetics, function and feeding growth promotants can change muscle fiber

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type composition in pigs (Lefaucheur & Gerrard, 2000). In addition to the amount of myoglobin in the muscle, the state of the myoglobin within the tissue is important. When myoglobin is in its native state, it reflects light in a particular manner and gives fresh meat a characteristic color. If this pigment is altered in anyway due to environmental effects, its ability to reflect, scatter or absorb light is impacted and meat presents a different color to the observer. Factors such as temperature, pH, ions, etc. impact myoglobin and its ability to interact with light. Finally, the unique structure and properties of meat contribute to the nature of color perceived by consumers. The ability of the tissue to absorb or scatter light is affected by the amount of moisture available in the tissue and the nature and organization of the underlying contractile tissue proteins in the meat. Because all of the aforementioned contribute to fresh meat color, these issues will be reviewed as they pertain to pork quality.

Conversion of muscle to meat and postmortem glycolysis

Muscle contraction requires ATP to interact with the myosin head and fuel the ATP-dependent pump on the sarcoplasmic reticulum, SR (Endo, 1985). Briefly, calcium is released from the SR during a twitch and binds to troponin C. This binding induces a confirmation change in thin filaments, which exposes the myosin binding site on actin. Once bound, ATP hydrolyzes, the myosin head releases and reattaches to the next actin. When the calcium is sequestered in the SR via an ATP-dependent pump, tropomyosin covers the actin-myosin binding site to inhibit cross-bridge formation (Weber & Murray, 1973). Muscle metabolism does not supply enough ATP to sustain muscle twitching past a few twitches (Scheffler & Gerrard, 2007). Therefore, additional energy is buffered from the phosphocreatine system, which phosphorylates ADP to ATP using creatine kinase, and from myokinase combining two ADP molecules into one AMP and one ATP molecule.

During times of reduced oxygen availability, oxidative metabolism is unable to supply enough energy to meet ATP consumption. As a result, anaerobic metabolism begins to metabolize glycogen to generate more ATP. Because anaerobic glycolysis is less efficient than oxidative metabolism, ATP levels fall (Bowker, Grant, Forrest, & Gerrard, 2000). Anaerobic glycolysis begins shortly after exsanguination and the majority of phosphocreatine is used within the first few minutes (Bendall, 1951). Glycolysis uses either glycogen or free glucose as the initial source of substrate. When using glycogen, glycogenolysis begins with the conversion of glycogen to glucose units (glucose 1-phosphate) by glycogen phosphorylase, then to glucose 6-phosphate by the enzyme phosphoglucomutase. Alternatively, free glucose is phosphorylated to glucose 6-phosphate by hexokinase, with the donation of a phosphate from ATP (Burleigh & Schimke, 1969). Phosphoglucoisomerase then rearranges glucose-6-phosphate into fructose-6-phosphate (does not require ATP). Phosphofructokinase (PFK) phosphorylates fructose-6-phosphate, with ATP, to generate fructose 1,6-bisphosphate.

Aldolase forms dihydroxyacetone phosphate and glyceraldehyde phosphate from fructose 1,6-bisphospate. Triose phosphate isomerase turns the molecule of dihydroxyacetone phosphate into glyceraldehyde phosphate, yielding two glyceraldehyde phosphates total. Both glyceraldehyde phosphates donate a hydrogen in the presence of triose phosphate dehydrogenase, to NAD+ forming NADH. This process forms two 1,3-bisphosphoglycerate molecules with the addition of a phosphate group from the cytosol. Phosphoglycerokinase removes the two phosphate groups, one from each of the two 1,3-bisphosphoglycerate molecules and transfers each phosphate molecule to an ADP, resulting in two ATP molecules and two 3-phosphoglycerate molecules. Phophosglyceromutase takes the remaining phosphate group on each 3-phosphoglycerate molecule and transfers it from the third carbon to the second, making

two 2-phosphoglycerate molecules. One molecule of water is then removed from each 2-phosphoglycerate molecule by enolase, forming phosphoenolpyruvic acid (PEP). Pyruvate kinase (PK) removes a phosphate group from each PEP and places it on ADP, making two ATP molecules and two pyruvate molecules. In summary, glycolysis nets a total of 2 ATP, 2 pyruvates, 2 NADH, and 2 water molecules when starting from glucose. If the glucose moiety is derived from glycogenolysis, one less ATP is consumed, bringing the overall ATP net production to three.

During this process PFK and PK can become inactivated if certain conditions are present. PFK is temperature and pH labile in ranges associated with normal pH decline (England, Matarneh, Scheffler, Wachet, & Gerrard, 2014), with its activity changing with temperatures (Freed, 1971). England et al. (2014) concluded that once postmortem pH dropped below 6.0, PFK started to lose function, with complete inactivation occurring after pH of 5.5. PK is also a rate limiting glycolytic enzyme in glycolysis, its activity can be altered by available ATP concentrations (England, Scheffler, Kasten, Matarneh, & Gerrard, 2013). At lower pH environments (PSE – Pale, soft, and exudative pork), PK is known to have greater activity while also existing in three different isoforms. This is unlike normal pH muscle environments (5.4-5.6) at which PK exhibits only two isoforms (Bowker et al., 2000).

Anaerobically, each pyruvate is converted to lactic acid's conjugate base, lactate, and H+, by lactate dehydrogenase and NADH. The hydrogen ion is stripped from NADH, forming NAD+. NAD+ forms two 1,3-bisphosphoglycerate molecules (as described above). Because the ability of the tissue to remove lactate and hydrogens is disrupted, lactate and H+ accumulates (Scheffler, Park, & Gerrard, 2011), which causes the pH to fall from 7.4 to 5.4-5.7 in 24 hours (Bowker et al., 2000). The rate of ATP hydrolysis eventually overwhelms ATP synthesis and

rigor ensues. Once ATP concentration is depleted, rigor mortis is complete (Scheffler & Gerrard, 2007). The permanent actomyosin cross-bridges provide rigidity to the muscle tissues postmortem.

Factors affecting metabolism

ATPase activity and Calcium levels

Consumption of ATP in muscle directly paces the rate of glycolysis by altering the concentrations of available cellular ATP. ATP is hydrolyzed by ATPases for use by phosphorylase, PFK and PK (Scheffler et al., 2011). The rate of glycolysis is directly related to the consumption of ATP that occurs in muscle (Scopes, 1974b) and is independent of the enzyme concentration (Scopes, 1974a). The decline of pH is faster when more ATP turnover occurs (Bowker, Botrel, Swartz, Grant, & Gerrard, 2004).

Turnover of ATP increases through increased sarcoplasmic calcium levels that activate ATPases (Bowker et al., 2000). Halothane sensitive pigs genetically code for a defective sarcoplasmic reticulum calcium release channel release the ion at much higher levels than those without the defect (Cheah & Cheah, 1976). The calcium is released from the SR in an aberrant manner, causing the Ca-ATPases in muscle, which are ATP-mediated, to work more, decreasing ATP levels more rapidly. Because ATP levels fall faster, glycolysis starts sooner, is more antagonistic and thereby decreases pH earlier postmortem. In addition, as calcium levels approach those needed for contraction, ATP utilization is also increased through actomyosin ATPases. ATP utilization from any of the muscle ATPases affects the overall pool of available ATP and the amount and rate of turnover, which in turn affects glycolysis. Muscle from these halothane positive pigs exhibit as much as a five-fold increase in PSE occurrences, resulting

from that increase in the rate of glycolysis, which accumulates lactate faster (Bowker et al., 2000).

Warner, Kauffman, and Greaser (1997) showed that PSE pork had significantly lower ATPase activity, when compared to all other quality classes of fresh pork. This agreed with previous findings that ATPase activity significantly decreased in PSE pork postmortem (Greaser, Cassens, Briskey, and Hoekstra, 1969), even though PSE pork is often associated with higher glycolytic muscles. It is important to note that sampling in these studies occurred 24 h postmortem. A significant decrease in ATPase activity 24 hours after death in poor quality pork occurs because of denaturation of the ATPase from the rapid pH decline (Bowker et al., 2004).

Fiber Type composition

Muscle is a collection of muscle fibers differing in their contractile speed and predominate type of energy metabolism. The contractile protein most responsible for conveying contractile speed is the myosin heavy chain (MyHC) isoform. In pig muscle, like in many other species, four adult isoforms can exist in muscle fibers: MyHC Type I, IIa, IIx, and IIb (Schiaffino et al., 1989). It is important to note, however, that additional isoforms are possible in regenerating and early neonatal muscle (Lefaucheur & Gerrard, 2000). Fibers are classified or 'typed' based on their predominate MyHC isoform. Type I fibers are slower contracting fibers, contain mainly type I MyHC compared to their faster contracting counter parts. These fibers are also smaller in diameter and redder in color. Type II, on the other hand, contain predominately type IIa, IIb or IIx MyHC isoforms and are faster contracting and paler in appearance than type I fibers (Schiaffino & Reggiani, 1994). Histological protocols are designed to stain for either mitochondrial- or glycolytic-based enzymes, such as succinate or nicotinamide dehydrogenases

or lactate dehydrogenase. Using this approach, type I and IIa fibers contain mainly oxidative-based metabolisms, while type IIx and IIb have higher glycolytic enzymes (Klont, Brocks, & Eikelenboom, 1998). Characterization using these approaches allows for identification of adult fiber types in pig muscle, type I, IIa, IIx(d) or IIb (Lefaucheur & Gerrard, 2000).

Pig muscle contains types I, IIa, IIx(d) and IIb (Henckel, Oksbjerg, Erlandsen, Barton-Gade, & Bejerholm, 1997), with the proportions of these fiber types varying between and within muscles (Armstrong, Delp, Goljan, & Laughlin, 1987). Of particular significance to the pork industry, the *longissimus dorsi* (LD) muscle, contains a high proportion of type IIb fibers (Karlsson, Klont, & Fernandez, 1999) while muscles of the ham, including the *semimembranosus (*SM), are more oxidative in nature. While the SM has a higher proportion of type IIb fibers, it is numerically lower than the proportion found in the LD (Gentry, McGlone, Miller, & Blanton, 2004). Organization of fiber types are predicated by motor unit organization (Beermann & Cassens, 1977). One motor unit, and all associated fibers, has similar metabolic and contractile properties (Karlsson et al., 1999). Histochemical staining of porcine muscle shows fibers are arranged in a unique rosette formation with slow contracting fibers toward the center of the rosette, while those more glycolytic in nature surround the oxidative fibers (Beermann & Cassens, 1977). The exact reason for this arrangement likely serves no functional purpose, as no other species to date exhibits such order in muscle fiber arrangement.

A number of factors influence fiber type composition, including muscle function, genetics, sex, age, exercise, and growth promotants. In terms of muscle function, muscles that are used for posture tend to have more fatigue-resistant oxidative fibers while muscles used predominately for power and quick movements have more type IIb fibers (Choi & Kim, 2009).

Muscles with a high percentage of type IIb fibers often require more ATP as they potentially will need to produce energy past the homeostatic levels provided by oxidative metabolism, creatine kinase, and myokinase (Totland & Kryvi, 1991). This would require glycolytic fibers as the source to provide the needed energy levels to do so (Klont et al., 1998). More type I clusters are found in deeper muscle areas, closer to bone, than their outer counterparts (Rosser, Norris, & Nemeth, 1992). Totland and Kryvi (1991) concluded that the increase in glycolytic fiber types distal to the bone was due to a distinct mechanical advantage. Being farther away from the bone allows the glycolytic muscle fibers more torque on the bone when more power (or speed) is needed in porcine muscle.

Swine genetic programs have selected for leaner animals with higher growth rates. This selection has resulted in a greater proportion of type IIb fibers in most highly selected lines (Weiler, Appell, Kremser, Hofäcker, & Claus, 1995) causing changes in fiber size (Rehfeldt, Fiedler, Dietl, & Ender, 2000). This change in the proportion of fiber types results in changes in pH decline postmortem (Ashmore & Doerr, 1971). Perhaps the best example of fiber type associations with muscle growth and meat quality development is observed when comparing domestic and wild hogs. Wild hogs have a higher proportion of type I and IIa fibers than domestic hogs (Ruusunen & Puolanne, 2004). In current commercialized hog breeds, like Yorkshires and Landraces, a higher concentration of glycolytic enzymes (Essén-Gustavsson & Fjelkner-Modig, 1985) and more type IIb fibers (Ryu & Kim, 2005) are found by comparison to their less selected, heritage breed counterparts, i.e., Berkshires. Rehfeldt et al. (2000) demonstrated that two thirds of the phenotypic standard deviation in muscle fiber size and number is due to genetic origin. Thus with domestication, porcine muscle fiber type has become more glycolytic in nature.

Age and sex individually contribute negligible amounts to changes in fiber type composition and ham color development. In the commercial U.S. meat industry, older pigs (greater than market age) are typically slaughtered for use in sausage, as opposed to whole muscle ham leaving only market-age animals (5.5 – 6 months old) to be further processed (NPB, 2014). Fiber type composition changes drastically from birth to market-age with the proportion of oxidative fibers decreasing and glycolytic fiber proportion increasing (Karlsson et al., 1999). Literature on the contribution of sex towards fiber type composition is conflicting. Miller, Garwood, and Judge (1975) found no differences in fiber type composition between hog sexes, while Karlsson et al. (1993) found small, but significant differences in cross-sectional area of the fibers, but no change in the actual fiber type composition.

Sex and slaughter age of hogs do not affect fiber type composition but they do affect the growth rate of the animal, weight at slaughter, and subsequent overall carcass composition of the animal. Latorre, Lázaro, Valencia, Medel, and Mateos (2004) showed that gilts have higher gain to feed ratios that results in leaner animals with higher dressing percentages. Conversely, barrows have a higher average daily feed intake and average daily gain, but produce carcasses that are fatter and yield fewer pounds (Latorre et al., 2004). Simultaneously as both sexes mature into market-age animals, their muscle fibers increase in size, eventually plateauing. The half month variation in market-age allows hogs to be slaughtered at different stages in fiber growth; either prior to plateauing where their fibers would be at different sizes, or after where they start to accumulate more fat (Rehfeldt et al., 2000). The variable age at slaughter coupled with the differences in growth rates known between sexes contribute to the variation in carcass composition and weight described by DeVol et al. (1988) at commercial hog facilities.

Fiber type composition can be affected by physical activity and growth promotant status. Past research shows conflicting results with Petersen, Henckel, Maribo, Oksbjerg, and Sørensen (1997) concluding that exercise influences glycolytic and oxidative enzyme activity in muscle while Gentry, McGlone, Blanton, and Miller (2002) found no difference in fiber type composition when pigs were exercised. For growth promotant status, Oksbjerg, Henckel, Rolph, and Erlandsen (1994) found that β-agonist growth promotants increased the proportion of type IIb fibers in pork. This agreed with later work showing that ractopamine based growth promotants increase type IIb gene expression in hogs (Gunawan, Richert, Schinckel, Grant, & Gerrard, 2007). Both instances can accelerate the growth curves within growing hogs.

There are documented relationships correlating growth rate with both fiber number and size (Henckel et al., 1997; Rehfeldt et al., 2000; Rehfeldt, Stickland, Fiedler, & Wegner, 1999). Differences in such growth rates could indicate the collaboration of factors that work together to impact one-third of the phenotypic standard deviation found in muscle fiber number and subsequent size that does not come from genetic origin (Rehfeldt et al., 2000). Individual animal growth rates differ, allowing fiber size to be variable at time of slaughter. The variability of fiber size, growth, and number could be responsible for slight changes in fiber type proportions.

Glycogen concentration

Glycogen concentrations prior to slaughter can alter the rate and extent of glycolysis. Muscle fiber type and glycogen concentration are associated and faster contracting muscle fibers have greater glycogen content and more glycolytic enzymes (Wittmann, Ecolan, Levasseur, & Fernandez, 1994). Because of this relationship, muscles starting higher glycogen concentrations generally produce meat with a lower ultimate pH at 24 hours post-mortem. However this relationship is curvilinear (Figure 1). Henckel, Karlsson, Jensen, Oksbjerg, and Petersen (2002)

produced data showing a threshold of 53 mmol of glycogen/kg tissue is necessary for pigs to

reach a normal ultimate muscle pH (5.4-5.7). However, glycogen values greater than about 50

mmol/kg do produce lower muscle pH values. In this case, only those animals having an initial

muscle glycogen below 53 mmol/kg result in a significant linear relationship between (0.88, R^2)

ultimate pH (P < 0.001) and glycogen deposition. In contrast, little association is noted among

those individuals having glycogen contents above 53 mmol/kg (correlation of 0.005, R^2)

(Henckel et al., 2002).
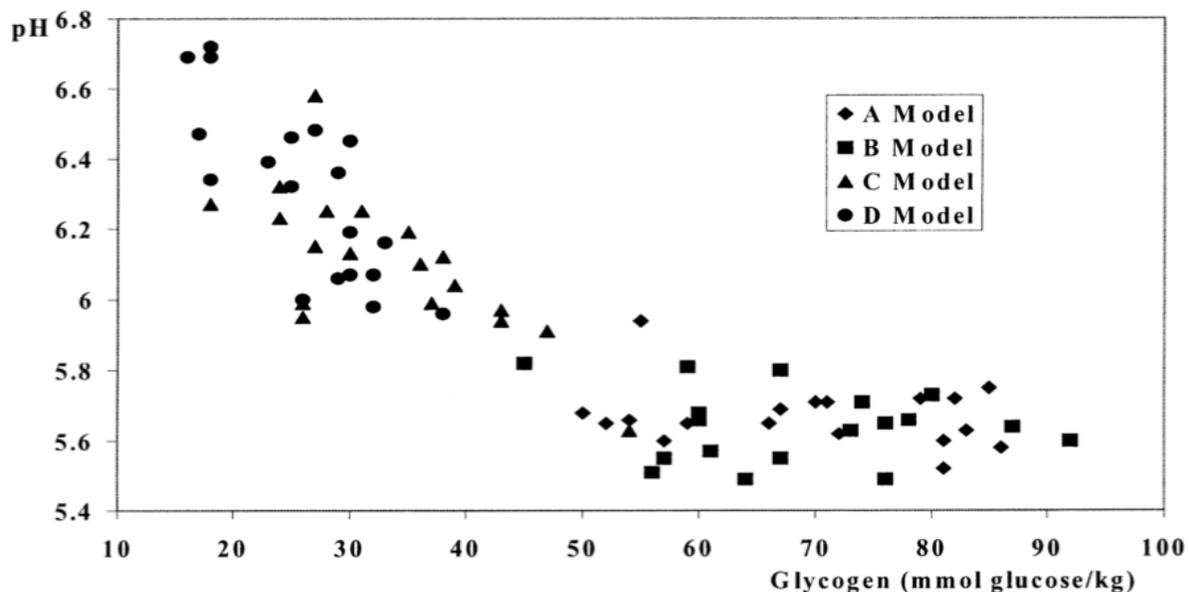
Figure 1: Relationship between ultimate pH and glycogen concentration immediately

before slaughter. Model C and D were injected adrenaline while A and B were not

(Henckel et al., 2002).

Glycogen is extremely vulnerable to antemortem stress or physical activity prior to

slaughter. Catecholamines are released in response to stress and mobilize muscle energy stores

(Kuchel, 1991). In contracting muscles, glycogenolysis increases through the activation of

phosphorylase, mobilizing substrates for glycolysis. Glycogenolysis increases in oxidative

muscle fiber types during extreme muscle contractions that result during stress, when more

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energy needs to be metabolized and the glycolytic fibers have already been maximized (Richter, Ruderman, Gavras, Belur, & Galbo, 1982). Henckel et al. (2002) simulated the catecholamine release that occurs in stressed hogs by injecting adrenaline (Figure 1), glycogen was metabolized prior to slaughter compared to the control, which resulted in higher ultimate muscle pH values. Subsequently less lactate was also produced in the adrenaline-injected animals. Altering the glycogen stores prior to slaughter does change the extent to which postmortem glycolysis occurs.

Glycolytic Flux

The overall biochemical and metabolic capacity of a muscle determines its potential glycolytic flux. The more oxidative fibers (types I and IIa) have decreased glycogen catabolism postmortem. The conversion of glycogen to lactate in these fibers occur at much slower rates (VØIllestad, Tabata, & MedbØ, 1992) because of their inherent differences in glycogenolytic and glycolytic enzyme concentrations and activity (Burleigh & Schimke, 1969). Their lower glycolytic capacity causes less lactate production (Karlsson et al., 1999) and thus a higher ultimate pH (Scheffler & Gerrard, 2007). When more glycogen was added to oxidative fiber types *in vitro*, pH did not drop any lower than without (England et al., Unpublished results), providing more evidence that flux relies on enzymatic capacity more than available substrate concentrations when the threshold has been met. Fiber types that have a higher glycolytic capacity, types IIx and IIb, have significantly faster glycolytic rates. An increased rate of glycolysis allows more substrate breakdown to occur, dropping pH faster from the increased production of lactate (Karlsson et al., 1999).

pH and temperature

Muscle pH ≤ 6.0, plays a role in the extent of glycolysis that occurs postmortem. Specifically, PFK activity is pH-dependent. England et al. (2014) showed that PFK activity is

responsible for the cessation of glycolysis, which results in an ultimate pH of 5.4-5.6. Using an *in vitro* simulated postmortem metabolism system, England et al. (2014) showed that PFK begins to lose activity at pH 5.9 and has complete loss of function at pH 5.5, while other enzymes remain functional to pH 5.0. This gradual loss of PFK activity from pH 5.9 to 5.5 is thought to drive the ultimate pH of all muscle tissue to a narrow ultimate pH range.

While pH is important in the conversion of muscle to meat, temperature is equally important. If ambient temperature is increased in muscle, the rate of pH decline increases (Liu, Ruusunen, Puolanne, & Ertbjerg, 2014). This increase in the rate of glycolysis is due to increased ATP hydrolysis within the muscle (Khan, 1971; McKee & Sams, 1998). When ATP hydrolysis occurs faster, so does the subsequent breakdown of glycogen in muscle (Bowker et al., 2004). In addition, postmortem reactions generate additional heat to raise the temperature of a hog carcass by three degrees (Bendall, 1973), compounding pH-mediated issues.

Meat color

Meat color is primarily determined by myoglobin, hemoglobin and cytochrome C. Hemoglobin is found in muscle at concentrations of 100-350 µM in µmol/kg of tissue (Wittenberg & Wittenberg, 1990) and represents only 5-14% of the total pigment in meat (Hunt & Hedrick, 1977). Cytochrome C resides in the mitochondria as part of the electron transport chain and is involved in mitochondrial apoptosis, after which it can also be found in the cytosol (Kroemer, Dallaporta, & Resche-Rigon, 1998). The pigment is found in meat at concentrations closer to 0.5 µmol/mg of protein (Cheah, 1973). Because cytochrome C and hemoglobin represent such a small fraction, their contributions to fresh meat color are often considered negligible.

Myoglobin

Myoglobin's ability to transport oxygen is facilitated by the presence of a heme ring with a centrally located iron that has a 6th ligand binding site (Wittenberg & Wittenberg, 1990). The state of the iron and the ligand bound to the 6th site determines the state of myoglobin and its contribution to color. When the 6th ligand site binds oxygen, the pigment is commonly known as oxymyoglobin. Oxymyoglobin produces a bright cherry-red color and is the most desirable form of myoglobin perceived by consumers. Deoxymyoglobin has water bound to the 6th ligand site with a ferrous central iron. Myoglobin can freely change between its deoxy- and oxy- forms depending on atmospheric oxygen (Mancini & Hunt, 2005). But, if the atmospheric oxygen is consumed, or there is low oxygen partial pressure, oxymyoglobin loses an electron from the central iron, oxidizing it to its ferric state (Wallace, Houtchens, Maxwell, & Caughey, 1982) and results in a brown pigment called metmyoglobin. Metmyoglobin is the most undesirable form of myoglobin in fresh meat for consumers (Mancini & Hunt, 2005). All three forms of myoglobin are physiologically water soluble (in saline) and contribute to the total myoglobin concentration (Lawrie, 1985). In general, muscles that are darker and redder have higher concentrations of myoglobin than those that are lighter and less red (Rickansrud & Henrickson, 1967).

Factors that affect myoglobin concentration

Protein denaturation

An increased glycolytic rate can result in an atypical low pH during periods of time postmortem when carcass or ambient temperatures are elevated. This results in protein denaturation in muscle and adverse meat quality development. Proteins most susceptible to denaturation include: myofibrillar proteins, ATPases and sarcoplasmic proteins. Myosin, the most abundant contractile protein in skeletal muscle (Yates & Greaser, 1983), denatures through

a series of 10 unfolding sequences, each having specific temperatures that cause each series or region to unfold. The specific temperature for each region's denaturation varies with pH. Muscles that vary in ultimate pH (6.0 versus 5.5) also vary in which domain of that myosin has been unfolded when aggregation of the myosin occurs, causing vast differences between muscle functional properties later (Vega-Warner & Smith, 2001). If the rate of glycolysis is increased, expected pH values would change, altering the temperature needed to unfold each of the 10 domains of myosin and result in the change of functional properties. Stabilization of myosin denaturation occurs after the onset of rigor, when actin irreversibly binds to myosin. When actomyosin cross bridges form, the ability of myosin to denature drastically decreases (Offer, 1991). Dutson (1983) reported other myofibrillar protein denaturation occurs between troponin, gap filaments, z-lines, and connectin, along with myosin postmortem. The denaturation increases when temperature increases during pH decline. Kim, Kerr, Geesink, and Warner (2014) provided evidence that ATPase activity decreases during periods of high pre rigor temperature in muscle, agreeing with Penny (1977) who indicated that meat exposed to 37 °C during pH decline, showed considerable decreased ATPase activity.

Scopes (1964) showed that as muscle pH comes closer to the isoelectric point and pre rigor temperature increases to 40 °C, precipitation of sarcoplasmic proteins is maximized. Sayre and Briskey (1963) agreed by finding a larger reduction in soluble protein content in muscles after exhibiting a low pH during onset of rigor, as compared to muscle samples with higher pH. Muscle constitutes many different proteins with unique vulnerabilities to denaturation (Smith, 1937), glycolytic enzymes representing the largest fraction of the sarcoplasmic protein (Clarke, Shaw, & Morton, 1980). Specifically meat color is highly affected by the concentration of myoglobin in the muscle (Seideman et al., 1984), though it makes up a smaller portion of the

sarcoplasmic fraction (Rhee & Ziprin, 1987). Myoglobin is one of the more stable components found in the sarcoplasmic fraction. It doesn't completely denature until temperatures of 70 – 80 $^{\circ}$C, but some researchers suggest partial denaturation starting at 40 $^{\circ}$C (Bernofsky, Fox, & Schweigert, 1959). Lawrie (1998) concluded that any "partial denaturation" of myoglobin below a temperature of 65 $^{\circ}$C was more likely due to increased precipitation than actual denaturation.

Loss of large amounts of sarcoplasmic proteins during postmortem pH decline is mainly due to the loss of water through increases in purge. The protein in purge is almost entirely sarcoplasmic proteins, including myoglobin, (Savage, Warriss, & Jolley, 1990) while meat is comprised mostly of water. Most water is found either within the myofibril space of individual muscle fibers, between myofibrils and the sarcolemma, or in the extra cellular space. Shortly after death, the muscle fiber network is capable of holding its integrity but after onset of rigor, myofibrils shrink, expelling water into endomysial and perimysial spaces that the fibrils and bundles once occupied (Offer & Cousins, 1992). Immobilized water is most affected during pre-rigor conditions, and although it is physiologically loosely bound to the protein molecule via a charge, it can escape as purge (Huff-Lonergan & Lonergan, 2005). Percent purge increases with increasing pre rigor temperatures (Farouk & Swan, 1998), subsequently increasing sarcoplasmic protein loss within the muscle. Increases in purge, caused by protein denaturation, increases the amount of native myoglobin lost.

Metabolism

As outlined above, changes in glycolysis form the basis of postmortem muscle metabolism and the transformation of muscle to meat. Two drastically different meats illustrate this phenomenon, dark, firm, and dry (DFD) pork and PSE pork. In the case of DFD pork, Adzitey and Nurul (2011) showed when muscle glycogen levels are compromised from long-

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term stress to the animal prior to slaughter and ultimate pH is increased a darker color results.

These data strongly implicate starting glycogen level as a driver of ultimate muscle pH in certain

situations. A reduction in the initial substrate level therefore can change the extent of glycolysis,

and result in an increase in the ultimate pH of meat and a darkening of the overall visible color.

Because the ultimate pH does not decline as much, muscle proteins remain in their native state

and water is held tightly in the tissues. In contrast, acid meat, which is much paler in color, is

associated with animals that produce significantly more lactate, (Monin & Sellier, 1985)

providing evidence of increased flux and extended postmortem glycolysis. Their diminished

quality is similar to that of PSE pork. PSE pork has an expedited rate of glycolysis causing an

atypical high temperature and low pH environment that leads to protein denaturation. The

denaturation then causes the exudative and other deleterious effects associated with PSE meat,

through increases in purging proteins.

Fiber type composition

Different types of muscle fibers contain inherently different concentrations of myoglobin.

Oxidative fiber types have more myoglobin than glycolytic fibers because they rely heavily on

oxidative metabolism (Klont et al., 1998). Evidence for this is found in meat color lightness

values (L*). Ryu et al. (2008) found muscle containing a higher proportion of type I fibers had

significantly lower L* measurements, or a darker color. Muscles with higher starting glycolytic

capacity and high glycolytic flux early postmortem produce significantly lighter meat (Choe et

al., 2008).

Muscle structure integrity

The intensity of the pigments in meat perceived by consumers is based on the integrity of

the muscle's structure, and is measured by the amount of light scattering collected by an

observer. When light hits an object it is either absorbed, transmitted, or reflected. If reflected, a detector can capture the light and interpret the color (Young & West, 2001). During peri-mortem protein denaturation, the muscle cell's integrity is disrupted, and sarcoplasmic proteins precipitate onto contractile proteins. This disruption increases the amount of light scattering by letting less light penetrate the structure (Bendall & Swatland, 1988). But, the light scatter is highly disorganized and does not reach the observing lens, therefore the red wavelengths that were not absorbed or penetrated are not interpreted, thus making the cut appear to have a less intense color.

Pork processing environments and thermodynamics

Whole hams are comprised of lean tissue with varying fat thickness surrounding it subcutaneously, which may affect fresh ham color. The *semimembranosus* (SM) muscle has sections of lean that differ in their fat thickness, while additively showing less fat covering than the *biceps femoris* (BF) muscle. Fat has a lower thermal conductivity than muscle, allowing regions of the hog insulated with more fat to exhibit smaller degrees of heat change from either of the extreme hot or cold processing environments it is subjected to (van der Wal, van Beek, Veerkamp, & Wijngaards, 1993).

Variation within carcass composition will affect the heat transfer properties of the ham (van der Wal et al., 1993), and is correlated to differences in slaughter weight and growth rate (Correa et al., 2006). According to Correa et al. (2006), ham fat thickness is significantly different amongst animals with different growth rates and of different sexes. Ham fat thickness was not significantly different in animals of different slaughter weights, but muscle yield was different, indicating that slaughter weight affected muscle mass while growth rate affected fat accumulation. Both work together to comprise the overall composition of the carcass, and

influence the rate of heat transfer and overall probability of high pre-rigor processing temperatures affecting the ham during pH decline.

Gardner, Huff-Lonergan, Rowe, Schultz-Kaster, and Lonergan (2006) compared processing environments and their effects on pork quality and found that dwell times and scalding times significantly affected temperature and pH of the SM and BF. Hunter L* scores did not differ, but scores were only taken on the LD muscle, which did not exhibit changes in temperatures. Longer scalding times that increased the ham's exposure to extreme heat, increased SM and BF temperatures two hours postmortem, which decreased pH measurements in both the SM and BF during the first 24 hours postmortem. Shorter dwell times only increased temperature in the SM two hours postmortem. After this two hour mark, temperatures for all treatments did not differ. Researchers concluded that muscles which were insulated with a higher degree of fat, like the LD, can therefore resist changes in muscle temperature more than those with less fat cover. Although all temperatures equilibrated after 24 hours, their respective temperature increase occurred during a vulnerable time of postmortem activity.

Conclusion

A number of factors can affect ham quality development. Of particular interest is the amount of pigment in the tissues, as well as the rate and extent of postmortem metabolism in the tissues. While pH in the tissues postmortem is driven by changes in muscle fiber type, myoglobin content, glycogen deposition and glycolytic flux, denaturation of muscle proteins is pH- and temperature- dependent. When exposure to high pre-rigor temperature increases, a decreased L* value in the SM suggests protein denaturation (Kim et al., 2014). Subsequently, hams with different compositions would be affected differently by processing temperature aberrations, and aid in the variation of color development. In addition, inherent myoglobin

concentrations that relate to differences in fiber type composition also produce variability in

fresh ham color development. After the curing process, all variability in fresh ham color is

exacerbated (McDonagh et al., 2005). Looking at consumer panels, research has shown that even

a difference of five L* units can negatively impact a consumer's intent to buy pork (Norman,

Berg, Heymann, & Lorenzen, 2003). Therefore, understanding the cause of fresh ham quality

development variations is necessary so management processes can be optimized to produce hams

that are uniform.

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CHAPTER 2. Manuscript
Biochemical basis of fresh ham color development

Abstract

Commercial hams display variation in color uniformity across the cut surface, especially the *semimembranosus (SM)* muscle. This variation in fresh ham color, or two-toning, persists through further processing and contributes to production of a less desirable end product. In an attempt to understand the underlying source of this color variation, we evaluated the differences in muscle fiber-type composition and glycolytic metabolism in the SM muscle of fresh hams differing in color uniformity. Fifty-eight fresh SM muscles, ranging in color, were selected at 24 h postmortem and each partitioned into four distinct regions and three color classes based on color uniformity in the caudal region. The L* (lightness) values were greatest and a* (redness) values lowest in the most caudal portions of the muscle. The caudal portion also exhibited the lowest ultimate pH ($P < 0.0001$), lowest myoglobin ($P < 0.05$), greatest glycolytic potential (GP) ($P < 0.0001$) and the lowest myosin heavy chain type I isoform ($P < 0.0001$) abundance of all regions in "normal" colored hams. After segregating based on L* values, the caudal region had identical pH, GP, LDH, and MyHC-I, despite significant differences in L* ($P < 0.0001$). These data show the most caudal aspects of the SM are indeed more prone to adverse postmortem metabolism and suggest that inherent differences in muscles of the ham may make some areas of the ham more vulnerable to temperature abuse during harvesting.

Introduction

Consumers use meat color as an indicator for product quality when making purchasing decisions (Troy & Kerry, 2010). Some suggest as little as a 5 unit difference in lightness (L*) score can negatively influence a consumers intent to purchase fresh pork (Norman, Berg, Heymann, & Lorenzen, 2003). Typically pork carcass lean color is assessed on the fresh

longissimus muscle (LM) when exposed between the 10th and 11th rib, yet little information is known how this assessment reflects overall ham color. The majority of hams are sold as a cured product (NPB, 2014). Any variation in fresh ham color is only exacerbated during the curing process (McDonagh, Troy, Kerry, & Mullen, 2005). Therefore, understanding variations in fresh ham color may help better understand how processed meat quality can be improved.

Ham color is primarily derived from the heme pigment myoglobin (Rhee & Ziprin, 1987). Many factors influence myoglobin concentrations in muscles, with muscle fiber type composition contribution being the most influential. Pork muscle is a heterogeneous mixture of glycolytic and oxidative muscle fiber types containing different levels of myoglobin and enzymes responsible for metabolizing energy. This unique heterogeneity of muscle fiber types dictates the overall nature of the postmortem metabolism in muscle tissue (Ryu & Kim, 2005). Red muscles consist of a greater proportion of slow-contracting, oxidative fibers. These fibers contain more myoglobin than fast-twitch glycolytic muscle fiber types (Karlsson, Klont, & Fernandez, 1999) and are most often positioned deeper in the animal closer to the skeleton (Armstrong, Delp, Goljan, and Laughlin (1987). On the other hand, however, the relative difference in the proportion of fiber types in muscle alone may be responsible for color gradients that exist across the face of a ham, described as "two-toning" by Wilson, Ginger, Schweigert, and Aunan (1959).

Changes in fiber type composition also indirectly impact the rate of postmortem metabolism. A greater composition of glycolytic fibers would result in greater concentrations of glycolytic enzymes in the muscle allowing for enhanced glycogen breakdown postmortem as compared to those muscles with a greater proportion of red muscle fiber types (Karlsson et al., 1999). Such a change in muscle fiber type composition would result in greater glycolytic flux,

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greater hydrogen ion accumulation and a more dramatic and profound change in muscle pH. Choe et al. (2008) previously showed regions of muscle with greater glycolytic capacity produce significantly lighter meat. Even so, however, it is not readily apparent whether muscle fiber type composition or the collective metabolic capacity of the tissue is responsible for all variations observed in fresh meat color across the SM muscle.

Some muscles, or aspects of muscles, are more available to insults in temperature during the harvesting process. For instance muscles of the ham like the SM or *biceps femoris* (BF), which are not as well insulated with fat like the LD, rise in temperature when the postmortem processing in altered (Gardner, Huff-Lonergan, Rowe, Schultz-Kaster, & Lonergan, 2006). Moreover, the rate of glycolysis changes with temperature (Liu, Ruusunen, Puolanne, & Ertbjerg, 2014). When highly glycolytic muscles are subjected to increases in ambient temperature, pH drops quicker. A lower pH at an elevated temperature would result in greater protein denaturation and greater moisture loss from the tissues. Scopes (1964) showed that sarcoplasmic proteins, like myoglobin, precipitate in a temperature-pH dependent manner. Precipitation of myoglobin would lower the concentration of myoglobin within the muscle, affecting its interaction with light, and ultimately affecting color. This is particularly germane for this muscle as Gardner et al. (2006) showed that the SM was vulnerable to temperature changes in ambient pork processing environments. The purpose of this study was to investigate the changes in muscle fiber type composition, energy metabolism, myoglobin content and color across the SM in fresh hams.

Materials and Methods

Muscle Sampling

Fifty-eight trimmed and fresh SM muscles were collected at 24 h postmortem in two

replicates from a commercial hog processing facility. Approximately 2.5 cm thick section was cut across the thickest portion of the muscle. Three equally spaced zones ranging cranial to caudal were labeled A, B, and C, respectively. Subsequent analyses were conducted on the most cranial ~ 1 cm strip on the face of each zones (A, B, and C), and the most caudal tip of zone C, which was labeled zone D (Figure 1). After color analysis, samples were snap frozen in liquid nitrogen and stored at either -80 or -20 ℃.

Color Analysis

Objective color measurements were performed using a portable Minolta CR300 colorimeter (Ramsey, NJ, USA), Illuminant D, 0° observer angle. Three random measurements were collected at separate locations on the surface of the muscle in an area free of any noticeable color defects. The three readings were averaged and the color for each of the four zones was expressed in terms of Commission Internationale de l'Éclairage (CIE) L* (lightness) and a* (redness). In addition, images of the SM were captured with a Powershot SX170 IS camera (Canon, Melville, NY)*,* equipped with a 16 megapixel sensor and a 28 mm wide-angle lens. Images were edited using Paint.NET software (dotPDN LLC, Spokane, WA, USA) by removing the background and any large sections of fat or connective tissue. Matlab image analysis software (The Mathworks, Inc., Natick, MA, USA) was used to evaluate color data from images collected from the second replicate (n = 29). Adapted from O'Sullivan et al. (2003), SM images were collected using a light stand (Bencher, Antioch, IL) and lamps (Sylvania DVY 650W 120V halogen/tungsten bulbs, Interlight, Hammond, IN) positioned 45 degrees to the surface of the meat. Lighting conditions are conducted in accordance with the American Meat Science Association guidelines for meat color evaluation (AMSA, 2012). A Matlab software script (Appendix A) was used to convert the red, green, blue image values to the CIE L* and a* color

space. Raw data sets from each image were truncated to include only those data points no greater than two standard deviations from the initial raw data set averages. These excluded data were likely generated from the pure white background, fat, connective tissue, and light scattering events.

Color Grouping

After collecting the color data, hams were sorted based on the L* measurements in zone D. The Least Squares Means (LSM) of zone D ± 2 standard deviations created the boundaries for the groups. Hams with a zone D L* value above the LSM + 2 standard deviation were designated as "light", those falling in below 2 standard deviations from the mean were referred to as "dark," and those in the middle were "normal."

Sample Preparation

Muscles were ground using a mortar and pestle. During grinding, liquid nitrogen was added frequently to ensure sample integrity. Samples were weighed into 100 mg and 50 mg aliquots and stored frozen at -80 C.

Total Protein Analysis

Frozen muscle samples (~ 50 mg) were lysed for 2 min in 1 ml buffer containing 8 M urea, 2 M thiourea, 3% SDS (wt/vol), 75 mM dithiothreitol, and 50 mM Tris-HCl (pH 6.8) (Warren, Krzesinski, & Greaser, 2003). After heating to 95 ºC for 10 min, samples were diluted 1:20 in water. Protein concentration was determined using a RC DC Protein Assay (BioRad Laboratories, Hercules, CA, USA) using bovine serum albumin for standard curve construction (Fisher Scientific, Pittsburg, PA, USA). Samples were evaluated spectrophotometrically at 750 nm, as reported above.

Ultimate pH

Muscle pH samples were prepared using the iodoacetic acid method as described by Bendall (1973) with a slight modification. Briefly, muscle samples (~ 100 mg) were lysed in 0.8 ml of ice-cold neutralized 5 mM iodoacetic acid and 150 mM KCL buffer. After centrifugation at 13,000 x g for 5 min, samples were equilibrated to 25 °C and pH was immediately determined using an Orion Ross Ultra pH electrode (Thermo Scientific, Pittsburg, PA).

SDS-PAGE and Western Blotting

Western blots were run from both the total protein extracts. After the determination of total protein concentration, 0.05% bromophenol blue was added, and samples were diluted using the same solubilization buffer (Warren et al., 2003) to a final concentration of 5 µg/µL and heated to 90 °C for 5 min.

For gel electrophoresis, 60 µg, 20 µg, and 15 µg of muscle protein were loaded per lane for detection of myoglobin, lactate dehydrogenase (LDH) and myosin heavy chain type I isoform (MyHC-1) with β-actin as the loading control, respectively. SDS-PAGE was performed using methods adapted from Warren et al. (2003). Myoglobin and LDH were separated using 15% and 10% acrylamide gels, respectively, while MyHC isoforms were resolved through a 4-15% final acrylamide gradient gel (Criterion TGX, Bio-Rad Laboratories, Hercules, CA, USA). Myoglobin and LDH electrophoretically separated for 20 min at 60 V and 2 h and 45 min at 120 V, while MyHC type I isoform was separated for 2 h at 150 V.

After separation, Myoglobin and LDH were transferred to nitrocellulose membranes for 1 h at 50 V and MyHC- I isoform transferred for 1 h at 45 V. All membranes were blocked for 1 h in blocking buffer (PBS with 3% milk casein and 0.1% Tween-20). Blots were incubated with mouse anti-Myoglobin and anti-MyHC-1 overnight at 4 °C. Blots were incubated with anti-LDH

and anti-β-actin for 1 h at room temperature. All blots were then washed three times with TBS containing 0.1% Tween-20 (TBS-T). Antibody dilutions were; anti-Myoglobin (1:2000, ab77232, Abcam, Cambridge, MA, USA), anti-LDH (1:2000, Novus 48336, Novus Biologicals LLC, Littleton, CO, USA), and anti-MyHC-1 (1:2000, A4.840, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), while the loading control antibody was β-actin (1:2000, NB600-503, Novus Biologicals LLC, Littleton, CO, USA). Then, blots were incubated with the appropriate IRDye 680- or 800-conjugated anti-IgG or anti-IgM secondary antibody (LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature. Lastly, all blots were washed three times with TBS-T.

Images of all blots and band intensity quantification were conducted using an Odyssey Infrared Scanner (Li-Cor, Inc., Lincoln, NE, USA). Ponceau (0.1% Ponceau S in 5% acetic acid) staining was used for equal loading assessment of myoglobin and LDH blots with images taken using a Universal Hood III and Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA). MyHC-1 intensities were normalized to β-actin bands within the lane.

Glycolytic Intermediates

Glycogen content was determined using powdered muscle samples ~ 100 mg that were lysed in 1 ml 1.25 M HCl, heated to 90 ^{o}C for 2 hr, then centrifuged at 13,000 x g for 5 min. Supernatants were neutralized with equal volume of 1.25 M KOH (Bergmeyer, 1984) and stored at -80 ^{o}C until analysis. The glycogen extract was added to the triethanolamine (TEA) buffer described by Hammelman et al. (2003) and aliquoted onto the 96-well plate to read the Optic Density (OD) 1. Glucose-6-phosphate dehydrogenase, hexokinase, and ATP were added and incubated for 15 min. Another aliquot was pipetted onto the plate and OD2 read. Total glycogen concentration was determined by subtracting OD1 into OD2 and comparing to standard curves

(Bergmeyer, 1984; Hammelman et al., 2003).

Lactate, glucose-6-phosphate, and glucose each were determined in another 100 mg sample. These samples were homogenized in 1 M ice-cold perchloric acid. After incubating on ice for 20 min, samples were centrifuged at 13,000 x g for 5 min and supernatants were again neutralized with of 2 M KOH (Bergmeyer, 1984) and stored at -80 °C until analysis. Lactate concentration was determined using the reaction buffer (200 mM Tris-hydrazine, 0.4 mM nicotinamide adenine dinucleotide) described by Hammelman et al. (2003). Samples were pipetted onto the plate and OD1 was read. LDH was added and the samples were incubated for 2 h at 25 °C and OD2 read. Lactate concentration was determined by subtracting OD1 from OD2 and comparing to standard curves (Bergmeyer, 1984; Hammelman et al., 2003). An additional extract of the original supernatant was added to the TEA buffer described by Hammelman et al. (2003), and aliquoted onto the plate to read OD1. After which glucose-6-phosphate dehydrogenase was added and incubated for 15 min and OD2 read. Lastly, hexokinase and ATP was added, incubated for 15 min, and read OD3. Glucose-6-phosphate concentration was determined by subtracting OD2 from OD1 while glucose subtracted OD2 from OD3 (Bergmeyer, 1984; Hammelman et al., 2003).

All metabolites were analyzed at room temperature and measured spectrophotometrically at 340 nm wavelength light. Residual glycogen concentration was determined by subtracting glucose-phosphate and glucose concentrations from the total glycogen concentration determined above. All metabolite concentrations were reported as µmol/g of tissue. Glycolytic potential (GP) was calculated using the equation by Monin and Sellier (1985): GP (µmol/g) = 2(glycogen concentration + glucose-6-phosphate concentration + glucose concentration) + lactate concentration, and reported as µmol lactate equivalent/g of tissue.

Statistical Analysis

Graphs are presented as means ± standard error mean (SEM). Data sets were analyzed

two ways, both using Fit Model in JMP (SAS Institute, Cary, NC, USA) with ham as the

experimental unit. Both statistical models used a mixed model with ham as a random effect, but

sliced either by zone or group. The LSM of all zones was evaluated using Tukey HSD with P

< 0.05 as significant. The blocking structure accounted for variation among blots when

applicable.

Results and Discussion

Color Analysis

Color variation across the cut surface of the SM muscle using either a colorimeter or

image processing are shown in Figures 1 through 9. Regardless of the methodology used or

group determination, color values show the more caudal zones (D and C) were lighter and less (P

< 0.0001) red than their more cranial counterparts. These data are consistent with Armstrong et

al. (1987) who showed muscles located closer to the bone are darker in color. Likewise,

Beermann and Cassens (1977) argued that the greater glycolytic fiber type composition,

consistent with a paler muscle color, in the superficial aspects of muscles would increase power

and speed of limb movement. In beef the opposite is observed about the SM, the deeper portions

are significantly lighter than the superficial (Kim, Lonergan, & Huff-Lonergan, 2010). This is

due in part to the larger mass of muscle on a beef round that takes longer to drop in temperature

causing protein denaturation to occur in the deep portion affecting overall color and

functionality. Regardless, these data show that the more proximal aspects of the pork SM muscle

are inherently darker and redder than those more distal.

More interestingly, just the outer zones showed significant differences in lightness

(Figures 3 and 7) and redness (Figures 5 and 9) in the light hams as compared to the normal grouped hams, while the cranial zones remained the same. This suggests that any variation in color found in the light group hams would be caused from factors only associated with the caudal zones. On the contrary, the dark grouped hams remained consistently darker across the entire face of the muscle (Figure 3) when analyzing color using the pinpoint technology of a colorimeter. But when Matlab analysis was conducted, differences in L* (Figure 7) and a* (Figure 9) readings were scarce between the normal and dark grouped hams. O'Sullivan et al. (2003) compared computer-assisted and colorimetric color analysis against consumer panel-based color evaluations. These investigations concluded that machine vision was more accurate in predicting human meat color evaluation than a colorimeter. While the exact reason for this difference is not known, it likely is in part due to the fact that computer-based image processing includes the entire region of interest while a colorimeter only samples a very small portion of the total. Increased sampling could resolve this shortcoming. For those reasons discussed, it was concluded that the dark and normal ham color were appropriately consistent with one another, while the light grouped ham color varied.

Drastic differences in meat color across a muscle and within a muscle can cause consumer discrimination, and is described as "two-toning" by Wilson et al. (1959). The cause of this phenomenon appears to be directly related to myoglobin concentrations across and between different muscles of the ham (Wilson et al., 1959). Toldrá (2004) was convinced that ham color improved with animal age, since myoglobin concentrations increased. But, discussion was lacking on whether or not the "two-toning" affect actually dissipated in older hogs, or was just harder for consumers to notice based on differences in color sensitivities as the color hue approached the end of the visible spectrum. Myoglobin concentration does increase with age in

hogs, from birth to one year of age (Lawrie, 1985), but it is not known how age affects the uniformity of color across an entire cut surface of a muscle.

Information regarding color development in which the entire face of the ham muscles are compared to each other is scarce. Researchers tend to take muscle measurements from only one location or average the score across the entire face (Tikk, Lindahl, Karlsson, and Andersen, 2008; Gentry, McGlone, Blanton, and Miller, 2002; Gentry, McGlone, Miller, and Blanton, 2004) leaving discussions to focus on how the treatment affects the color development of a specific muscle, but not how that color compares to other parts of that muscle. This type of sampling leaves many of the conclusions drawn about extrinsic and intrinsic factors tested against meat color to be confounded. For instance, Tikk et al.,(2008) found that sex had a significant effect on $L*$ scores within the SM when they averaged scores from across the entire face, but Self, Bray, and Reierson (1957) concluded that sex did not contribute to two toning when uniformity of color was actually quantified.

Myoglobin Concentration

Relative myoglobin concentrations were determined on all zones and groups using Western blotting. Consistent with color values reported across the muscle, normal, light, and dark grouped muscles had less ($P = 0.0028$, $P = 0.0012$, and $P = 0.0032$ respectively) myoglobin detected in the more caudal regions of the muscle than in cranial portions (Figure 10). Additionally, myoglobin concentration between the light and normal grouped hams, and the dark and normal grouped hams are not significant across the majority of the muscle. Significance in myoglobin concentration amongst like zones only lies between the light and dark grouped ham themselves (Figure 11). The inherent concentrations differences from zone A to D are the same across different groups, but not from like zones of hams in different groups. It is these

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differences in myoglobin concentrations that likely causes the difference in L* in the caudal zones of the light grouped hams, and leads to an increase in overall ham color variation. Factors that attribute to both color variation issues, the inherent color gradient from zone A to D and the significant lightness in zone D of the light hams, are explored throughout.

Physiologically myoglobin transports oxygen to muscle. But, once the muscle has been transformed into meat, myoglobin acts as the largest percentage of pigmentation found in meat (Lawrie, 1985). Many factors influence myoglobin concentrations in muscle, but all are mediated either through changes in fiber type composition (Karlsson et al., 1999) or differences in protein purge (Bendall & Swatland, 1988). Meat color often mimics fiber type composition due to the innate myoglobin concentration differences found between oxidative and glycolytic muscle. Oxidative fibers have more myoglobin than glycolytic fibers (Karlsson et al., 1999). Their blended composition within a muscle is determined by their motor unit topography (Beermann & Cassens, 1977).

Meat color pigment is primarily determined by native myoglobin concentration (Mancini & Hunt, 2005) found in the soluble protein fraction (Savage, Warriss, & Jolley, 1990). Soluble proteins can purge out or completely denature during times of increased protein denaturation and purge formation ocurring during the peri-mortem period (Bendall & Swatland, 1988). When meat protein approaches its isoelectric point, the myosin filament cross sectional area decreases 80-83%, increasing the sarcoplasmic space 1.6 fold. During that shrink, the myofilamentous water is mechanically forced out as purge (Offer & Cousins, 1992). In the process, moisture carries sarcoplasmic proteins, changing the concentration of proteins found in muscle (Savage et al., 1990). If severe perimortem conditions exist, additional protein denaturation can occur directly affecting native myoglobin concentration or cause increases to the formation of purge

and migration of sarcoplasmic proteins out of the muscle protein matrix. Both hypothesis are explored in the coming discussion.

Ultimate pH

Ultimate pH of meat is a valuable indication of pork quality (D'Souza, Dunshea, Warner, & Leury, 1998; England, Matarneh, Scheffler, Wachet, & Gerrard, 2014; Warner et al., 1997). For example, The ultimate pH of meat generally occurs below 6.0, regardless of species (Briskey & Wismer-Pedersen, 1961). While the exact reason for this is unknown, England et al. (2014), have shown that phosphofructokinase (PFK) is pH sensitive at or around pH 6.0. They further reported that the rate of flux through glycolysis may be important in lowering the ultimate pH of meat and this may be due to increasing depletion of AMP nucleotides that are mandatory to enable rephosphorylation.

In this study, pH signficantly dropped lower in the normal, light and dark grouped hams ($P < 0.0001$, $P < 0.0001$, and $P = 0.0021$ respectively) across the entire face of the muscle in the caudal zones (Figure 12), suggesting differences in rates or extents of postmortem glycolysis. When comparing like zones to one another, the pH values obtained between the light group and normal group of hams were not significantly different from another, while the dark hams had signficantly consistent higher ultimate pH values then both the light and normal hams (Figure 13). The pH values associated with the dark group of hams are well within the Dark, Firm, and Dry (DFD) pork pH value (Briskey, 1964), as they are above or around 6.0 after 24 hours postmortem (Figure 12C).

Differences in the rate, or extent of postmortem glycolysis affect the development of meat color (Scheffler & Gerrard, 2007). The inherent decline in ulitmate pH witnessed in all ham groups in the caudal zones do indicate potential differences in fiber type composition as they

relate to differences in the metabolic capacities of each zone to handle postmortem glycolysis. Subsequently, the dark group of hams alone indicate instances of DFD, that would be responsible for the darker and redder color quantified above. But, the nonsignificant pH values between the normal and light hams, specifically found in the caudal regions, do not match up with differences found in L* therein (Figure 3). The similar pH values suggest indentical metabolic capacities and fiber type compositions, arguing for the alternate hypothesis to explain the color divergencies found there.

Fiber Type Composition

Glycolytic muscles have a higher concentration of the glycolytic enzymes than oxidative muscles. Lactate dehydrogenase (LDH) is a glycolytic enzyme (Karlsson et al., 1999) that is water-soluble protein and responsible for converting pyruvate to lactate (Savage et al., 1990). LDH is capable of increasing its affinity to the actin filament during occasions of extreme glycolysis (Clarke, Shaw, & Morton, 1980). To validate differences in metabolic capacity, muscle fiber-specific proteins were analyzed in each region. Because LDH can associate with myofibrillar proteins, or remain localized in the cytoplasm, LDH abundance was measured in the total protein fraction (Figure 14 and 15). The light and dark grouped hams produced non significant relative LDH concentrations across the four zones (P = 0.4097 and P = 0.0941 respectively, Figure 14B and C), while the normal grouped hams barely approached significance (P = 0.024) between zones A through D (Figure 14A). In addition, like zones between the different grouped hams were not signficantly different (Figure 15), showing that there was no difference in metabolic capacity between hams in different groups.

Muscle fibers are characterized by speed of contraction, which is predicated on the predominate type of myosin heavy chain isoform in the fiber (Schiaffino et al., 1989). Myosin-

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heavy chain type I isform (MyHC-1) is one of four possible fiber types found in mammalian skeletal muscle, and is readily abundant in slower contracting, more oxidative, red muscles (Klont, Brocks, & Eikelenboom, 1998). Figure 16A shows there are greater amounts of MyHC-1 in the cranial zones versus caudal of normal grouped hams (P < 0.0001) which is indicative of differences in fiber type composition across the face of that muscle. The light and dark grouped hams give nonsignficant results (P = 0.0628 and P = 0.0522, Figure 16B and C respectively), but are represented in low replicated number (n=2). In Figure 17 nonsignficant differences exist between like zones of the different grouped hams indicating that this difference in MyHC-I found in the normal grouped hams can carrying throughout all hams evaluated.

Changes in fiber type composition across the cut surface of the SM would account for the innate myoglobin concentrations, causing the gradient of lightness and redness quantified in zones A through D for one of the color variation issues found herein. Muscles or regions of muscle with fiber type compositions that are more glycolytic have greater concentrations of glycolytic enzymes, thus have a higher glycolytic capacity, or the capacity of moving substrates through glycolysis. Glycolytic muscles accumulate more hydrogen ions quicker postmortem by producing more lactate (Scheffler & Gerrard, 2007) from greater ATP usage (Bottinelli, Canepari, Reggiani, & Stienen, 1994). This allows the pH of whiter muscles to drop quicker and to lower levels postmortem.

In this study, each group of hams had nonsignificant differences in LDH and MyHC-I amongst like zones, indicating that their potential to reach the same ultimate pH and observe the same color development was the same. Also, the MyHC-1 concentration showed differences in contractile protein abundance across zones, thereby suggesting differences in fiber type compositions, while LDH concentrations showed that energy metabolism can be uncoupled from

fiber type (Park, Gunawan, Scheffler, Grant, & Gerrard, 2009). But the dark group hams were still able to transmute into cases of DFD, while the caudal zones of the light grouped hams were able to also become signficantly lighter causing the second issue of color uniformity amongst these hams.

Glycolytic Flux

Glycolytic potential (GP) is used as a moderator of total flux occuring postmortem. Additionally, it is an estimate of glycolytic substrate, intermediate, and end product concentrations (Monin & Sellier, 1985), with glycogen being the main substrate for postmortem glycolysis (Hamm, 1977). But, this indicator is only useful if it is assumed that all glycogen and intermediates are headed towards lactate production. Monin and Sellier (1985) produced GP data through sheer calculations based upon glycolytic intermediate concentrations and said assumption.

In this study, portions of the SM are estimated to have more initial glycogen concentrations in their caudal zones, than cranial (Figure 18). Based on the aforementioned, more glycolytic flux could occur, producing more lactate and subsequently greater hydrogen production (Figure 20), and lower ultimate pH (Figure 12). Lactate formation should coincide with pH decline (Scheffler & Gerrard, 2007), as it does in this study (Figures 20 and 12). The zone consistently producing the most lactate across all groups, D, is also the zone with the lowest ultimate pH.

Lactate production declined significantly in the normal, light, and dark grouped hams (P = 0.0022, P < 0.0001, and P < 0.0001 respectively) on the SM, as the zone moved cranially, showing that less substrate was fluxed through before inactivation of PFK. Residual glycogen concentrations were negligible for the normal, light, and dark grouped hams (P = 0.4633, P =

0.5027, P = 0.9485 respectively). Glucose-6-phosphate (Figure 22) and glucose (Figure 24) followed the same pattern as lactate, allowing glycolytic potential to as well (Figure 18) for all groups. Numerically these figures are simmilar to those of Huff-Lonergan et al. (2002); Bee et al. (2006); and Fontanesi et al. (2008).

These data also support the hypothesis that the dark group hams are infact cases of DFD, as their GP (Figure 19), lactate (Figure 21), G6P (Figure 23), and glucose (Figure 25) concentrations are all signficantly below that of the normal and light hams, despite have identical metabolic capacity and fiber type concentrations. Their estimated starting muscle glycogen concentration would be within the concentration threshold where starting substrate would be the limiting factor of pH decline (Henckel, Karlsson, Jensen, Oksbjerg, and Petersen, 2002). In addition, there data herein conclude that the myoglobin concentration differences that exist across the cut surface of the ham are most likely, through differences in muscle fiber type composition. Again, muscle fibers with a higher glycolytic capacity and convert glycogen to lactate at much higher velocities (VØIllestad, Tabata, & MedbØ, 1992), due to their enzyme compositional differences, as compared to oxidative fibers (Klont et al., 1998). The higher velocity allows greater glycogen to be converted to lactate before postmortem glycolysis ceases. The increase in production of lactate aids in the lowering of postmortem ultimate pH as discussed previously. The rate of pH decline is not soley predicated on lactate formation, but instead a combination of lactate formation, the rate of ATP turnover, and the associated accumulation of hydrogen ions within the muscle (Scheffler & Gerrard, 2007). If ATP turnover is increased, postmortem flux increases prior to PFK inactivation. Increased lactate production, greater flux, and higher initial glycogen concentration, are indicative of a higher glycolytic fiber type composition (Fernandez & Tornberg, 1991; Karlsson et al., 1999).

In the case of the lightened caudal zones of the light grouped hams, muscle myoglobin can be denatured or signficantly lost in purge if the peri-mortem conditions present a higher prerigor temperature paired with a lower than normal pH decline (Bendall & Swatland, 1988). Glycolytic flux, as monitored by GP, was not signficantly different between the normal and light grouped hams (Figure 19), as well as neither was the concentrations of lactate (Figure 21), G6P (Figure 23), and glucose (Figure 25). The only other explaination for this divergency in color paired with identical metabolic capacities, flux, and ultimate pH, would be an expediated pH decline, causing the deleterious environment described above.

Increasing temperature increases the rate of glycolysis in muscle (Liu et al., 2014)..
Gardner et al. (2006) found that manipulating environmental temperature by changing scald and dwell times, increased temperature of the muscle early postmortem in the SM but not in the LD. In addition, commerical facilities do see large variation in carcass composition, such as fat thickness (DeVol et al., 1988), allowing differences in fat thickness over the SM to occur between animals. Changes in fat covering would allow heat to conduct differently within zones of muscles containing little or no fat, exhibiting a much larger degree of temperature change, than those with more fat (van der Wal, van Beek, Veerkamp, & Wijngaards, 1993). When a SM that contains little fat cover would be processed, the muscle would heat up to a higher temperature. The higher temperature coupled with lower pH would then create a deleterious environment known to increase the precipiation of sarcoplasmic proteins like myoglobin (Scopes, 1964).

During times of a high prerigor temperature and a lower pH, myofibrilar proteins denature and shrink, mechanically forcing water to gather in the increased sarcoplasmic space. The moisture that collects later purges with water soluble proteins, such as myoglobin (Offer &

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Cousins, 1992). This precipiation changes myoglobin concentration within the muscle and affects overall reflectance of light and ultimately the color of meat precieved by consumers.

<u>Conclusion</u>

Data presented herein show that the SM muscle of the ham varies in color in a caudal to cranial fashion. This variation is associated with changes in muscle fiber composition as measured by myoglobin content, amount of myosin heavy chain isoform, differences in flux and ultimate pH. Changes in color within like zones appear to result from changes in either starting substrate concentrations from DFD pork, or predicted differences in flux rate resulting in protein denaturation and a reduced capacity of the tissue to retain moisture. While changes in muscle fiber type and associated myoglobin suggest differences in color are manifested in the nature of the underlying tissues, interactions with processing enviroments cannot be ruled out. Additional work is needed to understand this relationship more thoroughly.
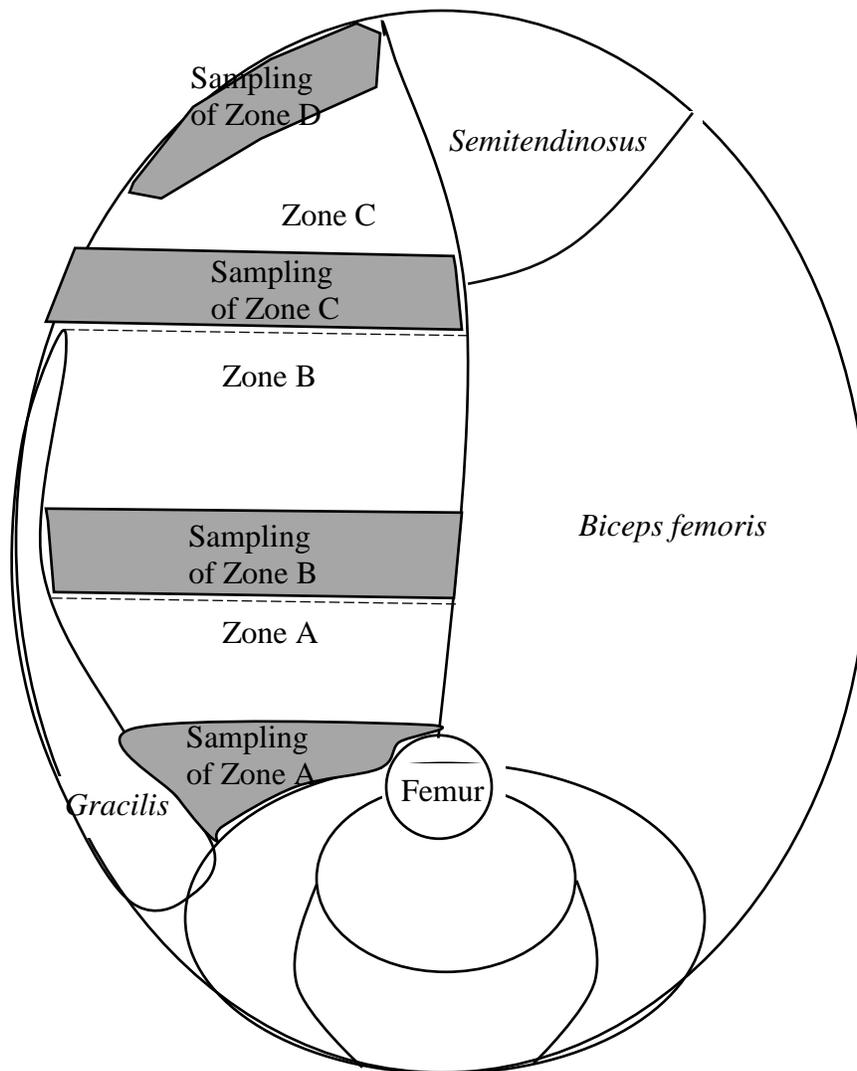
Figure 1: Sampling locations – from the cross-section cut of a porcine *semimembranosus* muscle. Zones A, B, and C are marked off with dashed lines, while the sampling area of all four zones is shaded in gray.
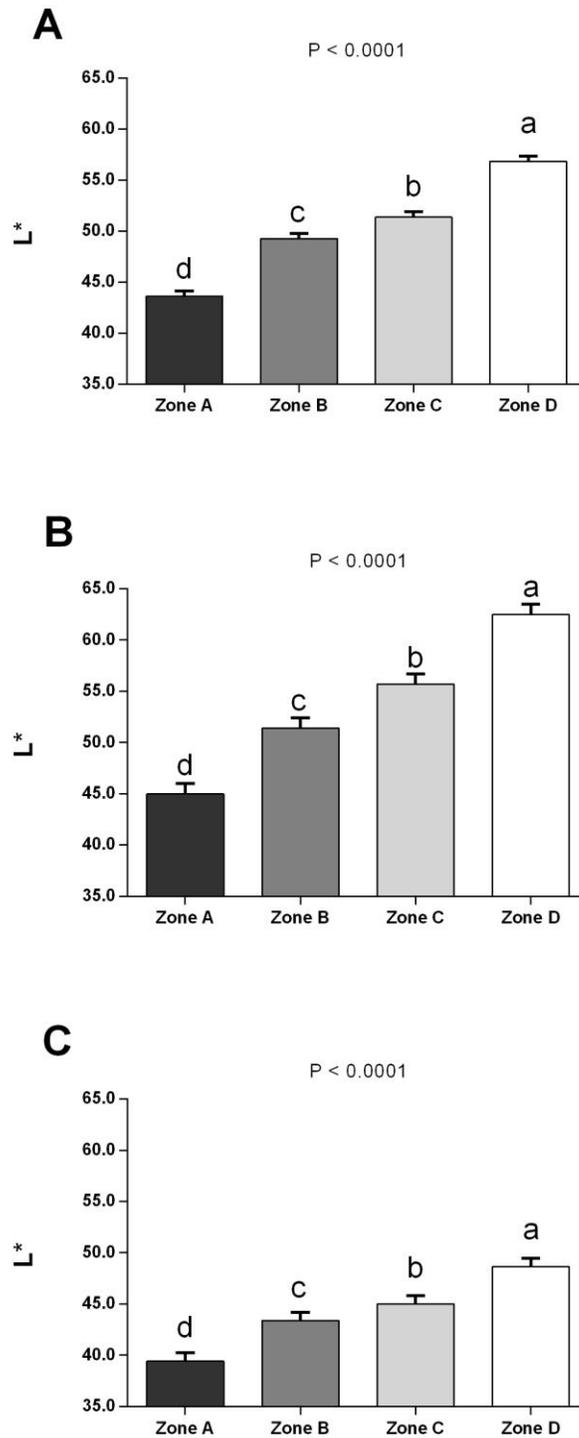
Figure 2: L* (Lightness) Minolta Colorimeter Data – sliced by group with (A) representing normal grouped hams, (B) representing light grouped hams, and (C) the dark grouped hams from across the cut surface of the SM muscle. Bars with different letters represents significant differences.
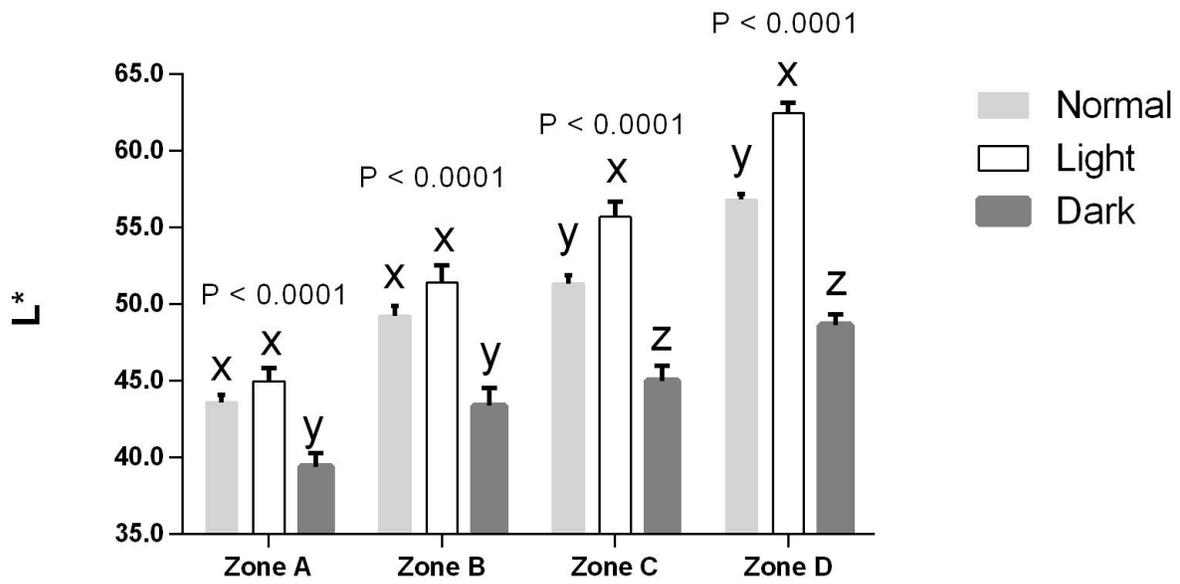
Figure 3: L* (Lightness) Minolta Colorimeter Data – sliced by zone with from across the cut surface of the SM muscle. Bars with different letters represents significant differences.
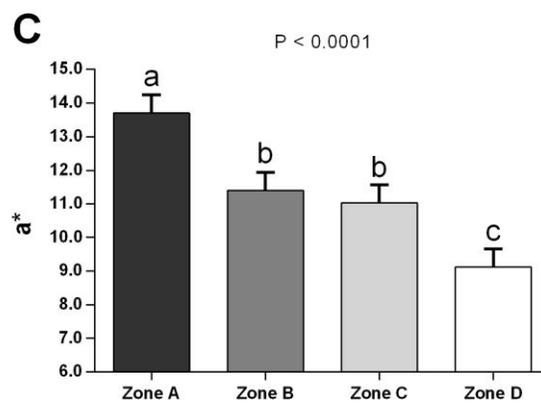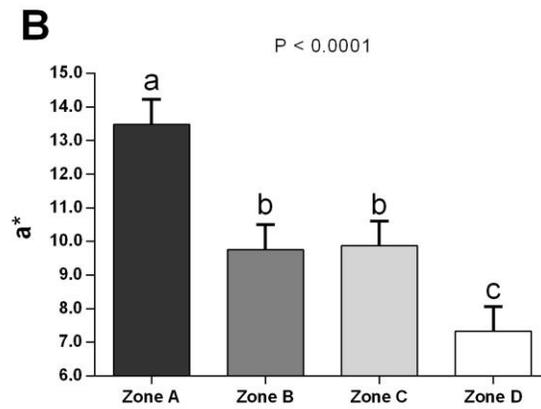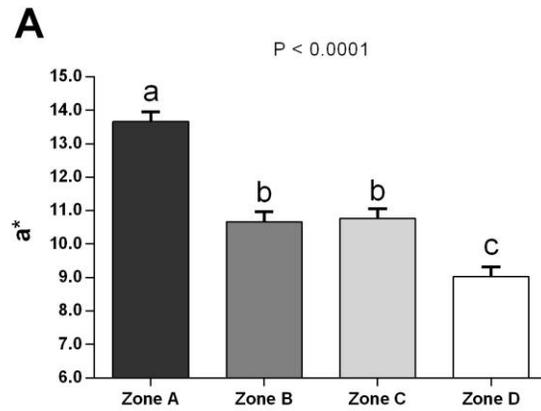
Figure 4: a* (Redness) Minolta Colorimeter Data – sliced by group with (A) representing normal grouped hams, (B) representing light grouped hams, and (C) the dark grouped hams from across the cut surface of the SM muscle. Bars with different letters represents significant differences.
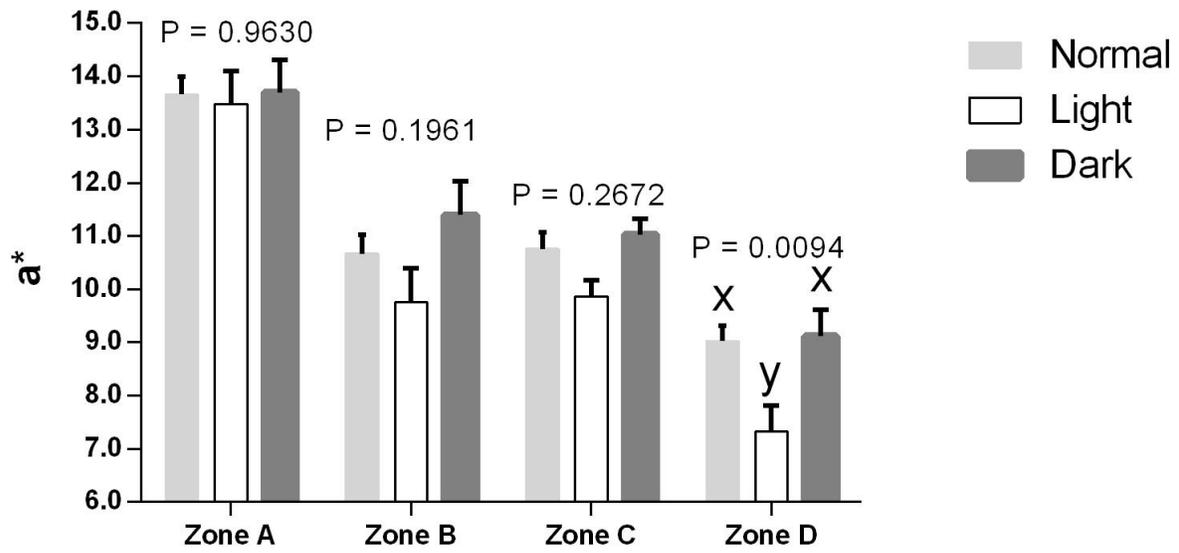
Figure 5: a* (Redness) Minolta Colorimeter Data – sliced by zone with from across the cut surface of the SM muscle. Bars with different letters represents significant differences.
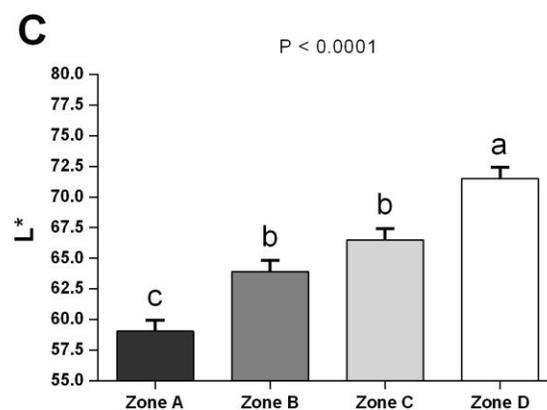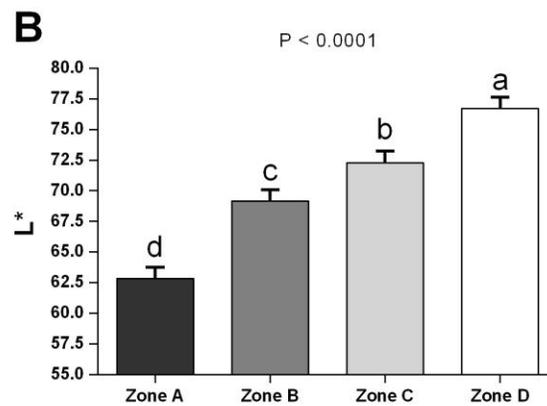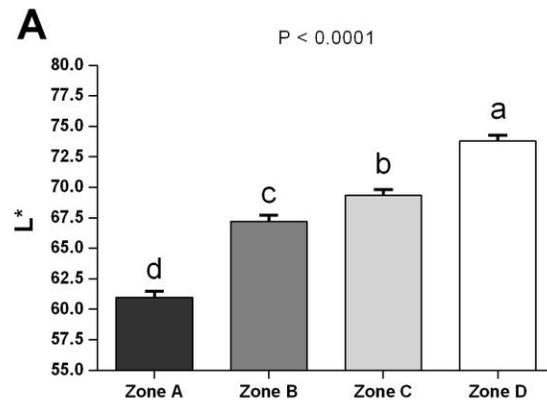
Figure 6: L* (Lightness) Matlab Image Processing Data – sliced by group with (A) representing normal grouped hams, (B) representing light grouped hams, and (C) the dark grouped hams from across the cut surface of the SM muscle. Bars with different letters represents significant differences.
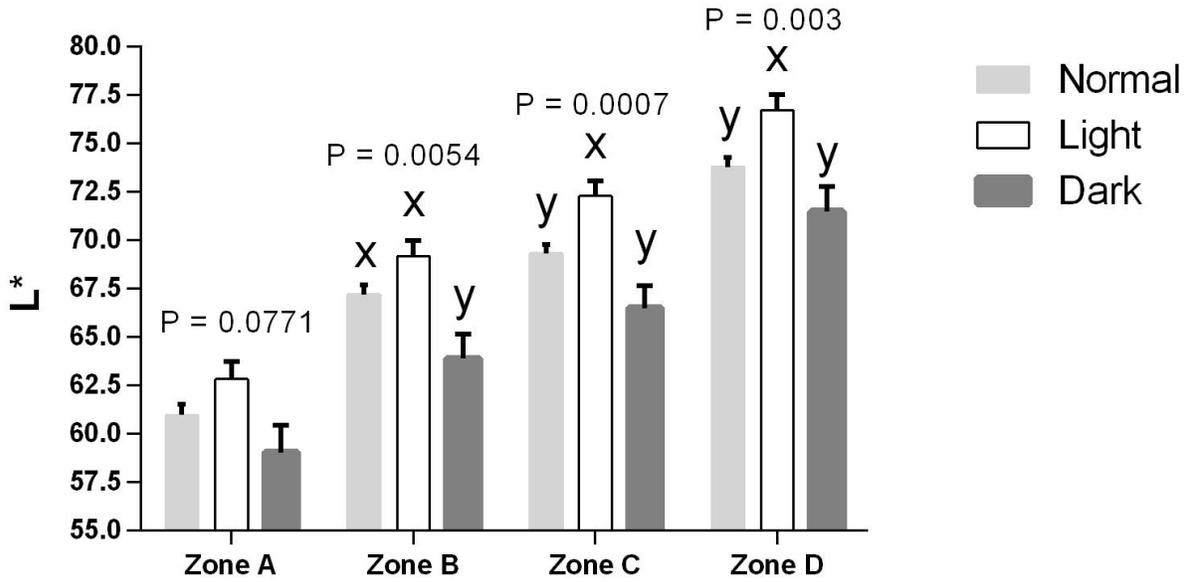
Figure 7: L* (Lightness) Matlab Image Processing – sliced by zone with from across the cut surface of the SM muscle. Bars with different letters represents significant differences.
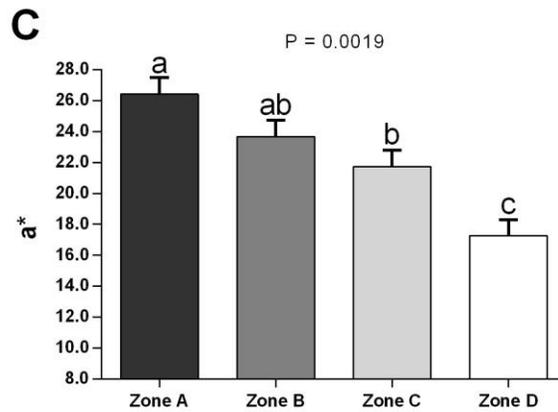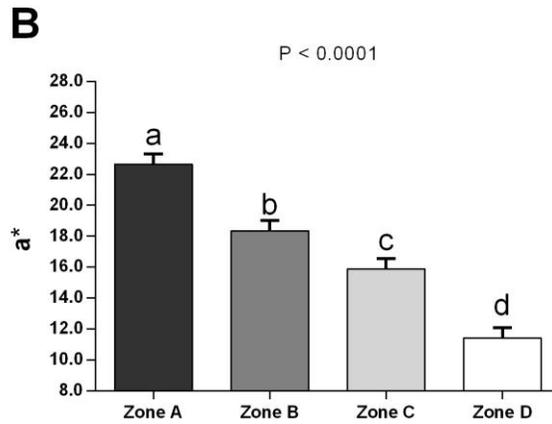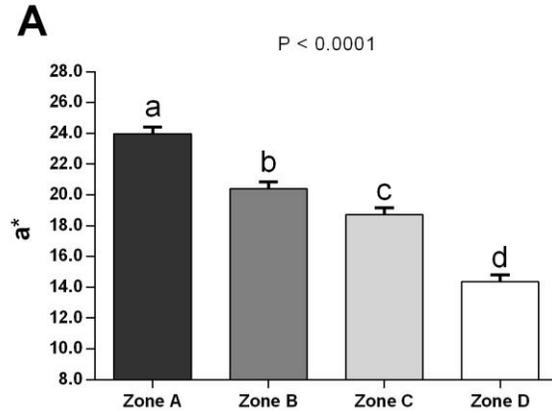
Figure 8: a* (Redness) Matlab Image Processing Data – sliced by group with (A) representing normal grouped hams, (B) representing light grouped hams, and (C) the dark grouped hams from across the cut surface of the SM muscle. Bars with different letters represents significant differences.
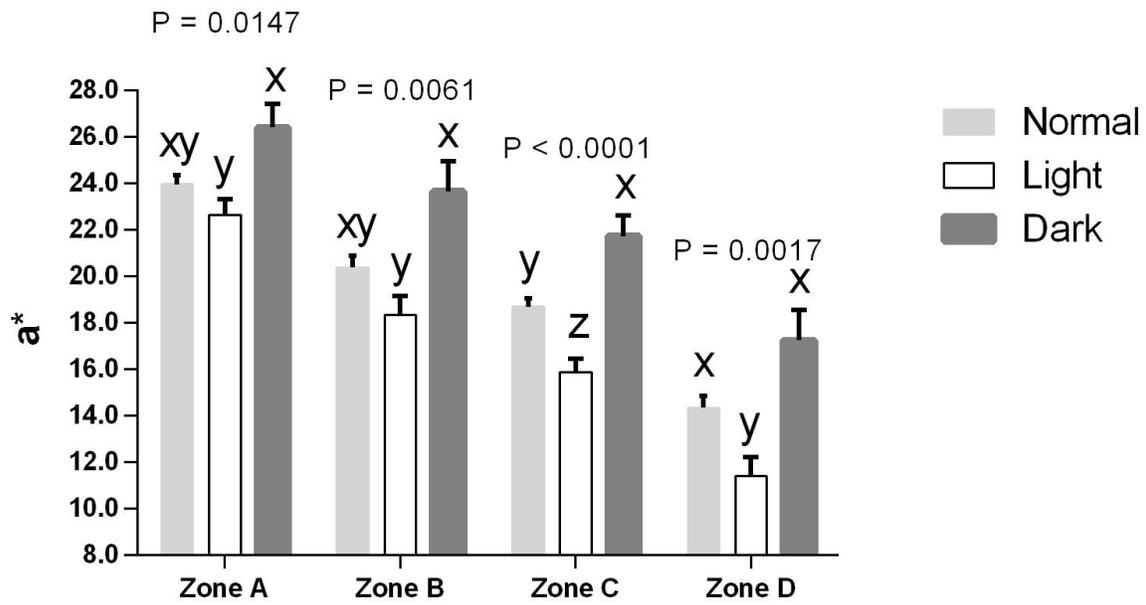
Figure 9: a* (Redness) Matlab Image Processing – sliced by zone with from across the cut surface of the SM muscle. Bars with different letters represents significant differences.
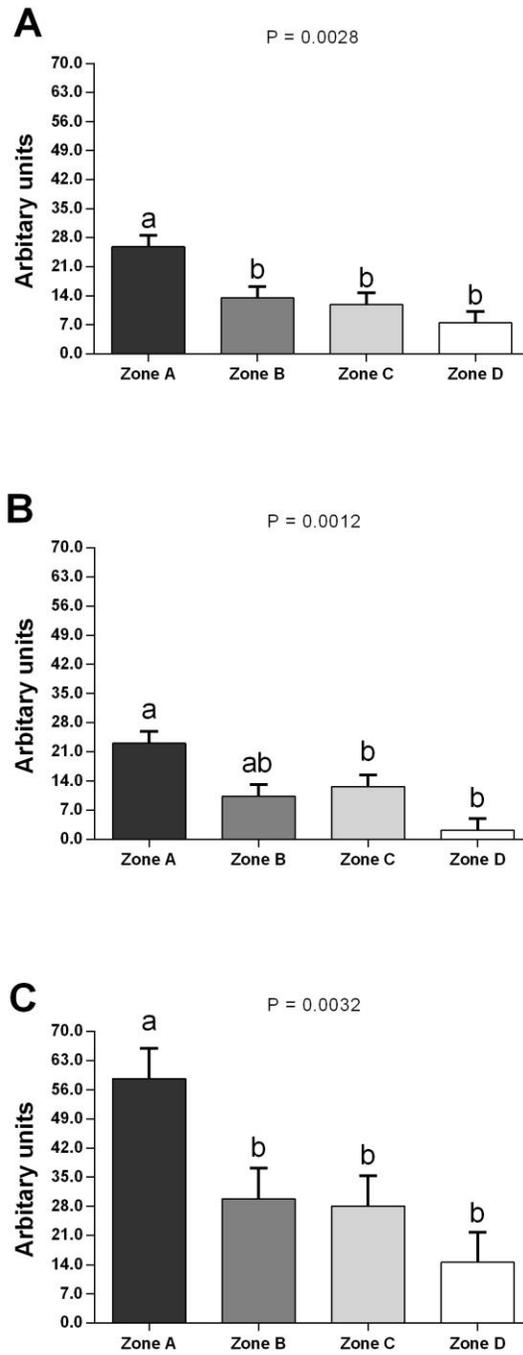
Figure 10: Relative Myoglobin concentration – sliced by group with (A) representing normal grouped hams, (B) representing light grouped hams, and (C) the dark grouped hams using Western blot techniques from across the cut surface of the SM muscle. Bars with different letters represents significant differences
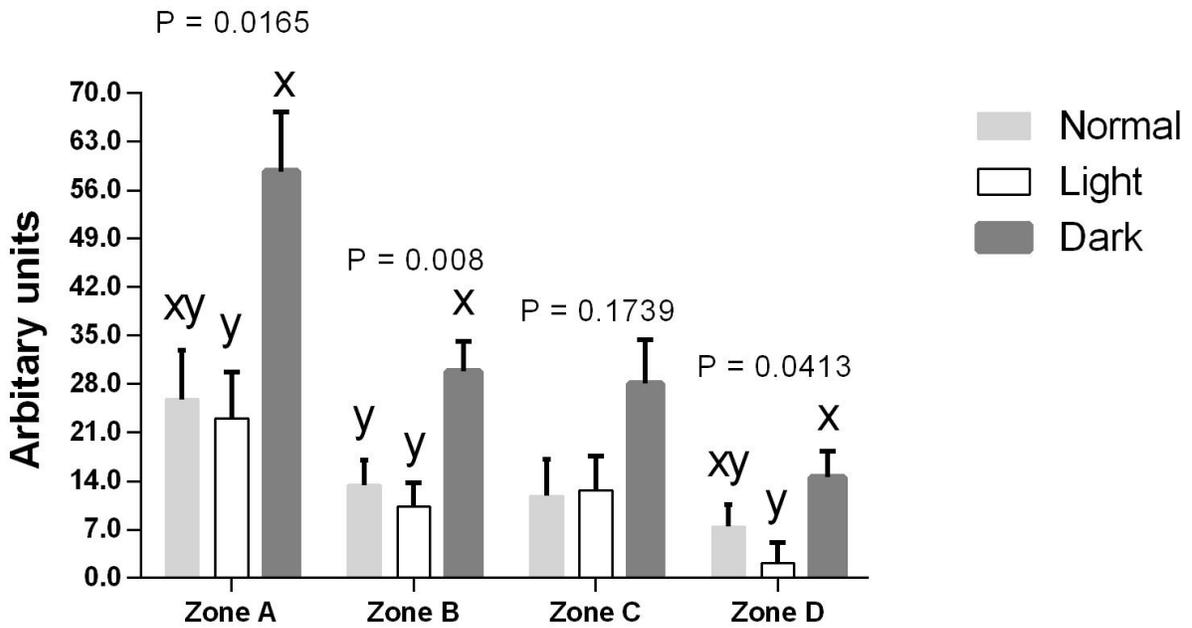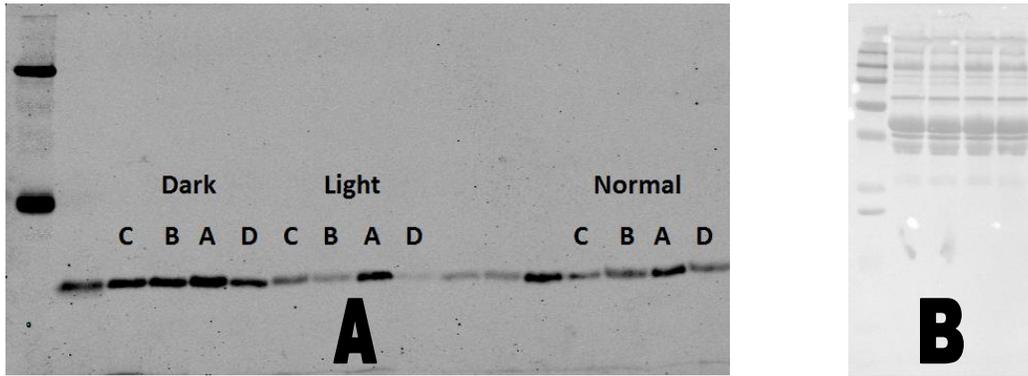
Figure 11: Relative Myoglobin concentration – sliced by zone from zones across the cut surface of the SM using Western Blot techniques. Bars with different letters represent significant difference. Blot (A) shows the intensity of the bands, while blot (B) shows the Ponceau S stain. Lanes are marked with their zone and group they represent..
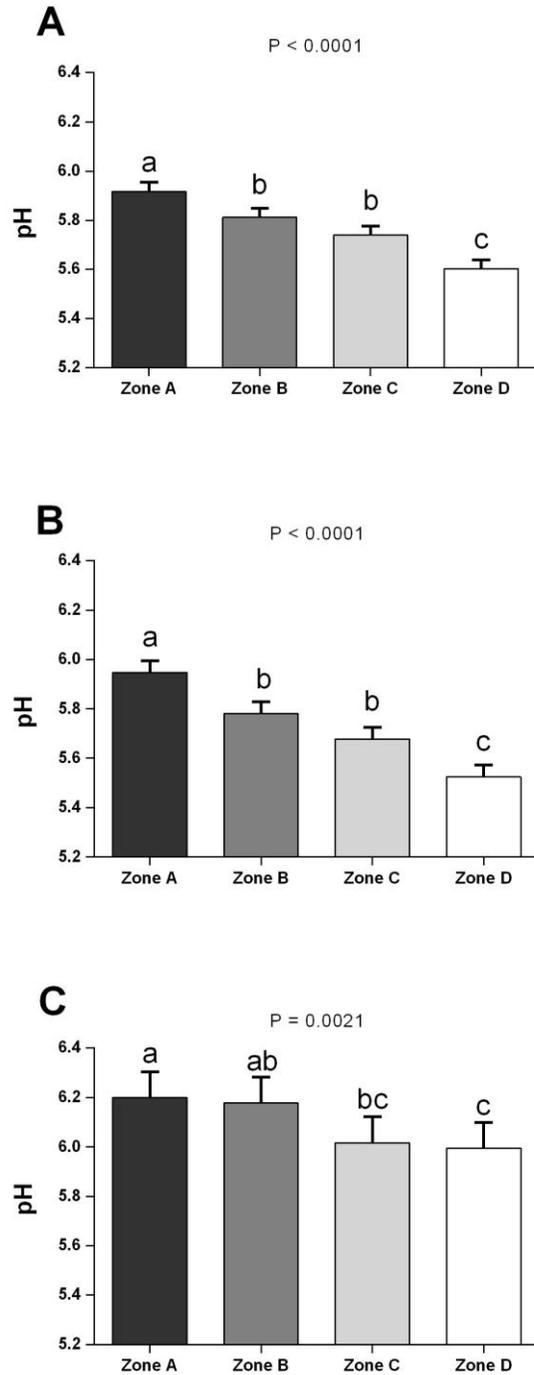
Figure 12: Ultimate pH – sliced by group from zones across the cut surface of the SM taken 24 hr postmortem with (A) representing normal grouped hams, (B) representing light grouped hams, and (C) the dark grouped hams. Bars with different letters represents significant differences
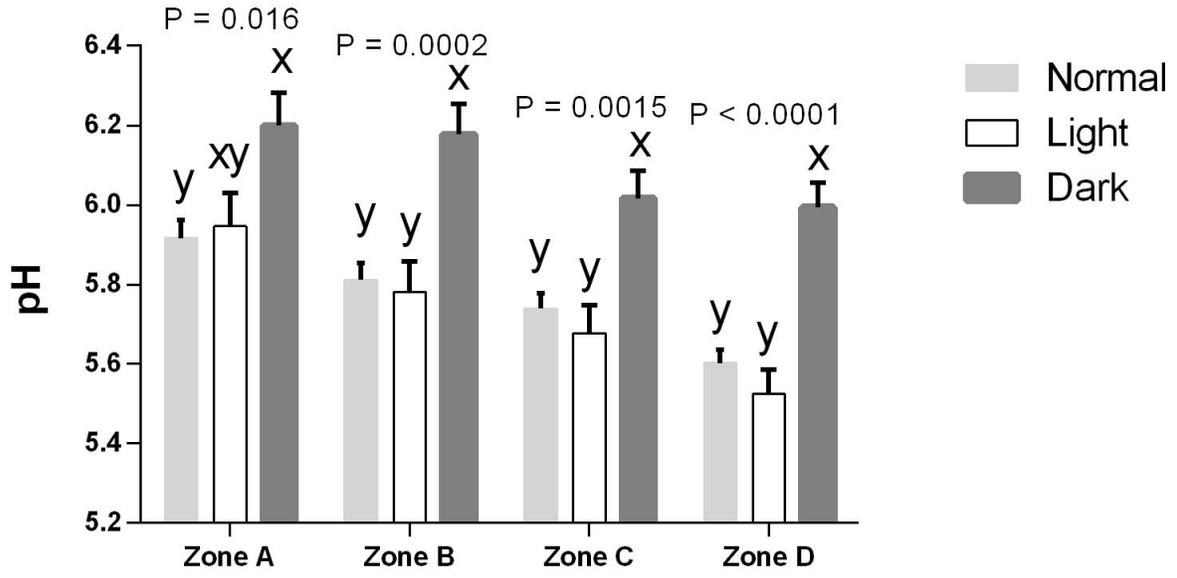
Figure 13: Ultimate pH – sliced by zone from zones across the cut surface of the SM taken 24 hr postmortem. Bars with different letters represent significant difference.
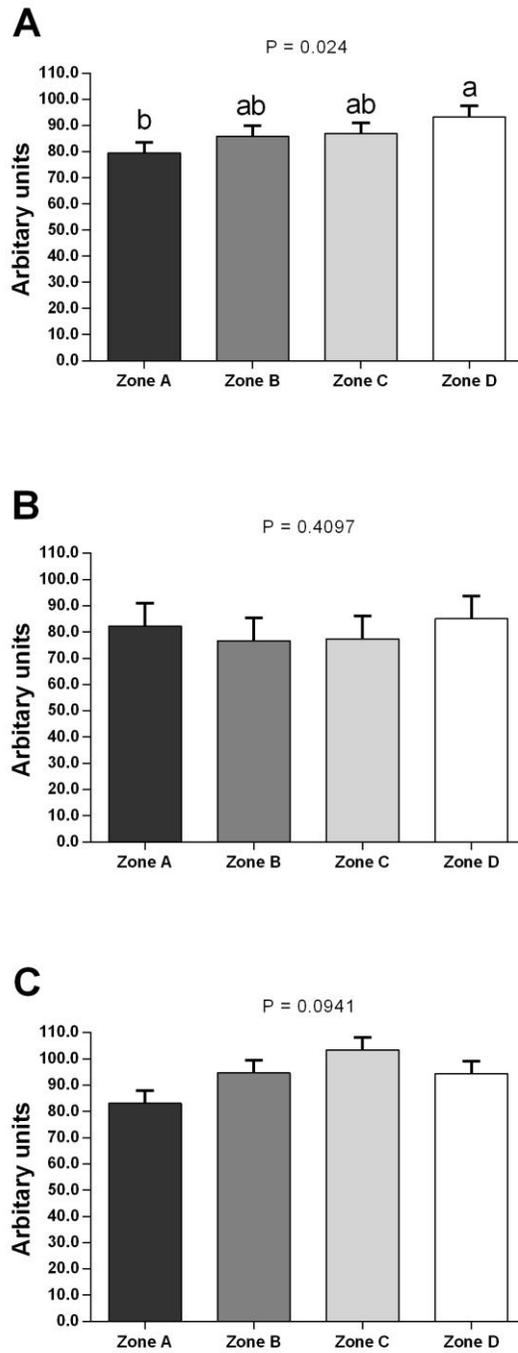
Figure 14: Relative LDH concentration – sliced by group from zones across the cut surface of the SM using Western Blot techniques with (A) representing normal grouped hams, (B) representing light grouped hams, and (C) the dark grouped hams. Bars with different letters represents significant differences.

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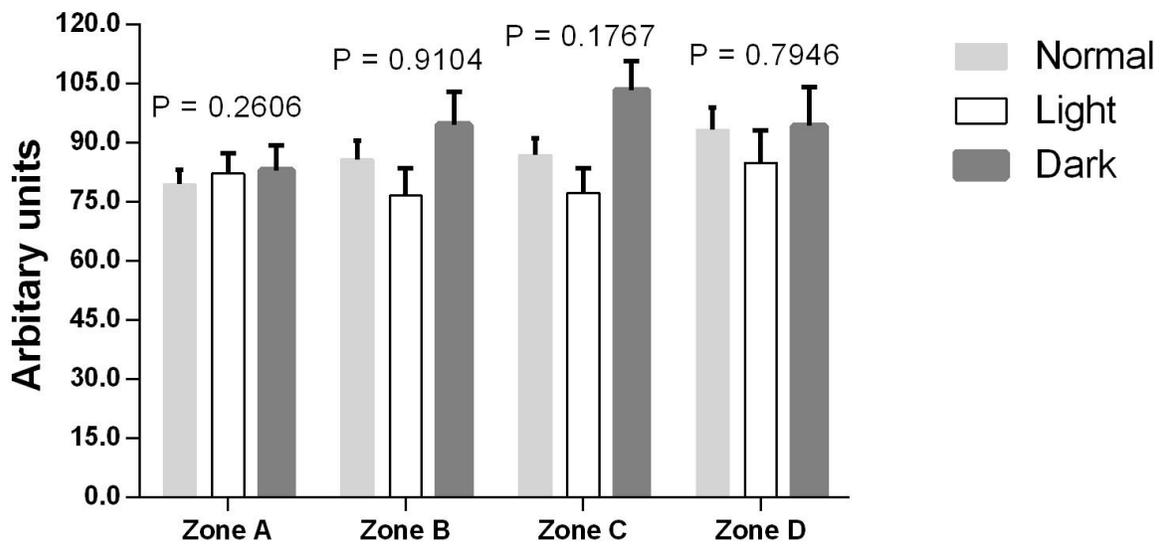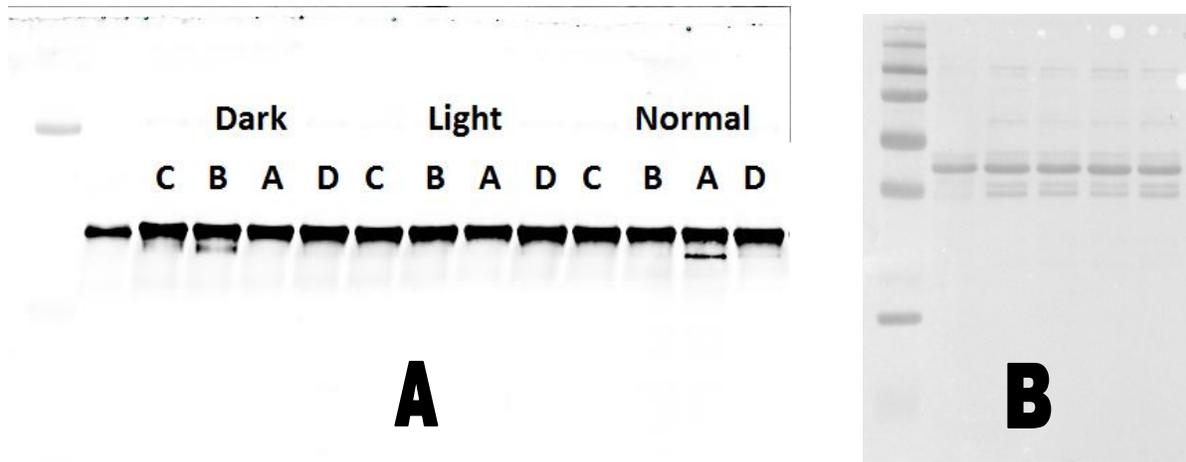
Figure 15: Relative LDH concentration – sliced by zone from zones across the cut surface of the SM using Western Blot techniques. Bars with different letters represent significant difference. Blot (A) shows the intensity of the bands, while blot (B) shows the Ponceau S stain. Lanes are marked with their zone and group they represent.
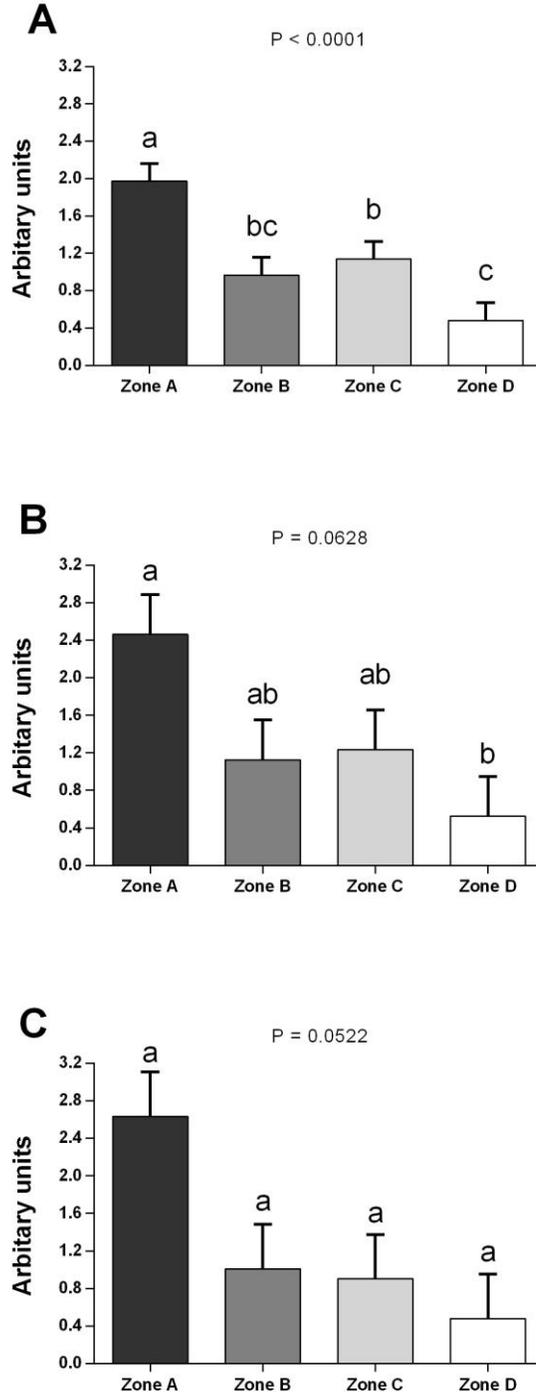
Figure 16: Relative MyHC-I concentration – sliced by group from zones across the cut surface of the SM using Western Blot techniques with (A) representing normal grouped hams, (B) representing light grouped hams, and (C) the dark grouped hams. Bars with different letters represents significant differences.
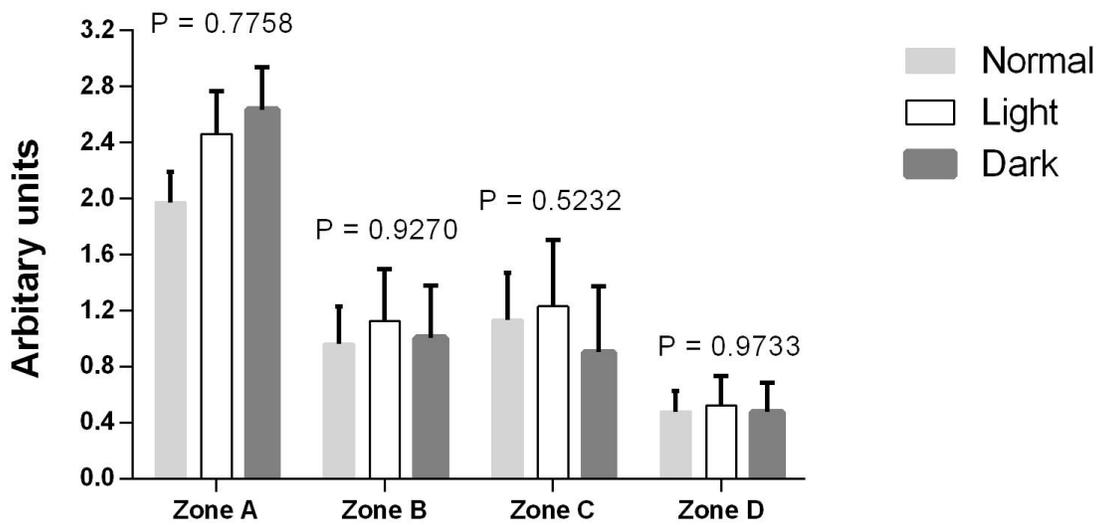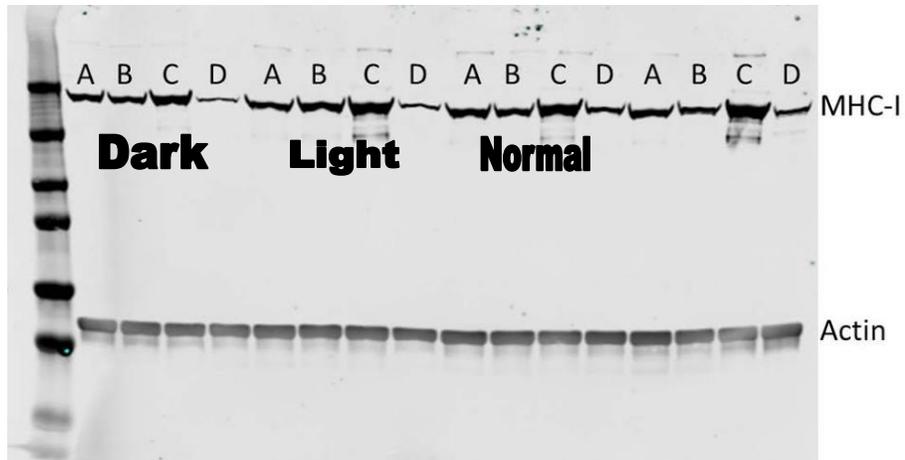
Figure 17: Relative MyHC-I concentration – sliced by zone from zones across the cut surface of the SM using Western Blot techniques. Blot shows the intensity of the bands of both the target protein and loading control. Lanes are marked with their zone and group they represent.
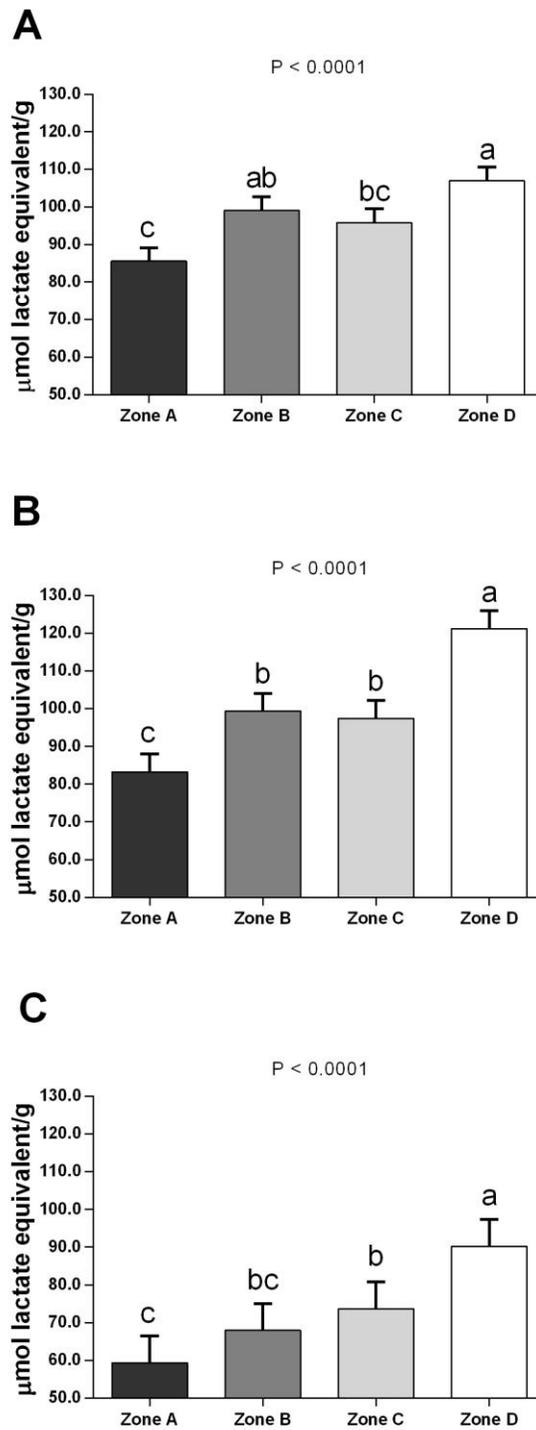
Figure 18: Glycolytic Potential – sliced by group from zones across the cut surface of the SM with (A) representing normal grouped hams, (B) representing light grouped hams, and (C) the dark grouped hams. Bars with different letters represents significant differences.
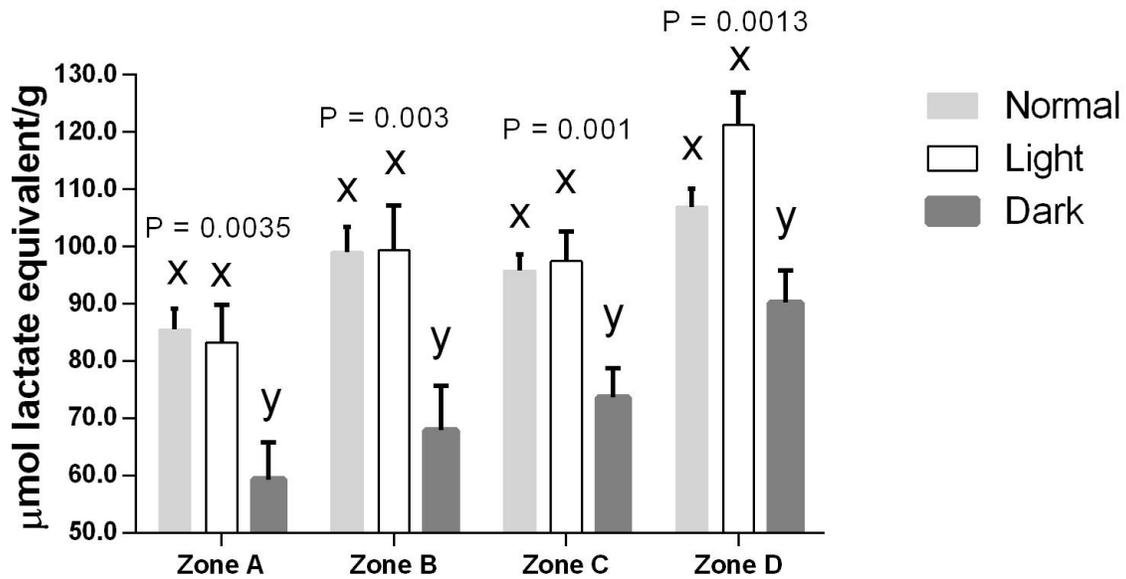
Figure 19: Glycolytic Potential – sliced by zone with from across the cut surface of the SM muscle. Bars with different letters represents significant differences.
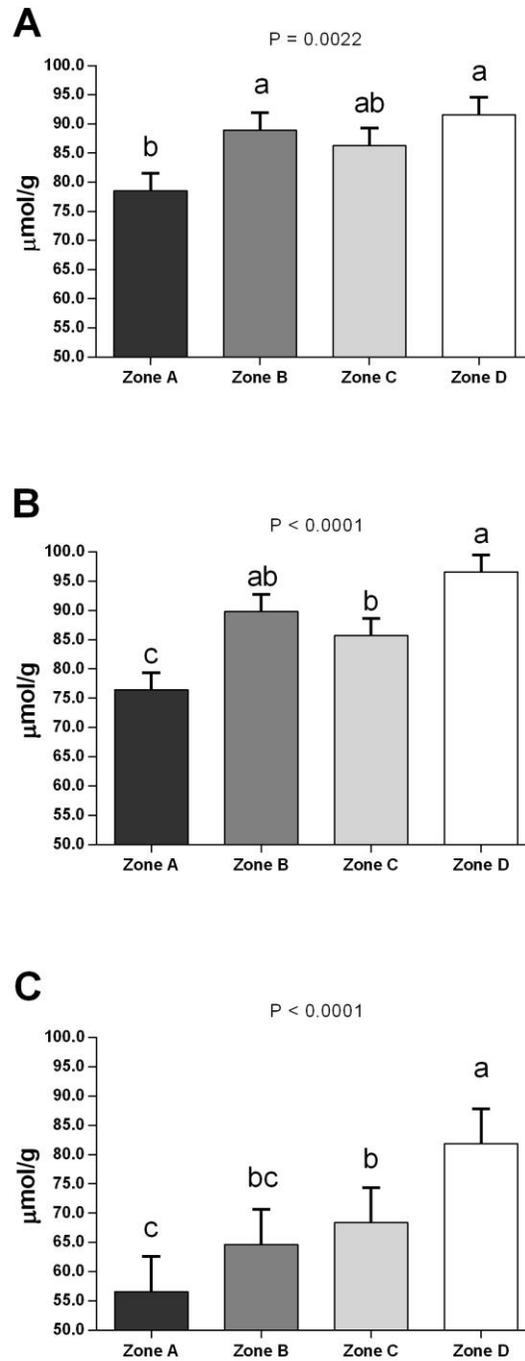
Figure 20: Lactate concentration – sliced by group with (A) representing normal grouped hams, (B) representing light grouped hams, and (C) the dark grouped hams from across the cut surface of the SM muscle. Data is expressed in μmol/g of tissue. Bars with different letters represents significant differences.

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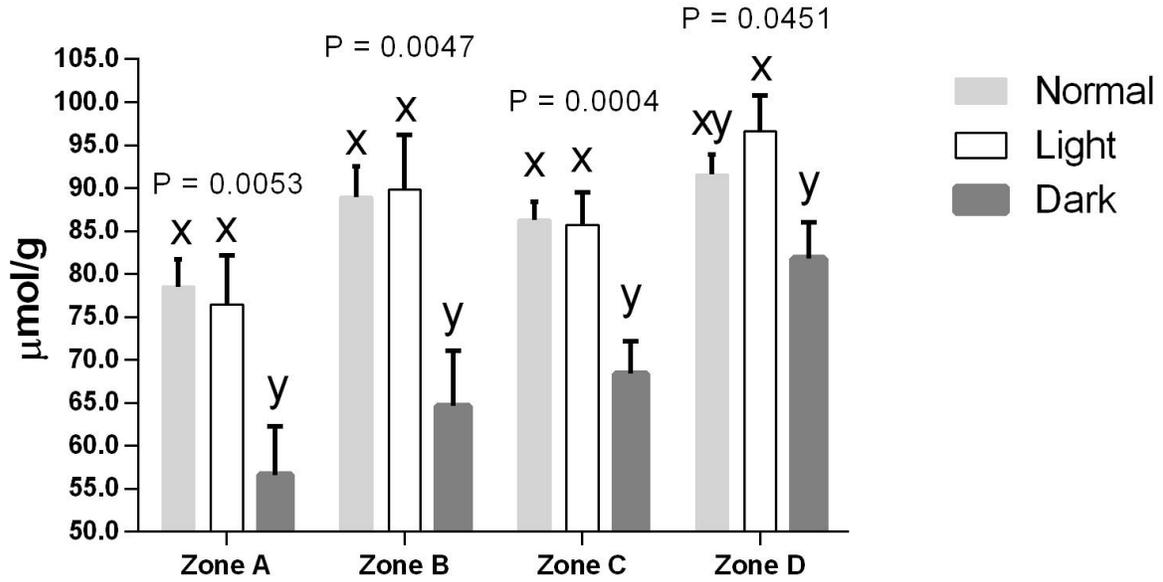
Figure 21: Lactate Concentration – sliced by zone with from across the cut surface of the SM muscle. Data is expressed in μmol/g of tissue. Bars with different letters represents significant differences.
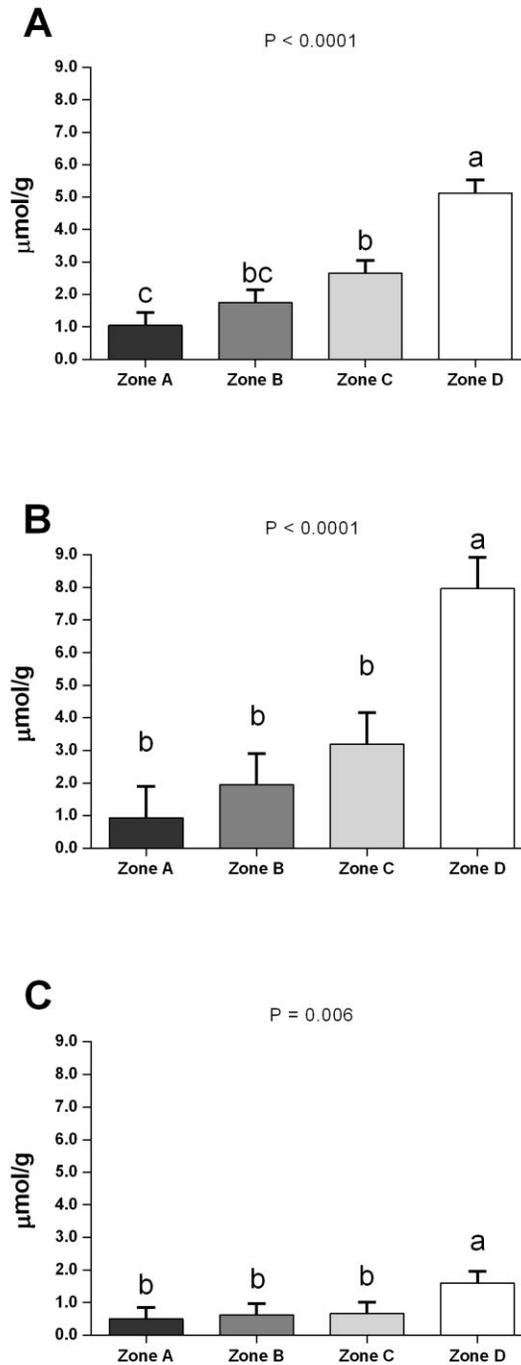
Figure 22: Glucose-6-Phosphate concentration – sliced by group with (A) representing normal grouped hams, (B) representing light grouped hams, and (C) the dark grouped hams from across the cut surface of the SM muscle. Data is expressed in µmol/g of tissue. Bars with different letters represents significant differences.
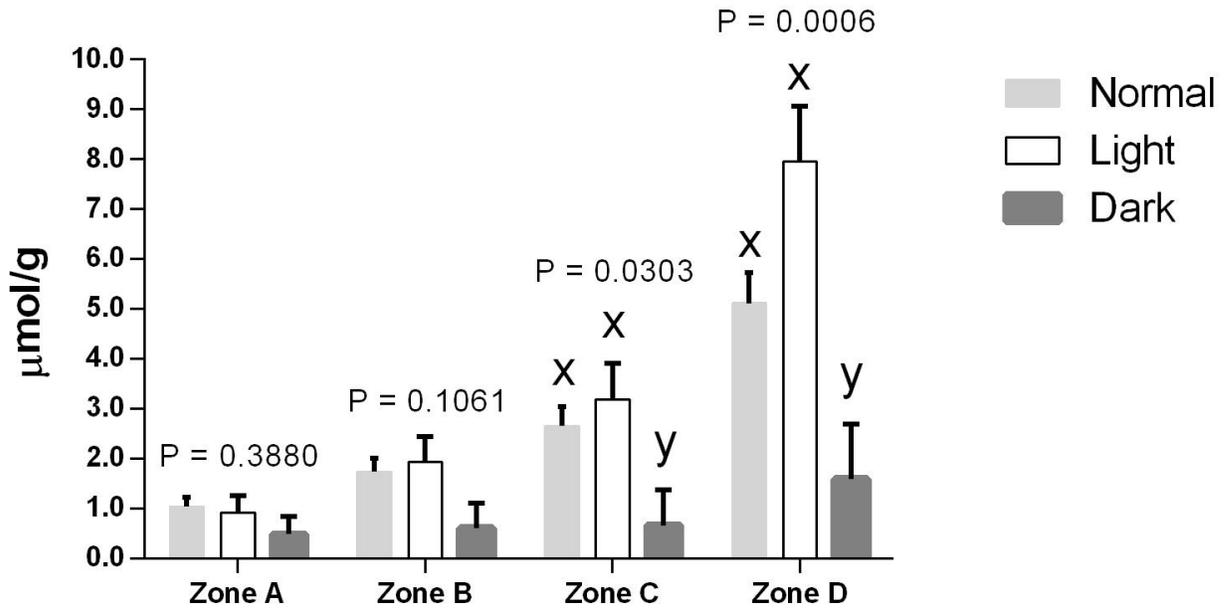
Figure 23: Glucose-6-Phosphate Concentration – sliced by zone with from across the cut surface of the SM muscle. Data is expressed in µmol/g of tissue. Bars with different letters represents significant differences.
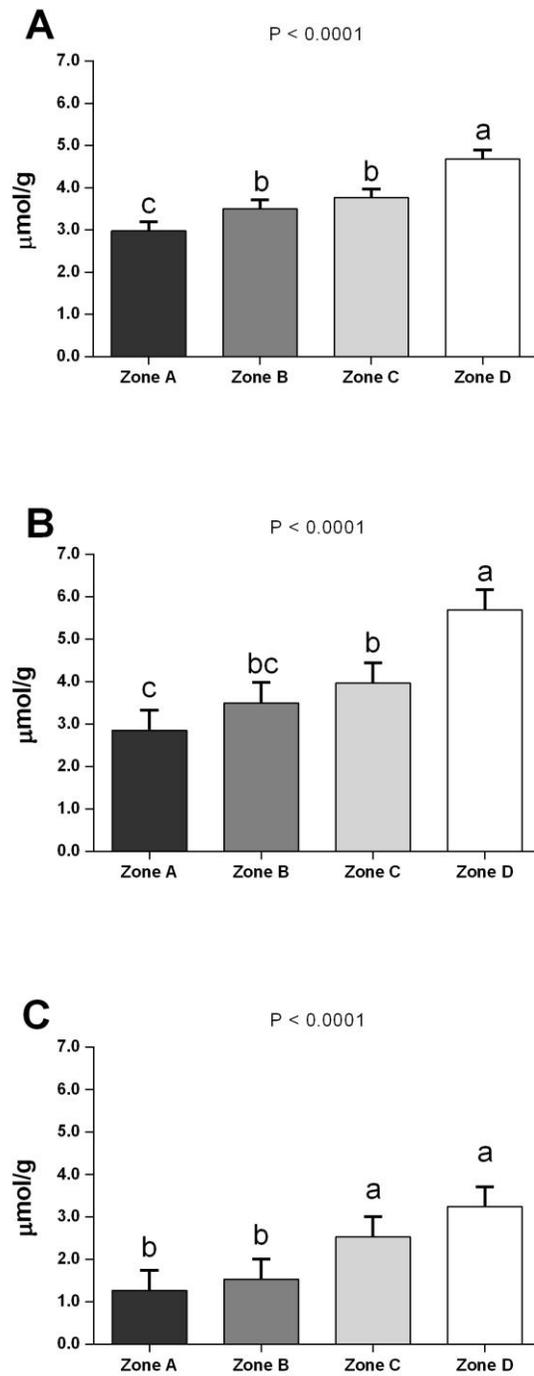
Figure 24: Glucose concentration – sliced by group with (A) representing normal grouped hams, (B) representing light grouped hams, and (C) the dark grouped hams from across the cut surface of the SM muscle. Data is expressed in μmol/g of tissue. Bars with different letters represents significant differences.
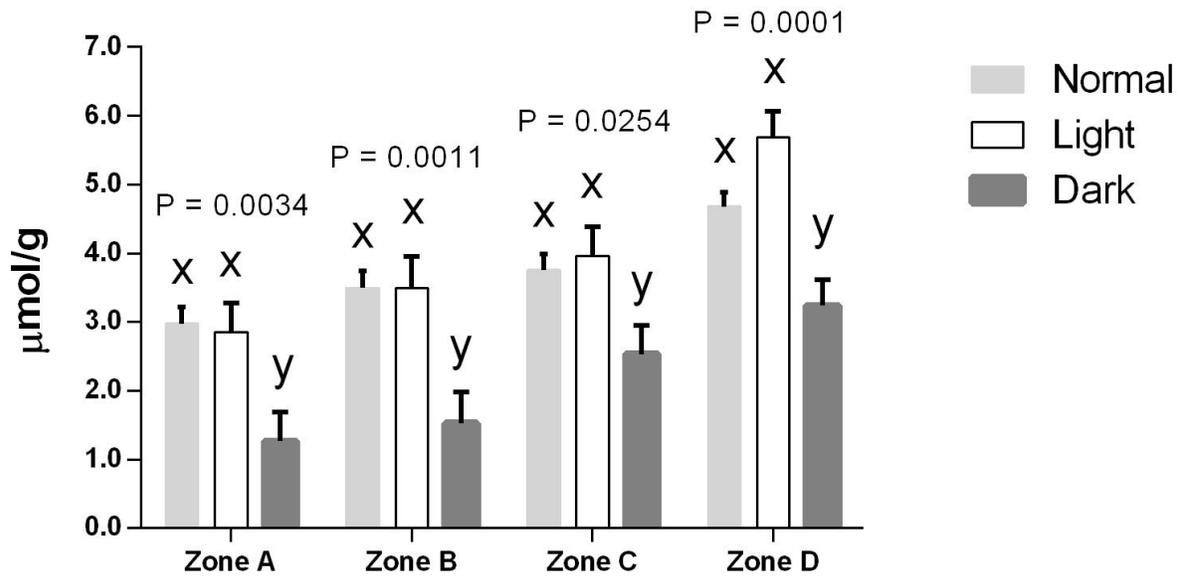
Figure 25: Glucose Concentration – sliced by zone with from across the cut surface of the SM muscle. Data is expressed in µmol/g of tissue. Bars with different letters represents significant differences.

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CHAPTER 3. Future Direction

Postmortem fresh ham color development has many different mechanisms working together. The vast number of mechanisms provides the same number of opportunities for an abberation to occur that changes phenotypic color. Many of those abberations result from a temperature and or pH deleterious environment causing some kind of protein denaturation. There are many documented instances of meat quality defects that quote protein denaturation as the source of the problem, not just in color development. But, knowledge of whether the muscle in question actually reaches the temperature environment needed for such denaturation does not extensively exist. Therefore, my future direction would focus on determining logistical ways of comprehesively mapping temperature decline in hams during pH decline. Then, apply that temperature decline topography to muscle proteins to determine when the denaturation of each protein occurs.

Doing such thing is almost impossible in a commerical setting, so to start, commerialized environmental conditions would be measured. These conditions would be programmed into a controlled atmospheric box, possibly a smokehouse, so they easily can be recreated with times that parallel postmortem pH decline. The ham would be filled with probes, capable of taking readings at multiple depths. The probes would be left in during the entire process to record muscle temperature. Use of an infrared camera that color maps heat lost would also be useful. This would be recreated on hams of varying size, genetic makeup, and fat composition to account for the variation seen in a plant setting. Being able to determine at what point certain protein functionality is lost would be instrumental to the future of meat quality control.

After determining when and where meat quality proteins denature, correlations can be determined relating the amount of potential protein denaturation to hams of different sizes,

lineages, and composition. Theoretically, these hams would thermodynamically respond to heat slightly differently due to their changes in muscle mass, fiber type composition, and fat insulation. For ham color development specfically, the end goal would be to create a selection process from this data. Plants could then sort out hams that would have more easily endured higher levels of protein denaturation, prior to complete fabrication or trimming.

```
if nargin == 1
 B = double(R(:,:,3));
 G = double(R(:,:,2));
 R = double(R(:,:,1));
end
if max(max(R)) > 1.0 || max(max(G)) > 1.0 || max(max(B)) > 1.0
 R = double(R) / 255;
 G = double(G) / 255;
 B = double(B) / 255;
end
% Set a threshold
T = 0.008856;
 [M, N] = size(R);
s = M * N;
RGB = [reshape(R,1,s); reshape(G,1,s); reshape(B,1,s)];
 % RGB to XYZ
MAT = [0.412453 0.357580 0.180423;
0.212671 0.715160 0.072169;
0.019334 0.119193 0.950227];
XYZ = MAT * RGB;
% Normalize for D65 white point
 X = XYZ(1,:) / 0.950456;
 Y = XYZ(2,:);
 Z = XYZ(3,:) / 1.088754;
 XT = X > T;
 YT = Y > T;
 ZT = Z > T;
 Y3 = Y.^(1/3);
 fX = XT .* X.^(1/3) + (~XT) .* (7.787 .* X + 16/116);
 fY = YT .* Y3 + (~YT) .* (7.787 .* Y + 16/116);
 fZ = ZT .* Z.^(1/3) + (~ZT) .* (7.787 .* Z + 16/116);
L = reshape(YT .* (116 * Y3 - 16.0) + (~YT) .* (903.3 * Y), M, N);
a = reshape(500 * (fX - fY), M, N);
b = reshape(200 * (fY - fZ), M, N);
if nargout < 2
L = cat(3,L,a,b);
end
```

Figure 1. Matlab script converting red, green, blue (RGB) color values to CIELAB color space.

```
function [xmean, xstd, xmin, xmax, xskew, xquan25, xquan75] = RunStats(x)

xmean = mean(x);
xstd = std(x);
xmin = min(x);
xmax = max(x);
xskew = skewness(x);
xquan25 = quantile(x,0.25);
xquan75 = quantile(x,0.75);
```

Figure 2. Matlab script producing statistical parameters for the raw data values.

```
clc; clear; close all
imname = 'insertfile.jpg';
[X] = imread(imname);
[L,a,b] = RGB2Lab(X);

[m,n] = size(L);
Lv =reshape(L,[1,m*n]);
Lv1=Lv(Lv<90);

figure
hist(Lv1,100);
[Lvmean, Lvstd, Lvmin, Lvmax, Lvskew, Lvquan25, Lvquan75] = RunStats(Lv1);

Lv_new=Lv1(Lv1<(Lvmean+2*Lvstd) & Lv1>(Lvmean-2*Lvstd));

figure
hist(Lv_new,100)
[Lvmean_trunc, Lvstd_trunc, Lvmin_trunc, Lvmax_trunc, Lvskew_trunc,
Lvquan25_trunc, Lvquan75_trunc] = RunStats(Lv_new);

[ma,na] = size(a);
av =reshape(a,[1,ma*na]);
av1 = av(av>0.5 | av<-0.5);

figure
hist(av1,100);
[avmean, avstd, avmin, avmax, avskew, avquan25, avquan75] = RunStats(av1);

av_new=av1(av1<(avmean+2*avstd) & av1>(avmean-2*avstd));

figure
hist(av_new,100)
[avmean_trunc, avstd_trunc, avmin_trunc, avmax_trunc, avskew_trunc,
avquan25_trunc, avquan75_trunc] = RunStats(av_new);

[mb,nb] = size(b);
bv =reshape(b,[1,mb*nb]);
bv1=bv(bv<-0.5 | bv>0.5);

figure
hist(bv1,100);
[bvmean, bvstd, bvmin, bvmax, bvskew, bvquan25, bvquan75] = RunStats(bv1);

bv_new=bv1(bv1<(bvmean+2*bvstd) & bv1>(bvmean-2*bvstd));

figure
hist(bv_new,100)
[bvmean_trunc, bvstd_trunc, bvmin_trunc, bvmax_trunc, bvskew_trunc,
bvquan25_trunc, bvquan75_trunc] = RunStats(bv_new);
```

Figure 3. Matlab script truncating the raw color values to eliminate values representing background, connective and fat tissues, and light scattering.

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