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The Influence of Diet and Oxidation on Calcium Retention of the Mitochondria in Fresh Beef

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THE INFLUENCE OF DIET AND OXIDATION ON CALCIUM RETENTION OF THE MITOCHONDRIA IN FRESH BEEF

by

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THE INFLUENCE OF DIET AND OXIDATION ON CALCIUM RETENTION OF THE MITOCHONDRIA IN FRESH BEEF

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Distillers grains (DG) contribute to cattle sarcoplasmic reticulum (SR) membrane instability by increasing poly-unsaturated fatty acid (PUFA) content in the SR membrane, possibly resulting in early postmortem calcium (Ca) leakage and improved tenderness. Mitochondria are relatively easy to isolate intact and provide the opportunity to study Ca in an SR model. The objective of this research was to determine the influence of diet and oxidation on Ca release from mitochondria. Cattle (n=48) were fed a corn-based finishing diet with or without deoiled, dried DG (50% DM basis). Steaks from each strip loin were aged for 2, 8, 14, and 21 days, powdered, and stored at -80ºC for lab analysis. Samples (n=12) were randomly sub-sampled from each diet group for all aging periods. Mitochondria were isolated from day 2, 8, and 14 day-aged samples using high speed ultracentrifugation. The SR was isolated from each day 2 sample. Both mitochondria and SR samples were analyzed for PUFA content using gas chromatography, and phospholipid content using thin layer chromatography. Mitochondria from days 2 and 8 were artificially oxidized using an iron and ascorbic acid mixture. In both organelles, the DG diet samples had higher 18:2 and total PUFA content ($P<0.05$) compared to corn samples. Day 14 mitochondria had higher 18:2 ($P<0.05$) and total PUFA ($P<0.05$)

contents compared to day 2 and 8 mitochondria, as expected from aging of unsaturated fats. Phospholipid contents (phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol) of the mitochondria and SR were unaffected by diet. Oxidized mitochondria retained significantly less Ca than non-oxidized ($P<0.05$) mitochondria. Day 2 mitochondria retained significantly less Ca than day 8 ($P<0.05$) mitochondria. Overall, mitochondria from cattle finished on corn tended ($P<0.10$) to retain more Ca than mitochondria from cattle finished on DG. Results indicate that greater PUFA content deposited in organelles by the DG diet may affect Ca flux by increased susceptibility to oxidation. A DG diet may influence Ca flux and ultimate tenderness by this mechanism.

Keywords: calcium, distillers grains, mitochondria

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TABLE OF CONTENTS

INTRODUCTION

This study seeks to understand the effects of dried distillers grains plus solubles on beef tenderness. Distillers grains are the major by-product of ethanol production. The process of ethanol production includes grinding corn kernels, then fermenting the starch to produce alcohol, and finally centrifuging the stillage to remove coarse grain particles. These left over grain particles are used to make distillers grains. Dried distillers grains plus solubles are made when a leftover solubles fraction is also dried and then mixed with the grain particles (Stock et al., 2000).

Compared to corn, distillers grains contain higher levels of protein, fat, and fiber. Therefore, distillers grains are a valuable by-product used to feed cattle (Klopfenstein et al., 2007). It has been reported by Senaratne (2012) that steaks from cattle fed a high wet distillers grain plus solubles (WDGS) diet have increased tenderness and higher levels of free calcium compared to steaks from cattle fed corn. Wet distillers grains are known to increase PUFA content, linoleic, and trans fatty acids while decreasing monounsaturated fatty acids (Mello et al., 2012). Furthermore, Chao (2015) demonstrated that cattle fed WDGS exhibited a greater calcium content in the sarcoplasm, a greater amount of PUFA in the sarcoplasmic reticulum, with altered phospholipid content in the sarcoplasmic reticulum (SR).

Our hypothesis is that an increase in muscle PUFA from distillers grain causes an increase in PUFA in the sarcoplasmic reticulum membrane, the organelle that sequesters calcium within the muscle. A greater amount of PUFA in the SR membrane will contribute to oxidation susceptibility, thereby causing the early post-rigor release of

calcium. An early release of calcium will more quickly activate calpains and other calcium-dependent proteolytic enzymes to promote beef tenderization.

The SR is a very difficult organelle to isolate intact, however. The structure of the sleeve-like organelle makes it exceptionally difficult to isolate without damaging its inherent structure. Therefore, mitochondria were used as a model system for the SR. Mitochondria are organelles within muscle known to sequester calcium, although not to the extent of the SR. They are relatively easy to isolate intact and offer the opportunity to study calcium release from an organelle under carefully controlled and tightly defined conditions.

The objectives of this research were:

1) To determine the effects of feeding distillers grains plus solubles to cattle on fatty acid composition of the membranes of the sarcoplasmic reticulum and the mitochondria.
2) Evaluate the effects of membrane fatty acid composition (PUFA content) on calcium release.
3) Determine the influence of oxidation on calcium release.
4) Relate fatty acid composition of whole muscle tissue to fatty acid composition of the sarcoplasmic reticulum and mitochondrial membranes.

The long-term goal of this research is to improve the tenderness of United States beef by better understanding the biology of beef tenderization.

LITERATURE REVIEW

Sarcoplasmic reticulum and calcium in muscle contraction.

Basal levels of calcium (in the μM range) are maintained in the sarcoplasm for various cellular metabolic events (Gorlach et al., 2006). However, the majority of calcium (Ca) in the muscle cell is sequestered in the sarcoplasmic reticulum (SR). Wray and Burdyga (2010) state that the main feature of the SR is the high Ca concentration within its lumen at rest, and the potential for large releases and uptake of this ion upon a stimulus from the cell.

Calcium manages muscle contraction (Du and McCormick, 2009). In the event of a nervous stimulus instigating contraction, t-tubules stimulate the release of calcium from the SR through gap junction signaling. When calcium is released from the SR, the sarcoplasmic levels of Ca can reach up to ~10 mM. The main purpose of calcium in a muscle contraction is to bind to troponin C, one of the three complexes on the regulatory protein that works in conjunction with tropomyosin to cover or uncover actin binding sites. Once troponin C is bound, the whole molecule of troponin “hunches” and causes tropomyosin to swivel and uncover the actin binding sites. With the actin binding sites exposed, myosin heads are able to swivel and attach. Then contraction occurs. Contraction will continue until enough calcium is removed from the system, reaching a level below the threshold activation level of troponin C. In order to remove calcium from the system, the nervous stimulus must cease. Then, the SERCA (sarco-endoplasmic reticulum calcium ATPase) pumps calcium back into the SR, with the help of ATP (Du and McCormick, 2009).

After death, circumstances change. When an animal is exsanguinated, oxygen is depleted, and when oxygen is depleted, ATP production cannot occur for very long. Without enough ATP in the muscle system, onset of rigor occurs with almost 100% isometric contraction of actin and myosin (Greaser et al., 1969). The lack of ATP in the system ultimately prevents Ca from being pumped back into the SR, allowing seepage into the sarcoplasm through calcium release channels (Hidalgo et al., 2000), or by the degradation of SR membranes (Ji and Takahashi, 2006). With the hindered function of the calcium pump, calcium levels in the sarcoplasm soar as rigor develops (Jeacocke, 1993).

The free calcium in the sarcoplasm facilitates activation of proteolysis postmortem with an increase in meat tenderness (Koohmaraie, 1994).

Mitochondria in the muscle cell.

Mitochondria are organelles well known as the "power house of the cell," because of their active and ample production of ATP (adenosine triphosphate), the energy molecules necessary for cellular metabolism and transport. Cellular signaling and growth, along with apoptosis, lipid and amino acid metabolism and interconversion, urea cycle propagation, insulin regulation, glucose sensing, and steroid hormone production are all additional processes that are regulated by mitochondria (Brookes, et al. 2004, Peterson et al., 2012). Mitochondria reside primarily in two regions of the cell: directly below the sarcolemma and within the myofibrillar complex – a strategic placement that allows for maximal efficiency in responding to transport and other metabolic functions requiring

ATP, including muscle contraction (Du et al., 2009). Important to this research, mitochondria function as secondary calcium buffers in the cell.

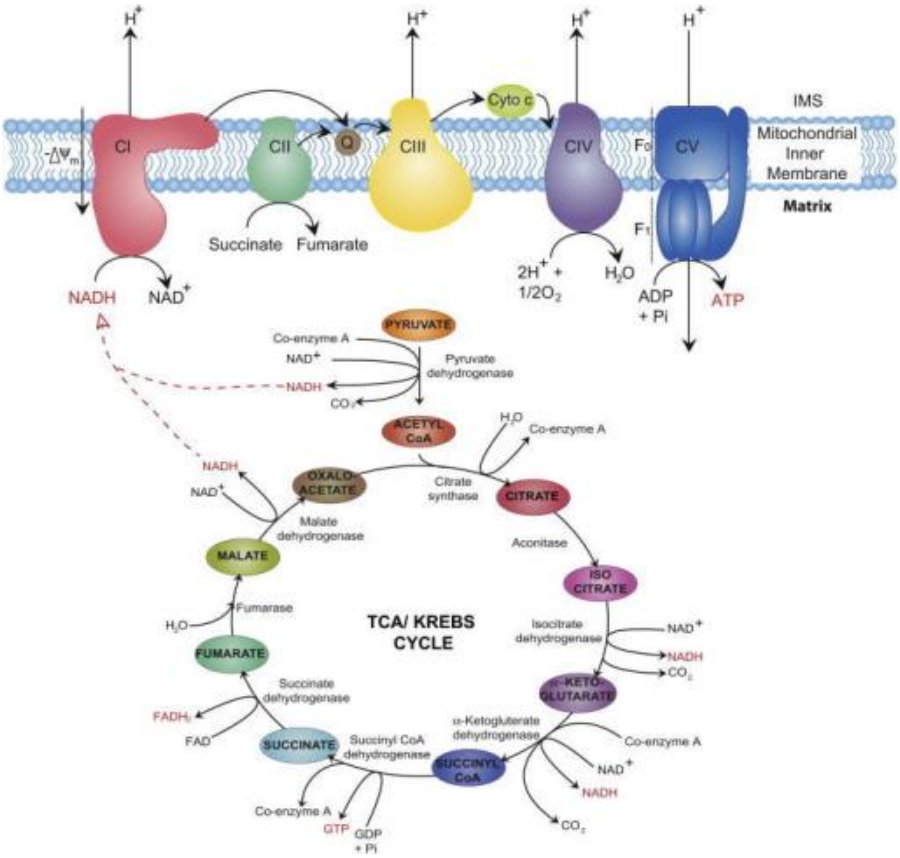
According to Alberts et al. (2002) and other researchers (Horvath and Daum, 2013; Monteiro et al., 2013; Mejia and Hatch, 2015; Schenkel and Bakovic, 2013; Subhashini and Jaimes, 2013), mitochondria have two plasma membranes: an inner mitochondrial membrane (IMM) and an outer mitochondrial membrane (OMM). The OMM is highly permeable and allows molecules up to 5000 Daltons to pass through it without much hindrance. Conversely, the IMM is highly impermeable, forms into cristae (a series of infoldings that increases surface area), and contains the electron transporting proteins involved in cellular respiration. The IMM and OMM create two sections of the mitochondria: the matrix (surrounded by the IMM) and the intermembrane space (positioned between the IMM and OMM).

The matrix serves as the buffer zone and can sequester or release different ions and molecules from and to the cytoplasm when needed to maintain membrane potential for the cellular respiration protein complexes on the IMM or to maintain homeostatic conditions within the cell. The intermembrane space acts as a bank with ions and molecules at the same concentration as the cytoplasm that await entry into the matrix, if permitted by the IMM (Alberts et al., 2002).

One other unique feature of mitochondria, again made evident by Alberts et al. (2002), is their ability to manufacture their own RNA (mitRNA), due to the matrix's capacity to house ribosomes. This is a direct result of their bacterial origin. However, as Sieber et al. (2011) noted, present day mitochondrial function depends mainly on the cell's nuclear genomic functions, as opposed to their own RNA coding; thus, the proteins

that are found in the mitochondria are imported mainly during biogenesis, but can be modified by mitochondrial RNA if cellular conditions call for that modification.

The primary function of the mitochondria is ATP synthesis via oxidative phosphorylation, also known as cellular respiration (Brookes et al. 2004, Rossi et al. 2009, Peterson et al. 2012). This process is controlled by the Tri-Carboxylic Acid (TCA) Cycle in the matrix, which produces a flux of reducing agents, NADH and $FADH_2$. These reducing agents then travel through the electron transporting system, which consists of the multipolypeptide complexes, I-V, located on the IMM (Celsi et al., 2009). Simply put, in the electron transporting system, electrons are liberated from the NADH/$FADH_2$ and delivered to O_2, forming H_2O, through a series of H^+ pumps. Moreover, the transport of H^+ via the pumps create a gradient of -180 mV across the IMM (Nelson and Cox, 2005). This gradient serves as the ultimate driver of the ATP synthase complex, the final step (complex V) in ATP production. Creatinine kinase activity along with pre-existing amounts of ADP and the reducing substrates will influence the rate of ATP production (Rossi, et al. 2009). Following, an image from Osellame et al. (2012) displays the TCA cycle and electron transport system working congruently to manufacture ATP in the mitochondria.



Mitochondria and calcium.

Mitochondria were the first organelles to be associated with calcium flux, according to (Rizzuto et al., 2012). It is well known that calcium is a physiological stimulus for ATP synthesis in the mitochondria (Williams et al., 2013; Rizzuto et al., 2012; Santo-Domingo and Demaurex, 2010; Contreras et al., 2010) Rate-limiting enzymes in the TCA cycle are controlled, in part, by the amount of Ca in the matrix. The rate-limiting enzymes include the pyruvate dehydrogenase complex, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase. Calcium functions as an allosteric activator of TCA enzymes (Nelson and Cox, 2005), which means that without Ca, the enzymes would not be able to transform into functional units capable of reducing their

substrates. These enzymes function in the direct manufacture of NADH, the reducing agent directly linked to ATP production. Additionally, Ca activates the ATP synthetic capability of the F_1F_0-ATPase (complex V of the electron transport chain), the key enzyme in the final step of ATP production. Muscle contraction requires a high level of ATP (Szent-Györgyi, 1975). Thus, in the muscle cell, Ca provides the means by which optimal NADH and ATP levels can be reached in the mitochondria for muscle contraction.

Mitochondria and sarcoplasmic reticulum relationship.

The mitochondria and sarcoplasmic reticulum (SR) are intricately connected in bidirectional communication via a mitochondrial associated matrix (MAM). Csordás and Hajnóczky (2009) say this is due to their abundant presence throughout the cytoplasm, as well as their central roles in cellular metabolism, contraction in muscle cells, and their influences on Ca dynamics in the cell. Franzini-Armstrong (2007) and Csordás et al. (2006) have documented gaps between the SR and mitochondria as small as 10 nm. Not only are these two organelles in close proximity to each other, they are physically tethered to one another by proteins that are constantly reorganized, permitting collaboration and interdependence between the two organelles (Boncompagni et al., 2009). This allows for a direct Ca communication as well as phospholipid biosynthesis, according to Rusinol et al. (1994). Berman et al. (2008) believe the interactions between the two organelles are regulated by the fusion and fission events of the mitochondria, and that directly correlates to Ca flux, because Ca influences the fusion and fission rate of the mitochondria. Fusion is the act of mitochondria elongating due to the fusing of two or

more mitochondria together. Fission occurs when mitochondria fragment. Fusion and fission regulate programmed cell death and apoptosis.

The SR functions as the main storage of calcium in the cell, and serves as the generation of rapid Ca release and uptake in the cell from its membrane proteins for contraction; while the mitochondria responds to local signals and functions as a buffer for Ca (Rossi et al., 2009). This collaboration helps meet energy demands during muscle activity and other metabolic activities.

Local releases of Ca from the SR only produce a small quantity of the ion over a very short time frame (about 10 ms), according to Rossi et al. (2009). Short releases in Ca are favored by the mitochondria to upkeep ATP production, thus, these localized Ca releases from the SR are directly related to the Ca content and capacity of the mitochondria (Rudolf et al., 2004).The mitochondria's speed of Ca diffusion and buffering is slower than the Ca release and uptake by the SR, thus the two organelles, via the MAM, create intracellular gradients and microdomains. These microdomains serve as localized, very small regions next to the Ca channels of the two organelles, and have Ca concentrations that exceed the concentration of Ca in the cytoplasm by several fold, which serve to help meet the low-affinity standards of proteins in the mitochondria (Franzini-Armstrong, 2007). Thus, it can be concluded that the intricate relationship between the SR and the mitochondria can create problems when reactive oxygen species (ROS) produced by the mitochondria are translated to the SR and alter the functions of the organelle's membrane or proteins. In fact, Rossi et al. (2009) suggested that alterations in SR-mitochondrial coupling and local inter-organelle signaling could lead to defects in activity-dependent ATP production and mechanisms of Ca release or removal.

The ATP produced by the mitochondria can be used by the ATPases located on the SR membrane. The SR manufactures many of the phospholipids that compose the IMM of the mitochondria. The direct tethering between these two organelles allows for the proximal flow of ATP and phospholipids between the organelles (Garcia-Perez et al., 2008).

Therefore, bi-directional SR-mitochondrial communication provides a powerful local control mechanism for integrating Ca release and uptake as well as ATP utilization during muscle contraction, and has the potential to impact muscle bioenergetics and postmortem activities.

Mitochondrial proteins involved in calcium release and uptake.

Calcium signaling, as a means of maintaining homeostasis, is a major contributing function of the mitochondria in the cell (Williams et al., 2013; Rizzuto et al., 2012). Mitochondria are more than Ca sinks that store Ca ions in their matrices. Instead, mitochondria exhibit a very dynamic, sophisticated approach to calcium release and uptake, acting as buffers to form an orchestrated chorus among its Ca transporters on the IMM and OMM with the environment of the cytosol in the cell and its concentrations of Ca. This orchestra induces a flux of calcium across the plasma membranes of the mitochondria as well as other organelles, such as the sarcoplasmic reticulum (Santo-Domingo and Demaurex, 2010).Thus, the mitochondria are key players in the shaping of cellular Ca signals. The main reason mitochondria are involved with calcium flux, other than Ca mediation for homeostatic conditions of the cell, is due to the requirement for Ca in ATP production, as will be discussed further in “The role of calcium in mitochondrial

function" section of this literature review. The balance of calcium storage and release is managed by a few proteins, of which the most significant are: the mitochondrial calcium uniporter (MCU), sodium-calcium exchanger (NCX), the calcium-hydrogen antiporter (Letm1 or HCX), and the permeable transition pore (PTP). An overload with Ca in the mitochondria, paired with an overload of ROS, has been reported by O'Rourke (2000) and Di Lisa and Bernardi (2009) to cause organelle lysis and cellular death. Thus, the role of these proteins in the flux of calcium into and out of the organelle are extremely crucial to the viability of cellular function.

Mitochondrial calcium uniporter. The MCU is the primary transporter involved in bringing Ca into the matrix of the mitochondria, and blocking the MCU will render the mitochondria essentially useless in the uptake of Ca into the organelle (Raffaello et al., 2012). According to Rizzuto et al. (2012), because of this primary function, the MCU is highly regarded as the functional unit by which mitochondria act as buffers for Ca in the cell, as the MCU allows the concentration of Ca concentration in the mitochondria to reach 10^6 times the concentration of Ca in the cytoplasm. This concentration of calcium is necessary for proper flux of the TCA cycle and the production of electrons for the purpose of ATP production. The MCU resides on the IMM and works off of the negative potential of the membrane that is created by the electron transport system described earlier (Patron et al., 2013). The MCU maintains a low affinity for calcium, though it has a high selectivity for the ion (Kirichok et al., 2004). This means that it takes a high amount of Ca concentrated in one area to induce the activity of the MCU in sequestering the ion into the matrix of the mitochondria.

Sodium-calcium exchanger and calcium-hydrogen antiporter. The NCX and HCX are responsible for Ca release from the mitochondria (Carafoli et al., 1974; Gunter et al., 1983). Both transporters use ion exchange as a means for expelling Ca from the organelle. The NCX will exchange one Ca for three Na+, while the HCX will exchange one Ca for one H+. In this manner, a congruent effort between the two transporters will maintain an ionic charge balance (Crompton et al., 1977). These transporters are essential to mitochondrial function, as Ca cannot easily diffuse across the IMM or OMM. Thus, the NCX and HCX serve to prevent over-accumulation of Ca in the matrix of the mitochondria, which would otherwise cause the organelle to rupture and undergo apoptosis (Malli and Graier, 2010). It was found by Jung et al. (1995) that the NCX has a reversible role of Ca and Na+ flux. It is believed that this is a large factor in cell death, especially with metastasis (Malli and Graier, 2010).

Permeable transition pore. The PTP is a secondary check to ensure that the matrix does not overload with Ca. Though this transition pore is poorly understood, it is believed that the most important trigger for PTP opening is Ca concentrations in the matrix of the mitochondria (Bernardi and Forte, 2007). The PTP functions mainly in the release of Ca from the matrix. Di Lisa et al. (2001) found that a prolonged opening of the PTP caused complete collapse of the IMM's membrane potential along with compete Ca discharge, leading to utter loss of mitochondrial function and cellular death.

Sarcoplasmic reticulum proteins.

Calcium release, uptake, storage in the SR is achieved through proteins that reside on the SR membrane. Calcium release is performed largely by type one ryanodine receptors (RyR1) and inositol triphosphate (IP3). Calcium uptake is attained via the sarco-endoplasmic reticulum calcium-ATPase (SERCA) pumps. The storage of Ca in the SR is maintained by luminal calcium-binding proteins (Rossi and Dirksen, 2006).

Luminal calcium-binding proteins. In serving to store Ca in the SR, luminal binding proteins maintain a low affinity, but high binding capacity to promote a large load of calcium in the SR, but simultaneously allow a quick release of the ion when a stimulus from the cell calls for it (MacLennan and Wong, 1971). Calsequestrin is the most abundant luminal calcium-binding protein in the SR (Costello et al., 1986). Calsequestrin is comprised of many acidic amino acids, which support the binding of calcium due to their negative charge. Calsequestrin also is tethered to the SR membrane through interactions with the RYR1. This allows for relaying of information in Ca release (Zhang et al., 1997).

Type one ryanodine receptors and inositol triphosphate. The type one ryanodine receptor is the primary protein involved in release of Ca from the SR, and is located in close proximity to the t-tubules (Fill and Copello, 2002). When an action potential passes down the t-tubule, the dihydropyridine receptors (DHPR) on the t-tubule change conformation, initiating the activation of the RYR1. In this manner, the DHPR acts as a voltage sensor for the RYR1, and the DHPR and RYR1 interaction is commonly referred to as excitation-contraction coupling (Rossi et al., 2009).

The type one ryanodine receptor is regulated by the amount of calcium outside and inside the SR. Low levels (μM) of Ca in the sarcoplasm will initiate Ca release, while high levels (mM range) of Ca in the sarcoplasm will inhibit Ca release (Copello et al., 2007). Calsequestrin, in its tethering to RYR1, can also affect the activity if RYR1, causing an upregulation of RYR1 when high levels of luminal calcium cause a weakened effect on the binding capacity of calsequestrin (Gyorke et al., 2004). The type one ryanodine receptor has low selectivity, but high conductivity, which allows for a high passage rate of Ca into the sarcoplasm when needed, but also permits the interfering of the magnesium ion (Fill and Copello, 2002). The RYR1 can be affected by altered sulfur groups in its tertiary structure (due to ROS) that cause it to remain in the open state, thus releasing a large amount of calcium (Abramson and Salama, 1988; Abramson and Salama, 1989).

Inositol triphosphate protein is the other important calcium release mechanism on the SR. Inositol triphosphate receptors aid in sustained Ca elevations in the sarcoplasm and are activated by Ca levels and the presence of IP3. Inositol triphosphate is made by the hydrolysis of PI by phospholipase C (Michell, 1975). Phospholipase C can be activated by many other proteins, which provides a connection between IP3 and DNA material in the cell (Kasri et al., 2004). MacMillan et al. (2005) report that IP3 receptors participate in a calcium-induced-calcium-release, positive feedback mechanism, where high Ca levels in the sarcoplasm initiate a large production of IP3 via PI hydrolysis, thus increasing the rate of IP3 receptor Ca release.

Sarco-endoplasmic reticulum ATP-ase. Calcium uptake into the SR occurs primarily via SERCA, the ATP-dependent transporter on the SR membrane. Sarcoplasmic-endoplasmic reticulum ATP-ase requires phosphorylation to support calcium uptake, thus the requirement for ATP (Rossi and Dirksen, 2006). There are two main structures that make up SERCA: a headpiece that manages calcium binding and release and a transmembrane piece that binds to phosphatidylethanolamine (PE) when not bound to Ca (Toyoshima and Inesi, 2004). Giroud et al. (2013) showed that SERCA is sensitive to the phospholipid composition of the membrane it is imbedded in. Additionally, Hunter et al. (1999) demonstrated that an increase in PE make up in the SR membrane will increase the activity and effectiveness of SERCA in Ca sequestration. Sarcoplasmic-endoplasmic reticulum ATP-ase is inhibited by sarcolipin (a regulatory protein), nitric oxide (NO) a possible ROS, and luminal calcium binding proteins (MacLennan et al., 1973; Ishii et al., 1998; MacLennan and Wong, 1971).

General membrane structure.

Membranes consist of a phospholipid bi-layer with hydrophilic heads facing outward to aqueous solutions, while hydrophobic tails (fatty acid chains) occupy the interior (Harder et al., 1998). This structure makes membranes impermeable to water-soluble molecules, including ions such as Ca. This phenomenon has been described as the Fluid Mosaic Model of the cell, as defined by Singer and Nicolson (1972). Fluidity, also known as the lateral movement of membrane constituents, is maintained by cholesterol and the presence of unsaturated fatty acids (Stanley, 1991). Fatty acids that are unsaturated have one or more double bonds that make the tail crimp in such a way as to

prevent close packing, thus increasing fluidity of the membrane. Conversely, fatty acids that are saturated contain no double bonds and decrease fluidity as they are more likely to tightly pack together (Cooper, 2000). Cholesterol acts as a mitigating agent for fluidity. Cholesterol can increase the fluidity of a membrane by interfering with the packing interactions of fatty acids. Cholesterol can also decrease fluidity by fitting into gaps created by the crimps in unsaturated fatty acid tails and securing them (Presti, 1985).

Spector and Yorek (1985) state that membrane fluidity controls membrane function and is dependent, in part, on phospholipid composition. The types of phospholipids vary, depending on the function of the membrane. Five major phospholipids appear in cell membranes. Those are: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SP) (Cooper, 2000). Phosphatidylethanolamine, PS, and PI primarily exist in the inner layer of the membrane, facing the inside of the cell or organelle, while PC and SP make up the majority of the outer layer of the membrane, facing outside the cell or towards the cytoplasm (Alberts et al., 2002). Since many of the functions of the membrane change under various conditions, including metabolic stress, it has been suggested by Fagone and Jackowski (2009) and others that the exact phospholipid composition of the membrane is an important parameter in determining membrane function.

In addition to being comprised of phospholipids, membranes also house proteins involved in transport of molecules across its barrier along with other functions, such as signaling and secretions (Stanley, 1991). Two types of proteins exist in the membrane, integral and peripheral, according to Yeagle (1987). Integral proteins are anchored

securely in the membrane, whereas peripheral proteins are found in association with other proteins, indirectly related to the membrane. A special type of integral protein is the transmembrane protein, which spans both the inner and outer layer of the membrane, as opposed to some integral membranes that only span one of the layers. Integral proteins function mainly in transport of molecules, while peripheral proteins function as enzymes or catalysts for various needs of a cell or organelle (Von Heijne, 1992).

Mitochondrial membrane composition.

Though often depicted as stiff, oval-shaped organelles, mitochondria are actually very dynamic in their shape, often undergoing multiple fusions and fissions with other mitochondria to better facilitate the demands of cellular metabolism (Berman et al., 2008). Fusion and fission are directly correlated to the membrane fluidity and phospholipid composition of the IMM and OMM. Both the IMM and the OMM are double-layer membranes, similar to other membranes, and act to serve as barriers between two aqueous compartments as well as possessing the ability to selectively transport molecules (Schenkel and Bokavic, 2014). Daum and Vance (1997) stated that the OMM is comprised primarily of PC (about 54 percent), PE (about 29 percent), and PI (about 14 percent). The remaining phospholipids make up a very small fraction (about three percent) of the total OMM. The IMM has a much larger surface area, due to its many cristae. The IMM also has many more proteins that work to carry out cellular respiration, including calcium transporters, so the phospholipid to protein ratio is much lower (Mejia and Hatch, 2015). In terms of phospholipid composition, the IMM varies from the OMM. Phosphatidylethanolamine and PC together make up approximately 75

percent, with a lower PI percentage. However, the main difference between the IMM and OMM is the presence of cardiolipin (CL) phospholipid in the IMM. Cardiolipin makes up about 15 percent of the total phospholipids that make up the IMM. Cardiolipin is only found in the IMM, very rarely anywhere else in the cell (Houtkooper and Vaz, 2008). Phospholipids of the mitochondria are mainly manufactured in the sarcoplasmic reticulum (Fagone and Jackowski, 2009).

Cardiolipin. CL is primarily found in the IMM, with very trace amounts, being noted in the OMM by Daum and Vance (1997) and Hovius et al. (1993). Simbeni et al. (1991) and Ardail et al. (1990) have found that contact sites between the IMM and OMM are rich in CL, too. Unlike the other phospholipids, CL is unique because its structure is composed of three glycerol groups, two phosphate moieties, and four acyl chains (Cheng and Hatch, 1995), thus acting as a "double phospholipid". The CL exists in a conical shape that favors hexagonal phase formation (van den Brink-van der Laan et al., 2004). This structure and shape allow for curving of the membrane, which is an important feature in the IMM for its cristae and increased surface area. Hence, there is strong evidence that CL is a pivotal player in the formation and sustaining of cristae, and would answer the question why CL is found almost solely in the IMM as opposed to other membranes (Acehan et al., 2007). The biosynthesis of CL occurs on the matrix side of the IMM, via the cytidine-5'-diphosphate-1,2-diacylglycerol pathway, which utilizes the precursors phosphatidic acid and phosphatidylglycerol (Mejia and Hatch, 2015).

Cardiolipin has been found to play an important role in protein import and assembly, as well as fusion and fission, and apoptosis (Kubli and Gustafsson, 2012).

Cardiolipin helps maintain membrane potential by anchoring the proteins involved in oxidative phosphorylation to the IMM, as well as serving to provide support for other essential proteins involved in IMM functions. Remodeling of CL occurs to maintain membrane function, and is carried out by tafazzin, a transacylase (Schenkel and Bakovic, 2014). Houttkooper and Vaz (2008) state that several mitochondrial enzymes have been shown to require CL and CL post-remodeling for optimal activity. Many diseases, as identified by Gasparre et al. (2013), have been associated with the decrease in CL in the IMM. Additionally, Dudek et al. (2013) demonstrated that diminished levels of CL altered supercomplex formation of the electron transport system, destabilizing complexes III and IV, while increasing ROS production.

Phosphatidylcholine. Phosphatidylcholine's structure consists of a choline head group attached to a glycerol-3-phophate molecule which contains two saturated or unsaturated fatty acid groups (van Meer et al., 2008). The cylindrical shape of PC along with the nature of its fatty acid groups, allow PC to remain fluid at room temperature, thus maintaining membrane fluidity. Therefore, PC is key in maintaining a membrane's structural integrity. Phosphatidylcholine is imported into the mitochondria after being synthesized in the sarcoplasmic reticulum, via the Kennedy pathway (Kennedy and Weiss, 1965). Phosphatidylcholine is also thought to be a precursor to CL (Mejia and Hatch, 2015).

Phosphatidylethanolamine. Phosphatidylethanolamine's structure consists of a glycerol-3-phophate backbone that contains an ethanolamine head and two saturated or

unsaturated fatty acid tails (Daum and Vance, 1997). According to, Osman et al. (2011), the conical shape of PE allows it to organize itself into hexagonal phases, which introduces tension in the bilayer that could serve as the source of protein movement across membranes as well as fusion and fission. For this reason, there is more PE in the IMM than in the OMM. The lack of PE in mitochondrial membranes has proven to create fragmented and misshapen mitochondria that lack substantial function for the cell (Steenbergen et al, 2005). Like CL, a decrease in PE will result in a decreased respiratory capacity and ATP production from the mitochondria, as PE is a vital role-player in maintaining the mitochondrial membrane potential at -180 mV (Martin et al., 1991). PE is manufactured in the mitochondria via the decarboxylation of PS, and via the Kennedy pathway in the SR as well (Schuki and Daum, 2009; Kennedy and Weiss, 1965).

Phosphatidylinositol. Phosphatidylinositol is not as well understood as the previously described phospholipids. The structure of PI is that of a glycerol-3-phosphate backbone, with a head consisting of a hexahydroxycyclohexane (inositol) ring, and two saturated or unsaturated fatty acid tails (van Meer et al., 2008). Daum and Vance (1997) report that PI is found in a larger abundance in the OMM (at 10 percent) versus the IMM (at 5 percent). In 1999, Tolias and Cantley data found that PI serves as a precursor for phosphoinositides, which are molecules that are involved in actin rearrangement and calcium regulation across membranes. This gives credence to its important function in the mitochondrial calcium flux systems, which are further described in the “Mitochondrial proteins” section of this literature review. Phosphatidylinositol, like PC, is synthesized in the sarcoplasmic reticulum via PI-synthase activity (Paulus and Kennedy, 1960).

Sarcoplasmic reticulum membrane.

The SR membrane, much like the mitochondrial membrane, is made up of PE, PC, PS, SP, and PI. However, the SR does not contain CL. Also, the SR does not contain two membranes, only one. It is difficult to characterize the percent composition of each phospholipid in the SR, as extracting this organelle without compromising its structural integrity is difficult. However, percent compositions of SR membrane phospholipids have been compared according to diet.

Role of diet on sarcoplasmic reticulum and mitochondria phospholipids.

It is known that diet can affect the phospholipid composition of membranes (Küllenberg et al., 2012). Gould et al. (1987) and Mlekusch et al. (1993) demonstrated that high PUFA content in diets can upregulate the production of PC while down regulating the production of PE in the SR membrane, which correlated to higher Ca concentrations found in the sarcoplasm, as Beare and Kates (1964) found that PC can incorporate linoleic fatty acids better than PE can. This is in large part due to the association of PE and SERCA stability, as well. When PE is replaced by PC, the stability of SERCA is thwarted, and thus Ca is not as readily pumped back into the SR (Hunter et al., 1999).

Monteiro et al. (2013) showed that fatty acid composition of the diet can alter the acyl chain remodeling systems of mitochondrial phospholipids. In their study, mice were fed an oil-rich diet that caused both the fatty acids and phospholipid proportions to change, simultaneously affecting the bioenergetics of the mitochondria. In another study,

mitochondria had higher respiratory capacity with a diet rich in polyunsaturated fatty acids when fed to trout (Guderley et al., 2008). Specifically, the composition of CL is greatly affected by the amount and type of dietary fat. For example, CL is greatly decreased in rat heart muscle when no linoleic acid, an essential fatty acid, is fed (Schenkel and Bakovic, 2014). These effects are reversed when linoleic acid is resupplied in the diet. Additionally, it is believed that increasing the amount of polyunsaturated fatty acids (PUFAs) in the diet will increase the metabolic rate of the mitochondria (Pan and Storlein, 1993). Pehowich (1999) showed in his study that rats with an increased n-3 PUFA supplement had a lower incidence of proton leakage from the respiratory chain, with less ROS produced, as there were higher amounts of this n-3 PUFA attached to CL, PE, and PC. Therefore, one can conclude that increasing the amount of PUFA in a diet can lead to an altered mitochondrial capacity and function.

Calcium in myofibrillar degradation and meat tenderization.

Maltin et al. (2003) and many others demonstrated that tenderness is an imperative attribute to considering when deeming meat quality, according to consumers. Tenderness is, in large part, the result of myofibrillar and proteolytic degradation post-mortem via aging by endogenous enzymatic activity (Du and McCormick, 2009; Aberle et al., 1975). Soon after an animal is harvested, proteolytic degradation begins. This is due, in part, to a potential early postmortem release of calcium at a higher temperature and pH that activates proteolytic enzymes, such as calpains and calpastatins (Dransfield, 1994; Geesink et al., 2000). Calcium has a central role in this enzymatic activity,

especially in consideration of the calpain and calpastatin proteinase system, as it is required for the activation of these enzymes.

The calpain system has been studied in relation to tenderness quite extensively and has been attributed a great responsibility for meat tenderness (Koohmarie, 1994; Taylor and Koohmarie, 1998). There are two major isoforms of calpains: μ-calpain and m-calpain. Both isoforms contain a 28 kD and 80 kD subunit to their structure (Suzuki, 1990). Hosfield et al. (1999) suggests that because the 80 kD subunit contains four domains that function to bind calcium or in catalysis, the conformational states of both calpain isoforms could have a severe impact on their activity. Additionally, a calcium-binding domain on the 80 kD subunit helps in the binding of PI phospholipid (Tompa et al., 2001), suggesting that calpains could be activated when PI is oxidized and released from the membrane.

Boehm et al. (1998) demonstrated that only 10 percent of calpain activity remained in meat after 24 hours postmortem at 5°C with a pH of 5.5. At the same time, though, Koohmarie et al. (1986) showed that calpains kept about 25 percent of their activity at a pH of about 5.6 at five degrees Celsius and could still be detected at 56 days postmortem. Thus, one can conclude that there is a significant amount of calpain activity postmortem that can be attributed to tenderness.

Mu calpain requires between 5-65 μM calcium to elicit half-maximal activity, while m-calpain needs between 300-1000 μM for half-maximal activity (Goll et al., 1992). Thus, the higher the concentration of calcium available post-mortem, the greater achievement in the activation of calpains. The amount of free calcium in muscle

postmortem ranges, but Parrish et al. (1981) found that at 10 to 14 days postmortem, beef longissimus contained 638-970 μM of free calcium.

The main targets of postmortem calpain proteolysis are ultrastructural and costameric proteins near the z-disc of the sarcomere (Du and McCormick, 2009; Goll et al., 1991; Taylor et al., 1995). These proteins aid in the integrity of the sarcomere structure, serving to bolster the contractile proteins, especially actin and myosin. Titin, nebulin, and desmin are among these proteins and have been found to be directly correlated to tenderness and myofibrillar fragmentation when degraded (Taylor et al., 1995).

Calpastatin is the negative, endogenous inhibitor of calpains. As Huff Lonergan et al. (2010) demonstrated, though, it also requires Ca to bind to calpains, and has four domains, each of which can inhibit a calpain molecule. High amounts of calpastatin in muscle leads to a decreased tenderization effect, as demonstrated by Kent et al. (2004). The amount of calcium required to activate calpastatin activity is lower than the calcium required to activate calpains. Thus, calpastatin will bind to inactive calpains and prevent their proteolytic effects early postmortem. As Miller et al. (2011) observed, calpastatin will degrade over time, allowing the activation of calpains with increased free calcium concentration. Therefore, a tenderization effect still occurs by means of calpain-induced proteolytic degradation (Wendt et al., 2004).

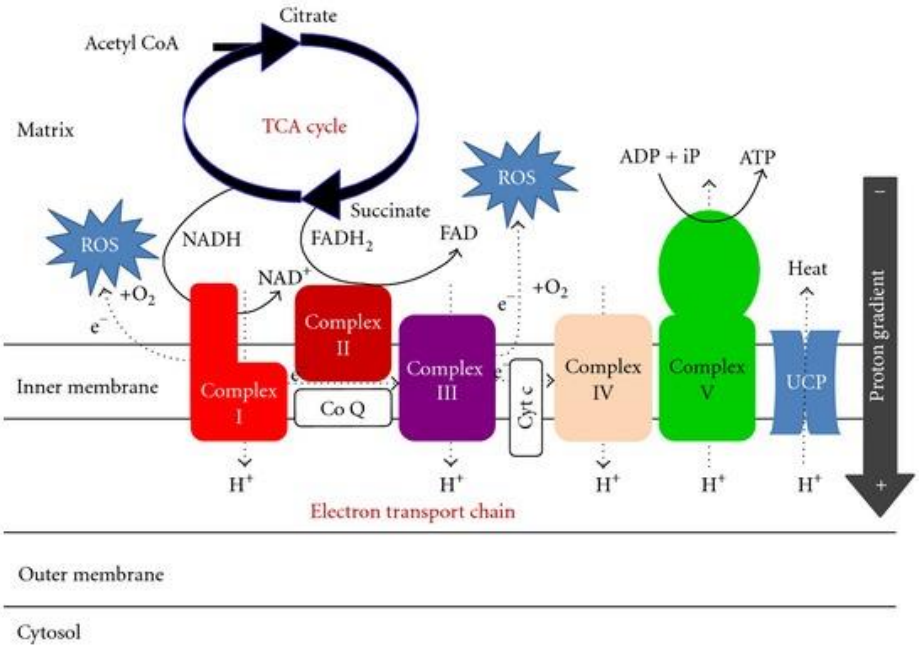
Reactive Oxygen Species.

Reactive oxygen species (ROS) are very reactive chemical compounds that contain oxygen. Some of the most recognized ROS are: superoxide (O_2^-), hydrogen

peroxide (H_2O_2), and hydroxyl radicals (OH^-). Reactive oxygen species are found in normal metabolic processes, and are maintained by endogenous enzymes at basal levels. Some of these endogenous enzymes are: superoxide dismutase which reduces O_2^- to H_2O_2, catalase which reduces H_2O_2 to H_2O and O_2, and glutathione reductase which reduces a disulfide complex to glutathione (Storz, 2007). It is believed that ROS are produced in the electron transport chain (ETC) of the mitochondria in the production of ATP. Electron leakage from the four complexes of the ETC react with molecular oxygen in addition to other reduced compounds containing oxygen, resulting in radical reactive oxygen species (Subhashini and Jaimes, 2013). Reactive oxygen species are extremely reactive with any substrate available in the cell, including proteins, lipids, and DNA (Alfadda and Sallam, 2012). Reactions with these substrates can induce cellular damage and rupturing of membranes.

Treberg et al. (2015) discussed that ROS production is a constant in healthy cells and is thought to help maintain membrane gradients. Mitochondria play a crucial role in maintaining proper balance of cellular redox potential, by managing normal ROS production versus excessive ROS production. Loss of mitochondrial function due to stress that is placed on the cell, causes a massive production of ROS (Subhashini and Jaimes, 2013).

The following diagram from Shinmura (2013) demonstrates the production of ROS from mitochondria ETC:



Oxidation of membrane lipids.

Oxidation refers to the production of ROS that can eventually alter the composition, and thus function, of lipids and proteins. Biological components, such as lipid membranes and proteins are very susceptible to oxidation.

The process of oxidation has been widely described in three main steps: initiation, propagation, and termination. The initiation phase refers to the formation of the free radicals, while the propagation phase describes the reactions that occur when these radicals interact with a lipid or protein (chemical bond of some sort), and termination refers to the formation of a non-radical product to cease the chain of events (Lund et al., 2011).

Reactive oxygen species can be produced for many reasons, including immune responses or stresses that are placed on the cell (Subhashini and Jaimes, 2013). How ROS are produced has been addressed in the “reactive oxygen species in mitochondria” section

of this literature review. Living muscle has the ability to mitigate negative effects associated with ROS production, including oxidation of proteins or lipids, as there are many mechanisms that produce checks and balances on the system (Santos et. al., 2009). Reactive oxygen species scavengers, better known as antioxidants, are examples of molecules that help mitigate ROS activity. However, postmortem, ROS endogenous prevention mechanisms breakdown, thus allowing ROS to accumulate and cause rapid oxidation of membrane phospholipids and proteins (Esposti, 2002). The presence of PUFA in a membrane structure predisposes that membrane to a higher risk for oxidation, just by the nature and presence of double bonds and their high reactivity with ROS. Machlin and Bendich (1987) demonstrated that lipid oxidation occurs at an accelerated rate in cell membranes containing a high amount of PUFA.

Oxidation of membranes leads to a whole slew of consequences for the integrity and function of that membrane. Some of the major consequences of oxidation on membranes include: increased permeability, decreased fluidity, deactivation of enzymes and proteins that are bound to the membrane, and cross-linking of proteins on the membrane (Stark, 2005). These consequences of oxidation can cause the stability of the membrane to decrease, thus allowing all of the contents bound by the membrane to be released. In the case of the sarcoplasmic reticulum and mitochondria, an oxidized membrane could possibly lead to a spillage of large quantities of calcium (Senaratne, 2012; Chao, 2015). Moreover, increased oxidation of lipids produces rancidity, and (Koger et al., 2010) demonstrated that PUFA content of phospholipids was positively correlated to rancidity and decreased consumer appeal in meat consumption. In this way, one sees both the benefits and risks of higher PUFA content in the membrane.

Effects of oxidation on calcium flux.

Oxidation indirectly affects Ca flux, whether through membrane instability or protein alteration (Hidalgo and Donoso, 2008). Between the mitochondria and the sarcoplasmic reticulum, considering the MAM and all of the communication that is required between the organelles for proper functioning and response to cellular signals and calcium levels, the effects of lipid oxidation and uncontrollable ROS production can develop into a vicious cycle of cell necrosis (Droge, 2002).

Specific ROS effectors between the sarcoplasmic reticulum and the mitochondria can affect the Ca homeostasis between these organelles. Isaeva et al. (2005) discovered that oxidative insult from the H_2O_2 ROS molecule accelerated the onset and frequency of Ca releases from the sarcoplasmic reticulum. It was reasoned that this occurred due to membrane and IP3 receptor instability. Likewise, Byrd (1992) found that Ca release was prolonged after fatiguing muscle activity, a process that produces many ROS. Another study by Hidalgo et al. (2005) showed that Ca release activity is increased under conditions that overwhelm the local redox control mechanism by the mitochondria and promote RYR1 oxidation.

More specific studies on the proteins of the sarcoplasmic reticulum and the mitochondria by Sharov et al. (2006) show that because SERCA has many cysteine amino acid residues, it is of greater risk for oxidative damage. The most common molecular targets for ROS are the cysteine thiol groups in proteins. These thiol groups will react with ROS and undergo multiple alterations that severely impact the function of the protein (Hidalgo and Donoso, 2008; Bishop et al., 1988). In the SERCA it has been

discovered that extensive exposure to massive quantities of ROS will inhibit the SERCA pump, causing a Ca depletion-induced stress on the sarcoplasmic reticulum (Li and Camacho, 2004). Postmortem, this serves as a prevention of calcium flux back into the sarcoplasmic reticulum and allows a great deal of calcium to be free floating in the sarcoplasm.

Additionally, RYR1 and IP3 contain cysteine residues in their tertiary structures, and the regulatory proteins that interact with these main calcium releasing proteins also have cysteine amino acid residues (Foskett et al., 2007; Meissner, 2002). Thiol oxidation of RYR1, as Hamilton and Reid (2000) state, generally increases channel activity by enhancing intersubunit binding and preventing the binding of the negative regulator, calmodulin, to the receptor. Inositol triphosphate receptors, when oxidized at their thiols, will become more sensitized to activation by IP3 (Joseph et al., 2006). Additionally, Bootman et al. (1992) found that thiol-oxidizing agents alone were able to elicit a spontaneous calcium release.

All of this oxidation is believed to be caused by mitochondrial production of ROS that is stimulated in part by the sarcoplasmic reticulum's calcium release quantity and frequency (Csordás and Hajnóczky, 2010). This mechanism and cycling of calcium can lead to increased ROS production from the mitochondria, meaning increased calcium release from the sarcoplasmic reticulum, which causes a higher uptake of calcium into the mitochondria, which then produces more ROS to counter act the activity, and so forth. In this manner, one can see how the cycle of events between ROS production in the MAM can serve as a detrimental whirlpool of necrosis and eventual apoptosis (Alfadda and Sallam, 2012).

The mitochondrial targets of ROS are the reducing agents along the electron transporting system. When less reducing agents exist in the chain of events, there is a progressive decrease in the membrane potential of the IMM, which will eventually lead to the acidification of the matrix contents as well as the loss of all function on the membrane and the activation of the PTP that will cause apoptosis (Csordás and Hajnóczky, 2010). Redox regulation of the MCU, HCX, or NCX is very limited. However, Bernardi et al. (2006) commented that there is evidence showing the PTP is activated by high ROS production and decreased membrane potential on the IMM and OMM. Once the PTP is opened and activated, there is high potential for apoptotic factors to excrete from the mitochondria and induce cellular death. Interestingly, Zorov et al. (2000) demonstrated that these ROS-induced activations of the PTP were dependent upon the calcium stored in the sarcoplasmic reticulum, and that local delivery of calcium to the mitochondria from the sarcoplasmic reticulum caused the persistence of the effects. Free fatty acids produced from oxidation events have also been shown to disrupt the membrane potential on the IMM and cause the opening of the PTP (Di Paola and Lorusso, 2006). Moreover, Martins et al. (2008) similarly reported an increase in calcium release where ROS was being generated from the mitochondria.

Player and Hultin (1978) investigated the effect of lipid peroxidation on the calcium flux in sarcoplasmic reticulum microsomes. It was found in this study that when introducing pro-oxidant effects via the Fenton reaction and ascorbic acid, the release of calcium from the microsomes was increased two-fold. Whether this can be replicated in mitochondria is not known. Still, it is easier to extract mitochondria in one piece as

opposed to SR. Therefore, it would be easier, and probably more reliable to measure oxidation and calcium flux in isolated mitochondria.

Distillers grains and the link between calcium flux and tenderness.

Distillers grains are a by-products of ethanol production. As Stock et al. (2000) describe; in the process of making ethanol, corn is distilled by allowing it to ferment with yeast to produce alcohol and then spun down to remove the remaining solids. The leftover solids are left to dry and the dried product is what is known as dried distillers grains. To produce dried distillers grains plus solubles (DDGS), a solubles fraction is also dried with the solids. To produce wet distillers grains plus solubles (WDGS), liquid solubles are added to the dried solids part. Klopfenstein et al. (2007) has shown that compared to corn, distillers grains contain higher levels of protein, fat, and fiber. Therefore, distillers grains are a valuable by-product used to feed cattle.

It has been reported by Senaratne (2012) that steaks from cattle fed a high wet distillers grain plus solubles (WDGS) diet had increased tenderness and higher calcium levels compared to cattle fed corn. Wet distillers grains are known to increase PUFA content, linoleic, and trans fatty acids while decreasing monounsaturated fatty acids (de Mello et al., 2012). Furthermore, Chao (2015) demonstrated that cattle fed WDGS exhibited a higher proteolytic degradation, greater calcium content in the sarcoplasm, and altering phospholipid content in the sarcoplasmic reticulum.

All of this data suggests that distillers grains have the ability to alter organellar membranes, specifically the SR, and therefore have the potential to impact the integrity of other organellar membranes, such as the mitochondria. With a higher PUFA content in

the membranes of the SR and mitochondria, we can hypothesize that these membranes will be more susceptible to oxidation postmortem and thus have a higher potential to release Ca into the sarcoplasm. Increasing Ca in the sarcoplasm will improve the chances for calpain activation, and, therefore, a greater amount of proteolytic degradation to promote tenderness. In this manner, we can seek to explain the basic mechanisms for tenderness between diets.

Conclusions.

This literature review supports the hypothesis that feeding cattle DDGS will increase PUFA composition of the sarcoplasmic reticulum and mitochondria membranes, increase the calcium flux and ROS production of the mitochondria, and result in higher proteolytic degradation, thus more tender meat. The exploration of this hypothesis will allow us to use the mitochondria as a model system for the sarcoplasmic reticulum, because the SR is very difficult to extract in one full piece.

Researching this hypothesis will prove valuable to the beef industry. If there is a link between these organelles and oxidation of membranes within the cell that promote tenderness, it could be possible to manipulate these factors to enhance tenderness, especially through finishing diets in the feedlot. Additionally, in dealing with the mitochondria, there is high potential to relate these factors genetically and select for cattle that have a greater oxidation potential and produce a more tender product without altering diet. There is still much to explore in this area.

MATERIALS AND METHODS

Animals. A total of 128 British-cross steers were used in a randomized complete block design. Steers were fed at University of Nebraska feedlot (Mead, NE). All animal use protocols were approved by the University of Nebraska-Lincoln's Animal Care and Use Committee (902). Steers were blocked by BW, stratified by BW within each block, and assigned randomly to pens. Steers received an initial implant with Ralgro® followed by a Revalor®200 implant. All diets were formulated on a dry matter basis using high-moisture corn (31.5%), alfalfa hay (5.5%), corn silage (4%), molasses (5%), and supplement (5%) containing Rumensin® (30g/ton) and Tylan® (90mg/steer/day). Steers were fed for 156 days. Feeding treatments were 50% dry-rolled corn or 50% de-oiled dry distillers grains plus solubles (DDGS; DM basis). Pens were randomly assigned to 1 of the 2 treatments with 8 steers per pen.

Sample collection and preparation. All steers were harvested at a commercial abattoir (Greater Omaha Pack, Omaha NE). After 48 h postmortem chilling, 48 out of 128 carcasses were selected (24 from each treatment, 4 from each pen). Carcass selection was based on quality grade. At the time of grading, any USDA Choice carcasses were selected until reaching four carcasses for each pen. Strip loins (*Longissimus lumborum*) from all selected carcasses were collected, vacuum-packaged, and transported to Loeffel meat laboratory at the University of Nebraska-Lincoln.

Strip loins from the left sides of the carcasses were fabricated on postmortem aging days 2 and 8. Strip loins from the right side of the carcasses were fabricated on postmortem aging days 14 and 21. Each strip loin was fabricated into 2 tenderness

samples (2.54cm) and 4 lab samples (1.27cm) for each aging period, from anterior to posterior ends. The remainder of the strip loins were immediately vacuum packaged in vacuum pouches (3 mm STD barrier, Prime Sources, St. Louis, MO) on a Multivac Packaging machine (Multivac C500, Multivac, Kansas City, MO) and aged to the next designated aging period.

Upon fabrication, steaks were packaged in Styrofoam trays (Styro-Tech, Denver, CO), overwrapped with oxygen permeable polyvinyl chloride film (PSM18, Prime Source, St. Louis, MO), and were subjected to retail display conditions (2 $\pm$ 2^{o}C, and exposed to continuous 1,000-1,800 lux warm white fluorescent lighting) for 0, 4, and 7 days.

Twelve samples were randomly sub-sampled from each diet group for all aging periods at 0 days of retail display (24 total, 2 from each pen). Sarcoplasmic reticulum membrane fatty acid and phospholipid as well as mitochondrial membrane fatty acid and phospholipid samples were obtained on day 0 of retail display for aging periods 2, 8, and 14 days. Mitochondrial artificial oxidation samples were obtained at day 0 of retail display for aging periods 2 and 8. All lab samples and were vacuum packaged and frozen at -80^{o}C until analysis. The lab samples were pulverized in liquid nitrogen with a blender (model 51BL32, Waring Commercial, Torring, CT).

Sarcoplasmic reticulum (SR) membrane extraction (Appendix I). The SR membrane was extracted according to the procedure described by Hemmings (2001) with modifications. Ten grams of powdered samples were suspended in 35 mL of 4^{o}C homogenization buffer (10 mM $NaHCO_3$, 2 mM NaN_3, 10 mM Tris-Cl , pH 7.5) and

homogenized using a Polytron homogenizer (Kinematica) at setting 6 for 15 sec, setting 7 for 15 sec, and setting 8 for 15 sec. Homogenate was filtered through cheesecloth and transferred into a 50 mL plastic centrifuge tube and centrifuged at 2,000 x *g* for 10 min at 4^{o}C. The supernatant was collected and centrifuged at 10,000 x *g* (Sorvall Ultracentrifuge, F37L-8x100, Thermo Scientific, 096-087056, Rockford, IL) for 30 min at 4^{o}C. Four and a half percent of KCl (w/v) was added to the supernatant and stirred for 30 min on ice. The supernatant was centrifuged at 100,000 x g for 60 min at 4^{o}C. The final supernatant was discarded, and the pellet was resuspended in 1 mL of 10mM tris buffer and stored at -80^{o}C until use.

Mitochondria Extraction (Appendix II). Mitochondria were extracted according to the procedure described by Smith (1967). Ten grams of powdered sample was suspended in 50 mL 4^{o}C maintenance buffer (70 mM sucrose, 200 mM mannitol, 5 mM HEPES, and 10 mM K_2PO_4, pH 7.5) and homogenized using a Polytron homogenizer (Kinematica, model CH-6010, Bohemia, NY) at setting 5 for two, 10 sec intervals. Homogenate was filtered through cheesecloth and transferred into a 50 mL plastic centrifuge tube and centrifuged at 650 x g for 10 min at 4^{o}C. The supernatant was collected and centrifuged at 7,750 x g (Sorvall Ultracentrifuge, F37L-8x100, Thermo Scientific, 096-087056, Rockford, IL) for 25 min at 4^{o}C. Multiples of the same sample were extracted to be used in each analysis. The final supernatant was discarded, and the pellet was resuspended in 1 mL maintenance buffer and stored at -80^{o}C until use. Multiples of the same sample were extracted to be used in each analysis.

SR and Mitochondria membrane lipid extraction (Appendix III). Membrane lipids were extracted following the procedure by Bligh and Dyer (1959) with modifications. Three hundred seventy-five µL of 1:2 chloroform:methanol was added to the SR or mitochondria from previous extraction. The tube was vortexed for five seconds, mixed on a shaker for 20 minutes, and stored overnight at 4°C. The next morning, 1.25 mL of chloroform was added, and the sample was mixed on a shaker for one minute. One hundred twenty-five µL of ddH_2O was added, and the sample was mixed again on a shaker for another minute. The homogenate was filtered through a Whatman #2 filter paper (Whatman, Clifton, NJ) into 13 x 100 mm screw cap tube. Samples were centrifuged at 1,000 x *g* for five minutes. Following centrifugation, the aqueous phase (top layer) was aspirated off. The remaining was evaporated to dryness under nitrogen at 60°C.

SR and Mitochondria Fatty Acids (Appendix IV). After extraction, lipids were converted to fatty acid methyl esters according to the procedures by Morrison and Smith (1964) and Metcalfe et al. (1966). Five hundred µL of 0.5 M NaOH in methanol was added to the extracted SR or mitochondria membrane lipid. The solution was vortexed for five s and heated for five minutes at 100°C. Five hundred µL of born trifuoride in 14% methanol was added to the solution, and the solution was vortexed for five seconds and heated for five minutes at 100°C. One milliliter of a saturated NaCl solution and 1 mL of hexane were added to the solution, and the solution was vortexed for five seconds. The solution was centrifuged at 1,000 x *g* for five minutes. Following centrifugation, the hexane layer (top layer) was removed and placed in a gas chromatography (GC) vial. The

prepared fatty acid methyl esters were further concentrated by drying at 60°C under constant nitrogen gas purging. One hundred µL hexane was added to the dry sample. The sample was then transferred to a 100 microliter spring bottom vial insert and put into GC vials. The fatty acids were analyzed using gas chromatography (Hewlett-Packard 6890 FID GC System; Agilent Technologies, Santa Clara, CA) with a capillary column (Chrompack CP-Sil 88 (0.25 mm x 100 m). Oven temperature was programmed for 140 to 220°C at 2°C/min and held at 220°C for 20 minutes. Injector temperature was maintained at 270°C. Detector temperature was maintained at 300°C. The carrier gas was Helium and had a flow rate of 30mL/min. Fatty acid classes were identified by comparing of retention times and peaks with five commercially known standards (GLC-68D, GLC-79, GLC-87, GLC-455, and GLC-458, Nu-Chek Prep, Inc., Elysian, MN).

SR and Mitochondria Phospholipids (Appendix V). After extraction, 30 µL of 2% methanol and 1% ddH_2O in chloroform was added to each lipid sample. Samples were separated into three lipid groups for SR and four lipid groups for mitochondria by one-dimensional thin-layer chromatography (TLC) described by Leray et al. (1987) with modifications. Whatman LK5 TLC plates (Whatman, Clifton, NJ) were prewashed by migration up to 1 cm from the top with chloroform/methanol (1/1, v/v) and wetted thoroughly with 2.3% (w/v) boric acid solution. All 30 µL of each extracted lipid sample was deposited on the plate and placed in the chromatography tank containing the following solvent: chloroform/ethanol/ddH_2O/trimethylamine (30/35/7/35, v/v). After migration was complete, phospholipid spots were quantified by the method described by Baron and Coburn (1984) with modification. Each plate was dampened with 10% (w/v)

cupric sulfate solution in 8% (w/v) phosphoric acid and placed in an oven at 180^{o}C for 10-15 minutes. The isolated fractions were identified by comparing their R_f values with known lipid standards (Medh and Weigel, 1989 and Allan and Cockcroft, 1982). The plate was scanned and fractions were quantified using Quantity One 1-D Analysis Software (BioRad, Hercules, CA). Each phospholipid was measured as a percentage of total phospholipids on one specific lane.

Mitochondrial Artificial Oxidation (Appendix VI). After measuring protein mitochondrial concentration, mitochondria were artificially oxidized following the procedure outlined by Martonosi and Feretos (1964) with modifications. Isolated mitochondria were transferred to a 5 mL ultracentrifuge tube (Thermo Fisher, 03127) and 2 mL loading buffer (0.1 M KCl, 5mM HEPES, 0.5 mM $CaCl_2$, 0.2 mM $MgCl_2$, 4 mM ADP) were added. Samples were vortexed and then placed into a 4^{o}C refrigerator for 30 mins. Two mL oxidation medium (0.1 M KCl, 5mM HEPES, 0.2 mM $MgCl_2$, 4 mM ADP, 0.1 mM $FeCl_3$, and 0.25 mM ascorbic acid) was added to half of the samples, vortexed, and rested for 5 mins while 2 mL loading buffer was added to the other half of the samples. All samples were centrifuged at 10,000 x *g* for 25 mins at 4^{o}C using a swinging bucket rotor (AH-650 Thermo Scientific, 54294). Supernatant was discarded and the pellet was resuspended in 2 mL extraction buffer (70 mM sucrose, 200 mM mannitol, 5 mM HEPES, and 10 mM K_2PO_4). All samples were sonicated at a 30% amplitude for 30 sec (Branson Digital Sonifier 450, 2004, 50/60Hz 4.0 A) and then transferred to a 2 mL Eppendorf tube. Samples were shipped to Ward Lab (Kearny, NE) for inductively coupled plasma optical emission spectrum (ICP-OES) calcium

concentration analysis. Calcium measurements were expressed as µM Ca^{2+}/µg mitochondrial protein.

Protein Concentration (Appendix VII). One hundred µL mitochondria stock sample was mixed with one hundred µL of maintenance buffer for diluted sample preparation. Protein concentration was determined using a Pierce bicinchoninic (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL). A concentration series (20 - 2,000 µg/mL) of bovine serum albumin (BSA) standards were prepared using the maintenance buffer as the diluent. Twenty-five µL of BSA standards and diluted mitochondrial samples were put into a 96 well microplate (Microtest III sterile 96-well flat-bottomed microplate; Becton Dickinson & Company). Two hundred µL of BCA working reagents (50:1 of Reagent A:Reagent B; Pierce Biotechnology, Rockford, IL) were added to all wells on the microplate. After all standards, samples, and reagents were added, the plate was incubated at 37°C for 30 mins. Absorbance was immediately read after 30 min incubation at 562 nm. Protein concentrations were expressed as µg/mL.

Statistical Analysis. Data for fatty acids, phospholipids, and oxidation were analyzed as a split plot design, with dietary treatment as the whole plot and aging period as the split plot. The GLIMMIX procedure in SAS (version 9.4, SAS Institute, Inc., Cary, N.C.) was used to analyze data. The experimental unit was the individual animal, with pen as the random variable. Means were separated using the LSMEANS statement with PDIFF options and the TUKEY adjustment with an alpha level of 0.05.

LITERATURE CITED

Aberle, E. D., J. C. Forrest, D. E. Gerrard, and E. W. Mills. 1975. Principles of Meat Science. 5th ed. Kendall Hunt Publishing. Dubuque, IA.

Abramson, J. J. and G. Salama. 1988. Sulfhydryl oxidation and Ca2+ release from sarcoplasmic reticulum. J. Mol. Cell Biochem. 82:81-84.

Abramson, J. J. and G. Salama. 1989. Critical sulfhydryls regulate calcium from sarcoplasmic reticulum. J. Bioenergetics and Biomembranes 82:81-84.

Acehan, D., Y. Xu, D. L. Stokes, and M. Schlame. 2007. Comparison of lymphoblast mitochondria from normal subjects and patients with barth syndrome using electron microscopic tomography. Lab Investigations 87:40-48.

Alberts, B., A. Johnson, J. Lewis, R. Martin, K. Roberts, and P. Walter. 2002. Molecular biology of the cell. 4th ed. Garland Science, New York.

Alfadda, A. A. and R. M. Sallam. 2012. Reactive oxygen species in health and disease. J. Biomed. Biotech. 2012:1-14.

Allan, D. and S. Cockcroft. 1982. A modified procedure for thin-layer chromatography of lipids. J. Lipid Res. 23: 1373-1374.

Ardail, D., J. P. Privat, M. Egret-Charlier, C. Levrat, F. Lerme, and P. Louisot. 1990. Mitochondrial contact sites: lipid composition and dynamics. J. Biol. Chem. 265:18797-18802.

Baron, C. B., and R. F. Coburn. 1984. Comparison of two copper reagents for detection of saturated and unsaturated neutral lipids by charring densitometry. J. Liq. Chrom. 7: 2793-2801.

Beare, J. L. and M. Kates. 1964. The deposition of linoleic acid in rats fed corn oil. Canadian J. Biochem. 42:1477-1486.

Berman, S. B., F. J. Pineda, and J. M. Hardwick. 2008. Mitochondrial fission and fusion dynamics: the long and short of it. J. Cell Death Differentiation 7:1147-1152.

Bernardi, P. and M. Forte. 2007. The mitochondrial permeable transition pore. Novartis Symposium 287:157-164.

Bernardi, P., A. Krauskopf, E. Basso, V. Petronilli, E. Blachly-Dyson, F. Di Lisa, and M. A. Forte. 2006. The mitochondrial permeability transition from in vitro artifact to disease target. FEBS Lett. 273:2077-2099.

Bishop, J. E., T. C. Squier, D. J. Bigelow, and G. Inesi. 1988. (Iodoacetamido) fluorescein labels a pair of proximal cysteines on the Ca2+-ATPase of sarcoplasmic reticulum. J. Biochem. 27:5233-5240.

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Canadian J. Biochem. Physiol. 37:911-917.

Boehm, M. L., T. L. Kendall, V. F. Thompson, and D. E. Goll. 1998. Changes in the calpains and calpastatin during postmortem storage of bovine muscle. J. Anim. Sci. 76:2415-2434.

Boncompagni, S., A. E. Rossi, M. Micaroni, G. V. Beznoussenko, R. S. Polishchuk, R. T. Dirksen, and F. Protasi. 2009. Mitochondria are linked to calcium stores in striated muscle by developmentally regulated tethering structures. J. Mol. Biol. Cell 20:1058-1067.

Bootman, M. D., C. W. Taylor, and M. J. Berridge. 1992. The thiol reagent, thimerosal, evokes Ca2+ spikes in HeLa cells by sensitizing the inositol 1,4,5-triphosphate receptor. J. Biol. Chem. 267:25113-25119.

Brookes, P. S., Y. Yoon, J. L. Robotham, M. W. Anders, and S. Sheu. 2004. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. J. Cell Physiol. 287: C817-C833.

Byrd, S. K. 1992. Alterations in the sarcoplasmic reticulum: a possible link to exercise-induced muscle damage. J. Med. Sci. Sports Exercise 24:531-536.

Carafoli, E., R. Tiozzo, G. Lugli, F. Crovetti, and C. Kratzing. 1974. The release of calcium from heart mitochondria by sodium. J. Mol. Cell. Cardio. 6:361-371.

Celsi, F., P. Pizzo, M. Brini, S. Leo, C. Fotino, P. Pinton, and R. Rizzuto. 2009. Mitochondria, calcium and cell death: a deadly triad in neurodegeneration. J. Biochim. Biophys. Acta 5:335-344.

Chao, M. 2015. Impact of wet distillers grains plus solubles and antioxidants on a basic mechanism of beef tenderization. Dissertation, University of Nebraska-Lincoln.

Cheng, P. and G. M. Hatch. 1995. Inhibition of cardiolipin biosynthesis in the hypoxic rat heart. J. Lipids 30:513-519.

Contreras, L., I. Drago, E. Zampese, and T. Pozzan. 2010. Mitochondria: the calcium connection. J. Biochim. Biophys. Acta 1797:607-618.

Cooper, G. 2000. Structure of the plasma membrane – the cell: a molecular approach. 2nd edition. Sinauer Associates, Sunderland, MA. 1-100.

Copello, J. A., S. Barg, H. Onoue, and S. Fleischer. 1997. Heterogenity of Ca2+ gating of skeletal muscle and cardiac ryanodine receptors. J. Biophys. 73:141-156.

Costello, B., C. Chadwick, A. Saito, A. Chu, A. Mauer, and S. Fleischer. 1986. Characterization of the junctional face membrane from terminal cisternae of sarcoplasmic reticulum. J. Cell Biol. 103:741-753.

Crompton, M., M. Kunzi, and E. Carafoli. 1977. The calcium-induced and sodium-induced effluxes of calcium from heart mitochondria. European J. Biochem. 79:549-558.

Csordás, G., C. Renken, P. Varnai, L. Walter, D. Weaver, K. F. Buttle, T. Balla, C. A. Mannella, and G. Hajnóczky. 2006. Structural and functional features and significance of the physical linkage between ER and mitochondria. J. Cell Biol. 174:915-921.

Csordás, G. and G. Hajnóczky. 2009. SR/ER-mitochondrial local communication: calcium and ROS. J. Biochem. Biophys. Acta 1787:1352-1362.

Daum, G. and J. E. Vance. 1997. Import of lipids into the mitochondria. J. Prog. Lipid Res. 36:103-130.

de Mello, A. S., C. R. Calkins, B. E. Jenschke, T. P. Carr, M. E. R. Dugan, and G. E. Erickson. 2012. Beef quality of calf-fed steers finished on varying levels of corn-based wet distillers grains plus solubles. J. Anim. Sci. 90:4625-4633.

Di Lisa, F. and P. A. Bernardi. 2009. Capful of mechanisms regulating the mitochondrial permeability transition. J. Mol. Cell Cardio. 46:775-780.

Di Lisa, F., R. Menabó, M. Canton, M. Barile, and P. Bernardi. 2001. Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD+ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. J. Biol. Chem. 276:2571-2575.

Di Paola, M. and M. Lorusso. 2006. Interaction of free fatty acids with mitochondria: coupling, uncoupling and permeability transition. J. Biochem. Biophys. Acta 1757:1330-1337.

Dransfield, E. 1994. Optimisation of tenderization, ageing and tenderness. Meat Sci. 36:105-121.

Droge, W. 2002. Free radicals in the physiological control of cell function. Physiol. Rev. 82:47-95.

Du, M., and R. J. McCormick. 2009. Applied Muscle Biology and Meat Science. Taylor and Francis Group, LLC. 1-337.

Dudek, J., I. F., Cheng, M. Balleininger, F. M. Vaz, K. Streckfuss-Bomeke, and D. Hubscher. 2013. Cardiolipin deficiency affects respiratory chain function and organization in an induced pluripotent stem cell model of Barth syndrome. Stem Cell Res. 11:806-819.

Esposti, M. D. 2002. Measuring mitochondrial reactive oxygen species. Cancer research campaign molecular pharmacology group. Methods 26:335-340.

Fagone, P. and S. Jackowski. 2009. Membrane phospholipid synthesis and endoplasmic reticulum function. J. Lipid Res. 50:S311-S316.

Fill, M. and J. A. Copello. 2002. Ryanodine receptor calcium release channels. Physiol. Rev. 82:893-922.

Foskett, J. K., C. White, K. H. Cheung, and D. O. Mak. 2007. Inositol triphosphate receptor Ca2+ release channels. Physiol. Rev. 87:593-658.

Franzini-Armstrong, C. 2007. ER-mitochondria communication. How privileged? J. Physiol. 22:261-268.

Garcia-Perez, C., G. Hajnóczky, and G. Csordás. 2008. Physical coupling supports the local Ca2+ transfer between sarcoplasmic reticulum subdomains and the mitochondria in heart muscle. J. Biol. Chem. 283:32771-32780.

Gasparre, G., A. M. Porcelli, G. Lenaz, and G. Romeo. 2013. Relevance of mitochondrial genetics and metabolism in cancer development. Cold Spring Harb. Sym. Perspective Biol. 1-17.

Geesink, G., A. Bekhit, and R. Bickerstaffe. 2000. Rigor temperature and meat quality characterisitics of lamb longissimus muscle. J. Anim. Sci. 78:2842-2848.

Giroud, S., C. Frare, A. Strijkstra, A. Boerema, W. Arnold, and T. Ruf. 2013. Membrane Phospholipid Fatty Acid Composition Regulates Cardiac SERCA Activity in a Hibernator, the Syrian Hamster (Mesocricetus auratus). PLOS. 8:1-9.

Goll, D. E., W. Dayton, I. Singh, and R. Robson. 1991. Studies of the alpha-actinin/actin interaction in the z-disk by using calpain. J. Biol. Chem. 266:8501-8510.

Goll, D. E., V. F. Thompson, R. Taylor, and J. Christiansen. 1992. Role of calpain system in muscle growth. J. Bichimie 74:225-237.

Gorlach, A., P. Klappa, and T. Kietzmann. 2006. The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. Antioxidant Redox Signal 8:1391-1418.

Gould, G. W., J. M. McWhirther, J. M. East, and A. G. Lee. 1987. Effects of diet on the function of sarcoplasmic reticulum. J. Biochem. 245:751-755.

Greaser, M., R. Cassens, W. Hoekstra, and E. Briskey. 1969. The effect of pH temperature treatments on the calcium accumulating ability of purified sarcoplasmic reticulum. J Food Sci. 34:633-637.

Guderley, H., E. Kraffe, W. Bureau. And D. P. Bureau. Dietary fatty acid composition changes mitochondrial phospholipids and oxidative capacities in rainbow trout red muscle. J. Comparative Physiol. 178:385-399.

Gunter, T. E., J. H. Chace, J. S. Puskin, and K. K. Gunter. 1983. Mechanism of sodium independent calcium efflux from rat liver mitochondria. J. Biochem. 22:6341-6351.

Györke, I., N. Hester, L. R. Jones, and S. Györke. 2004. The role of calsequestrin, triadin, and junctin in conferring cardiac ryanodine receptor responsiveness to luminal calcium. J. Biophys. 86:2121-2128.

Hamilton, S. L. and M. B. Reid. 2000. RyR1 modulation by oxidation and calmodulin. Antioxidant Redox Signal 2:41-45.

Harder, T., P. Scheiffele, P. Verkade, and K. Simons. 1998. Lipid domain structure of the plasma membrane revealed by patching of membrane components. J. Cell Biol. 141:929-942.

Hemmings, S. J. 2001. New methods for the isolation of skeletal muscle sarcolemma and sarcoplasmic reticulum allowing a comparison between the mammalian and amphibian beta(2)-adrenergic receptors and calcium pumps. Cell Biochem. Funct. 19:133-141.

Hidalgo, C., R. Bull, J. J. Marengo, C. F. Perez, and P. Donoso. 2000. SH oxidation stimulates calcium release channels (ryanodine receptors) from excitable cells. Biol. Res. 33:113-124.

Hidalgo, C. and P. Donoso. 2008. Crosstalk between calcium and redox signaling: from molecular mechanisms to health implication. Antioxidant Redox Signal 10:1275-1312.

Hidalgo, C., P. Donoso, and M. A. Carrasco. 2005. The ryanodine receptors Ca2+ release channels: cellular redox sensors? IUBMB Life 57:315-322.

Horvath, S. E., and G. Daum. 2013. Lipids of mitochondria. Progress in Lipid Research. 52: 590-614.

Hosfield, C. M., J. S. Elce, P. L. Davies, and Z. Jia. 1999. Crystal structure of calpain reveals the structural basis for Ca2+-dependent protease activity and a novel mode of enzyme activation. EMBO J. 18:6880-6889.

Houtkooper, R. H. and F. M. Vaz. 2008. Cardiolipin, the heart of mitochondrial metabolism. J. Cell. Mol. Life Sci. 65:2493-2506.

Hovius R., J. Thijissen, P. van der Linden, K. Nicolay, and B. de Kruijff. 1993. Phospholipid asymmetry of the outer membrane of rat liver mitochondria: evidence for the presence of cardiolipin on the outside of the outer membrane. FEBS Lett. 330:3059-3064.

Huff-Lonergan, E., W. Zhang, and S. M. Lonergan. 2010. Biochemistry of postmortem muscle: lessons on mechanisms of meat tenderization. Meat Sci. 86:184-195.

Hunter, G. W., S. Negash, and T. C. Squier. 1999. Phosphatidylethanolamine modulates Ca-ATPase function and dynamics. J. Biochem. 38:1356-1364.

Isaeva, E. V., V. M. Shkryl, and N. Shirokova. 2005. Mitochondrial redox state and Ca2+ sparks in permeabilized mammalian skeletal muscle. J. Physiol. 565:855-872.

Ishii, T., O. Sunami, N. Saitoh, H. Nishio, T. Takeuchi, and F. Hata. 1998. Inhibition of skeletal muscle sarcoplasmic reticulum Ca2+-ATPase by nitric oxide. FEBS Lett. 440:218-222.

Jeacocke, R. E. 1993. The concentrations of free magnesium and free calcium ions both increase in skeletal muscle fibers entering Rigor mortis. Meat Sci. 35:27-45.

Ji, J. R. and K. Takahashi. 2006. Changes in concentration of sarcoplasmic free calcium during post-mortem ageing of meat. Meat Sci. 73:395-403.

Joseph, S. K., S. K. Nakao, and S. Sukumvanich. 2006. Reactivity of free thiol groups in type-I inositol triphosphate receptors. J. Biochem. 393:575-582.

Jung, D. W., K. Baysal, and G. P. Brierly. 1995. The sodium-calcium antiport of heart mitochondria is not electroneutral. J. Biol. Chem. 270: 672-678.

Kasri, N. N., A. M. Holmes, G. Bultynck, J. B. Parys, M. D. Bootman, K. Rietdorf, L. Missian, F. McDonald, H. D. Smedt, and S. J. Conway. 2004. Regulation of InsP3 receptor activity by neuronal Ca2+-binding proteins. EMBO J. 23:312-321.

Kennedy, E. P. and S. B. Weiss. 1956. The function of cytidine coenzymes in the biosynthesis of phospholipids. J. Biol. Chem. 286:7841-7853.

Kent, M., M. Spencer, and M. Koohmarie. 2004. Postmortem proteolysis is reduced in transgenic mice overexpressing calpastatin. J. Anim. Sci. 82:794-801.

Kirichok, Y., G. Krapivinsky, and D. E. Clapham. 2004 . The mitochondrial calcium uniporter in a highly selective ion channel. Nature 427:360-364.

Klopfenstein, T. J., G. E. Erickson, and V. R. Bremer. 2007. Board-invited interview: Use of distillers byproducts in the beef cattle feeding industry. J. Anim. Sci. 86.5: 1223-1231.

Koger, T. J., D. M. Wulf, A. D. Weaver, C. L. Wright, K. E. Tjardes, K. S. Mateo, T. E. Engle, R. J. Maddock, and A. J. Smart. 2010. Influence of feeding various quanitites of wet and dry distillers grains to finishing steers on carcass characteristics, meat quality, retail-case life of ground beef, and fatty acid profile of longissimus muscle. J. Anim. Sci. 88:3399-3408.

Koohmarie, M. 1994. Muscle proteinases and meat aging. Meat Sci. 36:93-104.

Koohmarie, M., J. Schollmeyer, and T. Dutson. 1986. Effect of low-calcium-requiring calcium activated factor on myofibrils under varying pH and temperature conditions. J. Food Sci. 51:28-32.

Kubli, D. A. and A. B. Gustafsson. 2012. Mitochondria and mitophagy: the yin and yang of cell death control. Circ. Res. 111:1208-1221.

Küllenberg, D., L. A. Taylor, M. Schneider, and U. Massing. 2012. Health effects of dietary phospholipids. J. Lipids Health 11:3.

Leray, C., X. Pelletier, S. Hemmendinger, and J. P. Cazenave. 1987. Thin-layer chromatography of human platelet phospholipids with fatty acid analysis. J. Chrom. 420:411-416.

Li, Y. and P. Camacho. 2004. Ca2+-dependent redox modulation of SERCA 2b by ERp57. J. Cell Biol. 164:35-46.

Lund, M. N., M. Heinonen, C. P. Baron, and M. Estevez. 2011. Protein oxidation in muscle foods: a review. J. Mol. Nutr. Food Res. 55:83-95.

Machlin, L. J. and A. Bendich. 1987. Free radical tissue damage: protective role of antioxidant nutrients. FASEB J. 1:441-445.

MacLennan, D. H. and P. Wong. 1971. Isolation of calcium-sequestering protein from sarcoplasmic reticulum. Proceedings of the National Academy of Sci. 68:1231-1235.

MacLennan, D. H., C. Yip, G. Iles, and P. Seeman. 1973. Isolation of sarcoplasmic reticulum proteins. Cold Spring Harb. Sym. Quantitative Biol. 469-477.

MacMillan, D., S. Chalmers, T. C. Muir, and J. G. McCarron. 2005. IP3-mediated Ca2+ increases do not involve the ryanodine receptor, but ryanodine receptor antagonists reduce IP3-mediated Ca+ increases in guinea-pig colonic smooth muscle cells. J. Physiol. 569:533-544.

Malli, R. and W. F. Graier. 2010. Mitochondrial Ca^{2+} channels: great unknowns with important functions. FEBS Lett. 584:1942-1947.

Maltin, C., D. Balcerzak, R. Tilley, and M. Delday. 2003. Determinants of meat quality: tenderness. Proceedings of Nutr. Society. 62:337-347.

Martin, J., K. Mahlke, and N. Pfanner. 1991. Role of energized inner membrane in mitochondrial protein import. J. Biol. Chem. 266:18051-18057.

Martins, A. S., V. M. Shkryl, M. C. Nowycky, and N. Shirokova. 2008. Reactive oxygen species contribute to Ca2+ signals produced by osmotic stress in mouse skeletal muscle fibers. J. Physiol. 586:197-210.

Martonosi, A. and R. Feretos. 1964. Sarcoplasmic reticulum: the uptake of Ca++ by sarcoplasmic reticulum fragments. J. Biol. Chem. 239:648-658.

Medh, J. D. and P. H. Weigel. 1989. Separation of phosphatidylinositols and other phospholipids by two-step one-dimensional thin-layer chromatography. J Lipid Res. 30: 761-764.

Meissner, G. 2002. Regulation of mammalian ryanodine receptors. Front Biosci. 7:d2072-d2080.

Mejia, E. M., and G. M. Hatch. 2015. Mitochondrial phospholipids: role in mitochondrial function. J. Bioenergetics and Biomembranes 1-14.

Metcalfe, L. D., A. A. Schmitz, and J. R. Pelka. 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem. 38: 514-515.

Michell, R. H. 1975. Inositol phospholipids and cell surface receptor function. J. Biochim. Biophys. Acta 415:81-147.

Miller, R. K., S. B. Smith, and G. E. Carstens. 2011. New beef tenderness theory. Exec. summary prepared for NCBA.

Mlekusch, W., C. Celedin, R. C. Aloia, and R. Moller. 1993. Effect of a high fat diet on phospholipid class distribution and fatty acid composition in rat liver. Int. J. Biochem. 25:1539-1547.

Monteiro, J. P., P. J. Oliveira, and A. S. Jurado. 2013. Mitochondrial membrane lipid remodeling in pathophysiology: a new target for diet and therapeutic interventions. Prog. Lipid Res. 52:513-528.

Monteiro, J. P., C. V. Pereirra, and A. M. Silva. Rapeseed oil-rich diet alters hepatic mitochondrial membrane lipid composition and disrupts bioenergetics. Archives of Toxicol. 87:2151-2163.

Morrison, W. R. and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride – methanol. J. Lipid Res. 5:600-608.

Nelson, D. L., and M. M. Cox. 2005. Lehninger Principles of Biochemistry. 4th ed. W. H. Freeman and Company. 1-800.

O'Rourke, B. 2000. Pathophysiological and protective roles of mitochondrial ion channels. J. Physiol. 529:23-236.

Osellame, L. D., T. S. Blacker, and M. R. Duchen. 2012. Cellular and molecular mechanisms of mitochondrial function. Best Pract. Res. Cl. En. 26:711-723.

Osman, C., D. R. Voelker, and T. Langer. 2011. Making heads or tails of phospholipids in mitochondria. J. Cell Biol. 192:7-16.

Pan, D. A. and L. H. Storlien. 1993. Dietary lipid profile is a determinant of tissue phospholipid fatty acid composition and rate of weight gain in rats. J. Nutrition 120:1291-1296.

Parrish, F., C. Selvig, R. Culler, and M. Zeece. 1981. CAF activity, calcium concentration, and the 30,000-dalton component of tough and tender bovine longissimus muscle. J. Food Sci. 46:308-311.

Patron, M., A. Raffaello, V. Granatiero, A. Tosatto, G. Merli, D. De Stefani, L. Wright, G. Pallafacchina, A. Terrin, C. Mammucari, and R. Rizzuto. 2013. The mitochondrial calium uniporter (MCU): molecular identity and physiological roles. J. Biol. Chem. 288: 10750-10758.

Paulus, H. and E. P. Kennedy. 1960. The enzymatic synthesis of inositolmonophosphatide. J. Biol. Chem. 235:1303-1311.

Pehowich, D. J. 1999. Thyroid hormone status and membrane n-3 fatty acid content influence mitochondrial proton leak. J. Biochim. Biophys. Acta 1441:192-200.

Peterson, C. M., D. L. Johannsen, and E. Ravussin. 2012. Skeletal muscle mitochondria and aging: a review. J. Aging Res. 2012: 1-20.

Player, T. J. and H. O. Hultin. 1978. The effect of lipid peroxidation on the calcium-accumulating ability of the microsomal fraction isolated from chicken breast muscle. J. Biochem. 174:17-22.

Presti, F. 1985. The role of cholesterol in regulating membrane fluidity. Mem. Fluidity in Biol. 4:97-146.

Raffaello, A., D. De Stefani, and R. Rizzuto. 2012. The mitochondrial Ca^{2+} uniporter. Cell Calcium 52:16-21.

Rizzuto, R., D. De Stefani, A. Raffaello, and C. Mammucari. 2012. Mitochondria as sensors and regulators of calcium signaling. Mol. Cell Biol. 13:566-578.

Rossi, A. E., S. Boncompagni, and R. T. Dirksen. 2009. Sarcoplasmic reticulum-mitochondrial symbiosis: bidirectional signaling in skeletal muscle. Exercise Sport Sci. R. 37:29-35.

Rossi, A. E. and R. T. Dirksen. 2006. Sarcoplasmic reticulum: the dynamic calcium governor of muscle. Muscle and Nerve 33:715-731.

Rudolf, R., M. Mongillo, P. J. Magalhaes, and T. Pozzan. 2004. In vivo monitoring of Ca^{2+} uptake into mitochondria of mouse skeletal muscle during contraction. J. Cell Biol. 166:527-536.

Rusinol, A. E., Z. Cui, M. H. Chen, and J. E. Vance. 1994. A unique mitochondria-associated membrane fraction from rat liver has a high capacity for lipid synthesis and contains pre-golgi secretory proteins including nascent lipoproteins. J. Biol. Chem. 269:27494-27502.

Santos, C. X. C., L. Y. Tanaka, J. Wosniak, Jr., and F. R. M. Laurindo. 2009. Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. Antioxidant and Redox Signal 11:2409-2426.

Santo-Domingo, J. and N. Demaurex. 2010. Calcium uptake mechanisms of mitochondria. J. Biochim. Biophys. Acta 1797:907-912.

Schenkel, L. C., and M. Bakovic. 2013. Formation and regulation of mitochondrial membranes. J. Cell Biol. 2014:1-13.

Schuki, I. and G. Daum. 2009. Phosphatidylserine decarboxylases, key enzymes of lipid metabolism. IUBMB Life. 61:151-162.

Senaratne, L. S. 2012. Mechanism and control of beef toughening during retail display in high oxygen modified atmosphere packages. Dissertation, University of Nebraska-Lincoln.

Sharov, V. S., E. S. Dremina, N. A. Galeva, T. D. Williams, and C. Schoneich. 2006. Quantitative mapping of oxidation-sensitive cysteine residues in SERCA in vivo and in vitro by HPLC-electrospray-tandem MS: selection protein oxidation during biological aging. J. Biochem. 394:605-615.

Shinmura, K. 2013. Effects of caloric restriction on cardiac oxidative stress and mitochondrial bioenergetics: potential role of cardiac sirtuins. Oxid. Med. and Cell. Longev. 2013:1-11.

Sieber, F., A. M. Duchêne, and L. Maréchal-Drouard. 2011. Mitochondrial RNA import: from diversity of natural mechanisms to potential applications. Int. Rev. Cell Mol. Biol. 287:145-190.

Simbeni, R., L. Pon, L. Pon, E. Zinser, F. Paltauf, G. Daum. 1991. Mitochondrial membrane contact sites of yeast: characterization of lipid components and possible involvement in intramitochondrial translocation of phospholipids. J. Biol. Chem. 280:40032-40040.

Singer, S. J. and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. Science 175:720-731.

Smith, A. L. 1967. Preparation, properties, and conditions for assay of mitochondria: slaughterhouse material, small scale. Methods in Enzymology. 10: 81-86.

Spector, A. A. and M. A. Yorek. 1985. Membrane lipid composition and cellular function. J. Lipid Res. 9:1015-1035.

Stanley, D. W. 1991. Biological membrane deterioration and associated quality losses in food tissues. Critical Rev. in Food Sci. and Nutr. 30:487-553.

Stark, G. 2005. Functional consequences of oxidative membrane damage. J. Mem. Biol. 205:1-6.

Steenbergen, R., T. S. Nanowski, A. Beigneux, A. Kulinski, S. G. Young, and J. E. Vance. 2005. Disruption of the phosphatidylserine decarboxylase gene in mice causes embryonic lethality and mitochondrial defects. J. Biol. Chem. 266:10047-10049.

Stock, R., J. Lewis, T. J. Klopfenstein, and C. Milton. 2000. Review of new information on the use of wet and dry mill feed by-products in feedlot diets. J. Anim. Sci. 77:1-12.

Storz, P. 2007. Mitochondrial ROS – radical detoxification, mediated by protein kinase D. Trends in Cell Biol. 17: 13-18.

Subhasini, B. and E. A. Jaimes. 2013. Mitochondria and reactive oxygen species: physiology and pathobiology. J. Mol. Sci. 3:6306-6344.

Suzuki, K. 1990. The structure of calpains and the calpain gene. CRC Press, Boca Raton, FL.

Szent-Györgyi, A. G. 1975. Calcium regulation of muscle contraction. J. Biophys. 15:707-723.

Taylor, R. G., G. Geesink, V. Thompson, M. Koohmarie, and D. E. Goll. 1995. Is z-disk degradation responsible for postmortem tenderization? J. Anim. Sci. 73:1351-1367.

Taylor, R. G. and M. Koohmarie. 1998. Effects of postmortem storage on the ultrastructure of the endomysium and myofibrils in normal and callipyge longissimus. J. Anim. Sci. 76:2811-2817.

Tolias, K. F. and L. C. Cantley. 1999. Pathways for phosphoinositide synthesis. Chem. Phys. Lipids 98:69-77.

Tompa, P., Y. Emori, H. Sorimachi, K. Suzuki, and P. Friedrich. 2001. Domain III of calpain is a Ca2+-regulated phospholipid-binding domain. Biochem. Biophys. Res. Comm. 280:1333-1339.

Toyoshima, P. and G. Inesi. 2004. Structural basis of ion pumping by Ca2+-ATPase of the sarcoplasmic reticulum. J. Biochem. 73:269.

Treberg, J. R., D. Munro, S. Banh, P. Zacharias, and E. Sotiri. 2015. Differentiating between apparent and actual rates of H_2O_2 metabolism by isolated rat muscle mitochondria to test a simple model of mitochondria as regulators of H_2O_2 concentration. Redox Biol. 5:216-224.

van den Brink-van der Laan, E., J. A. Killian, and B. de Kruijff. 2004. Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure profile. J. Biochim. Biophys. Acta 1666:275-288.

van Meer, G., D. R. Voelker, and G. W. Feigenson. 2008. Membrane lipids: where they are and how they behave. Nature Rev. Mol. Cell Biol. 9:112-124.

Von Heijne, G. 1992. Membrane protein structure prediction: hydrophobicity analysis and the positive-inside rule. J. Mol. Biol. 225:487-494.

Wendt, A., V. F. Thompson, and D. E. Goll. 2004. Interaction of calpastatin with calpain: a review. J. Biol. Chem. 385:465-472.

Williams, G. S. B., L. Boyman, A. C. Chikando, R. J. Khairallah, and W. J. Lederer. 2013. Mitochondrial calcium uptake. PNAS. 110:10479-10486.

Wray, S. and T. Burdyga. 2010. Sarcoplasmic reticulum function in smooth muscle. Physiol. Rev. 90:113-178.

Yeagle, P. 1987. The Membranes of Cells. Academic Press, Orlando, Florida.

Zhang, L., J. Kelley, G. Schmeisser, Y. M. Kobayashi, and L. R. Jones. 1997. Complex formation between junctin, triadin, calsequestrin, and the ryanodine receptor proteins of the cardiac junctional sarcoplasmic reticulum membrane. J. Biol. Chem. 272:23389-23397.

Zorov, D. B., C. R. Filburn, L. O. Klotz, J. L. Zweier, and S. J. Sollott. 2000. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompyaning induction of the mitochondrial permeability transition in cardiac myocytes. J. Exp. Med. 192:1001-1014.

The Influence of Diet and Oxidation on Calcium Retention of the Mitochondria in Fresh Beef

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ABSTRACT

Distillers grains (DG) contribute to cattle sarcoplasmic reticulum (SR) membrane instability by increasing poly-unsaturated fatty acid (PUFA) content in the SR membrane, possibly resulting in early postmortem calcium (Ca) leakage and improved tenderness. Mitochondria, the secondary Ca-sequestering organelles in the muscle, are relatively easy to isolate intact and provide the opportunity to study Ca release under carefully controlled and tightly defined conditions. Our objective was to isolate mitochondria from cattle fed DG and corn to determine the influence of diet and oxidation on Ca release. Cattle (n=48) were fed a corn-based finishing diet with or without deoiled, dried DG (50% DM basis). After harvest, strip loins were collected and steaks from each loin were aged for 2, 8, 14, and 21 days, powdered, and stored at -80^{o}C for lab analysis. Samples (n=12) were randomly sub-sampled from each diet group for all aging periods. Mitochondria were isolated from day 2, 8, and 14 day aged samples using high speed ultracentrifugation. The SR was isolated from each day 2 sample. Both mitochondria and SR samples were analyzed for PUFA content using gas chromatography, and phospholipid content using thin layer chromatography. Mitochondria from days 2 and 8 were artificially oxidized using an iron and ascorbic acid mixture. In both organelles, the DG diet samples had higher 18:2 and total PUFA content ($P<0.05$) compared to corn samples. Day 14 mitochondria had higher 18:2 ($P<0.05$) and total PUFA ($P<0.05$) contents compared to day 2 and 8 mitochondria, as expected from aging of unsaturated fats. Phospholipid contents (phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol) of the mitochondria and SR were unaffected by diet. Oxidized mitochondria retained significantly less Ca than non-oxidized ($P<0.05$) mitochondria. Day 2 mitochondria

retained significantly less Ca than day 8 ($P<0.05$) mitochondria. Overall, mitochondria from cattle finished on corn tended ($P<0.10$) to retain more Ca than mitochondria from cattle finished on DG. Results indicate that greater PUFA content deposited in organelles may affect Ca flux by increased susceptibility to oxidation. A DG diet may influence Ca flux and ultimate tenderness by this mechanism.

Keywords: calcium, distillers grains, mitochondria

INTRODUCTION

Tenderness is one of the most important attributes of a consumer's eating experience and influences the economic value of beef (Miller et al., 2001). For this reason, understanding meat tenderization and what mechanisms can alter tenderness has high significance in research. Antemortem factors, such as diet, may impact the ultimate tenderness of beef, but further exploration of this topic is required to fully grasp the basic mechanism by which tenderness occurs.

Distillers grains are an economical and nutritious grain source for cattle. Distillers grains are widely used for finishing rations in feedlots, ranging in levels of dry matter basis. When compared to corn, distillers grains may contain up to three times the amount of protein, fiber, and fat (Klopfenstein et al., 2007). Interestingly, Senaratne (2012), Depenbusch et al. (2009), and Segers et al. (2011) showed that beef from steers fed wet distillers grains plus solubles was more tender than beef from steers fed corn. Chao (2015) explored the mechanism behind this phenomenon of increased tenderness and found that a greater amount of polyunsaturated fatty acids (PUFA) were deposited in the

sarcoplasmic reticulum (SR) membrane of cattle fed wet distillers grains, and beef from cattle fed wet distillers grains had a greater amount of troponin T degradation (an indication of proteolysis).

The hypothesis is that PUFA content in the SR membrane would increase as a consequence of feeding distillers grains, which would predispose the SR membrane to oxidation, and cause a greater postmortem release of calcium into the cytoplasm. Cytoplasmic calcium would activate proteolytic degradation via the calpain system and thereby enhance tenderness.

Studying calcium flux in the SR proves a very difficult challenge. The organelle has a very irregular structure and isolating fully intact SR is almost impossible. Mitochondria, secondary calcium sequestering organelles in the muscle cell, are relatively easy to isolate intact and offer the opportunity to study calcium release and uptake under carefully controlled and tightly defined conditions. The objective of this study was to evaluate the hypothesis, using mitochondria as a model system for the SR to better understand calcium flux via oxidation.

MATERIALS AND METHODS

All animal use protocols were approved by the University of Nebraska-Lincoln's Animal Care and Use Committee (902).

Animals and Diets

A total of 128 steers were used in a randomized complete block design. Steers were fed at University of Nebraska feedlot (Ithica, NE). Steers were blocked by BW,

stratified by BW within each block, and assigned randomly to pens. Steers received an initial implant with Ralgro® followed by a Revalor®200 implant. All diets were formulated on a dry matter basis using high-moisture corn (31.5%), alfalfa hay (5.5%), corn silage (4%), molasses (5%), and supplement (5%) containing Rumensin® (30g/ton) and Tylan® (90 mg/steer/day). Steers were fed for 156 days. Feeding treatments were 50% dry-rolled corn or 50% de-oiled dry distillers grains plus solubles (DDGS; DM basis). Pens were randomly assigned to 1 of the 2 treatments with 8 steers per pen.

Sample Collection, Fabrication, and Preparation

All steers were harvested at a commercial abattoir (Greater Omaha Packing Co., Omaha NE). After 48 h postmortem chilling, 48 out of 128 carcasses were selected (24 from each treatment, four per pen). Carcass selection was based on quality grade (low Choice). Strip loins (*Longissimus lumborum*) from all selected carcasses were collected, vacuum-packaged, and transported to Loeffel meat laboratory at the University of Nebraska-Lincoln.

Strip loins from the left sides of the carcasses were fabricated on postmortem aging days 2 and 8. Strip loins from the right side of the carcasses were fabricated on postmortem aging days 14 and 21. Each strip loin was fabricated into 2 tenderness samples (2.54 cm) and 4 lab samples (1.27cm) for each aging period, from anterior to posterior ends. The remainder of the strip loins were immediately vacuum packaged in vacuum pouches (3 mm STD barrier, Prime Sources, St. Louis, MO) on a Multivac Packaging machine (Multivac C500, Multivac, Kansas City, MO) and aged to the next designated aging period.

Upon fabrication, steaks were packaged in Styrofoam trays (Styro-Tech, Denver, CO), overwrapped with oxygen permeable polyvinyl chloride film (PSM18, Prime Source, St. Louis, MO), and were subjected to retail display conditions (2 $\pm$ 2^{o}C, and exposed to continuous 1,000-1,800 lux warm white fluorescent lighting) for 0, 4, and 7 days.

Twelve samples were randomly sub-sampled from each diet group for all aging periods (24 total), with two from each pen. Samples for SR membrane fatty acid and phospholipid as well as mitochondrial membrane fatty acid and phospholipid composition were obtained on day 0 of retail display for aging periods 2, 8, and 14 days. Mitochondrial artificial oxidation samples were obtained at day 0 of retail display for aging periods 2 and 8. All lab samples and were vacuum packaged and frozen at -80^{o}C until analysis. The lab samples were pulverized in liquid nitrogen with a blender (model 51BL32, Waring Commercial, Torring, CT).

SR Membrane Extraction

The SR membrane was extracted according to the procedure described by Hemmings (2001) with modifications. Ten g of powdered samples were suspended in 35 mL of 4^{o}C homogenization buffer (10 mM $NaHCO_3$, 2 mM NaN_3, 10 mM Tris-Cl , pH 7.5) and homogenized using a Polytron homogenizer (Kinematica) at setting 6 for 15 sec, setting 7 for 15 sec, and setting 8 for 15 sec. Homogenate was filtered through cheesecloth and transferred into a 50 mL plastic centrifuge tube and centrifuged at 2,000 x *g* for 10 min at 4^{o}C. The supernatant was collected and centrifuged at 10,000 x *g* (Sorvall Ultracentrifuge, F37L-8x100, Thermo Scientific, 096-087056, Rockford, IL) for

30 min at 4°C. Four and a half percent of KCl (w/v) was added to the supernatant and stirred for 30 min on ice. The supernatant was centrifuged at 100,000 x *g* for 60 min at 4°C. The final supernatant was discarded, and the pellet was resuspended in 1 mL of 10mM tris buffer and stored at -80°C until use.

Mitochondrial Extraction

Mitochondria were extracted according to the procedure described by Smith (1967). Ten g of powdered sample was suspended in 50 mL 4°C maintenance buffer (70 mM sucrose, 200 mM mannitol, 5 mM HEPES, and 10 mM K_2PO_4, pH 7.5) and homogenized using a Polytron homogenizer (Kinematica, model CH-6010, Bohemia, NY) at setting 5 for two, 10 sec intervals. Homogenate was filtered through cheesecloth and transferred into a 50 mL plastic centrifuge tube and centrifuged at 650 x g for 10 min at 4°C. The supernatant was collected and centrifuged at 7,750 x *g* (Sorvall Ultracentrifuge, F37L-8x100, Thermo Scientific, 096-087056, Rockford, IL) for 25 min at 4°C. Multiples of the same sample were extracted to be used in each analysis.
The final supernatant was discarded, and the pellet was resuspended in 1 mL maintenance buffer and stored at -80°C until use. Multiples of the same sample were extracted to be used in each analysis.

SR and Mitochondrial Membrane Fatty Acids

Membrane lipids were extracted following the procedure by Bligh and Dyer (1959). After extraction, lipids were converted to fatty acid methyl esters according to the procedures by Morrison and Smith (1964) and Metcalfe et al. (1966). The prepared fatty

acid methyl esters were further concentrated by drying at 60°C under constant nitrogen gas purging and mixed with 100 µL hexane. The fatty acid methyl esters were transferred to 100 µL spring-bottom vial inserts and placed into GC vials. The samples were analyzed using gas chromatography (Hewlett-Packard 6890 FID GC System; Agilent Technologies, Santa Clara, CA) with setups described by Mello et al. (2012). Fatty acid classes were identified by comparing of retention times and peaks with five commercially known standards (GLC-68D, GLC-79, GLC-87, GLC-455, and GLC-458, Nu-Chek Prep, Inc., Elysian, MN).

SR and Mitochondrial Membrane Phospholipids

Thirty µL of 2% methanol and 1% ddH_2O in chloroform was added to each lipid sample. Samples were separated into 3 lipid groups for SR and 4 lipid groups for mitochondria by one-dimensional thin-layer chromatography (TLC) described by Leray et al. (1987) with modifications. Whatman LK5 TLC plates (Whatman, Clifton, NJ) were prewashed by migration up to 1 cm from the top with chloroform/methanol (1/1, v/v) and wetted thoroughly with 2.3% (w/v) boric acid solution. All 30 µL of each extracted lipid sample was deposited on the plate and placed in the chromatography tank containing the following solvent: chloroform/ethanol/ddH_2O/trimethylamine (30/35/7/35, v/v). After migration was complete, phospholipid spots were quantified by the method described by Baron and Coburn (1984) with modification. Each plate was dampened with 10% (w/v) cupric sulfate solution in 8% (w/v) phosphoric acid and placed in an oven at 180°C for 10-15 min. the isolated fractions were identified by comparing their R_f values with known lipid standards (Medh and Weigel, 1989 and Allan and Cockcroft, 1982). The

plate was scanned and fractions were quantified using Quantity One 1-D Analysis Software (BioRad, Hercules, CA). Each phospholipid was measured as a percentage of total phospholipids based on percentages of total lipids on one specific lane.

Artificial Oxidation

Mitochondria were artificially oxidized following the procedure outlined by Martonosi and Feretos (1964) with modifications. Isolated mitochondria were transferred to a 5 mL ultracentrifuge tube (Thermo Fisher, 03127) and 2 mL loading buffer (0.1 M KCl, 5mM HEPES, 0.5 mM $CaCl_2$, 0.2 mM $MgCl_2$, 4 mM ADP) were added. Samples were vortexed and then placed into a 4^{o}C refrigerator for 30 mins. Two mL oxidation medium (0.1 M KCl, 5mM HEPES, 0.2 mM $MgCl_2$, 4 mM ADP, 0.1 mM $FeCl_3$, and 0.25 mM ascorbic acid) was added to half of the samples, vortexed, and rested for 5 mins while 2 mL loading buffer was added to the other half of the samples. All samples were centrifuged at 10,000 x *g* for 25 mins at 4^{o}C using a swinging bucket rotor (AH-650 Thermo Scientific, 54294). Supernatant was discarded and the pellet was resuspended in 2 mL extraction buffer (70 mM sucrose, 200 mM mannitol, 5 mM HEPES, and 10 mM K_2PO_4). All samples were sonicated at a 30% amplitude for 30 sec (Branson Digital Sonifier 450, 2004, 50/60Hz 4.0 A) and then transferred to a 2 mL Eppendorf tube. Samples were shipped to Ward Lab (Kearny, NE) for inductively coupled plasma optical emission spectrum (ICP-OES) calcium concentration analysis.

Statistical Analysis

Data for fatty acids, phospholipids, and oxidation were analyzed as a split plot design, with dietary treatment as the whole plot and aging period as the split plot. The GLIMMIX procedure in SAS (version 9.4, SAS Institute, Inc., Cary, N.C.) was used to analyze data. The experimental unit was the individual animal, with pen as the random variable. Means were separated using the LSMEANS statement with PDIFF options and the TUKEY adjustment with an alpha level of 0.05.

RESULTS

Results for SR membrane fatty acid profile analysis are shown in Table 1. Feeding DDGS decreased ($P < 0.05$) concentrations of 17:0, increased ($P<0.05$) concentrations of 18:2 fatty acid, and tended ($P<0.10$) to increase 20:3, 20:4, and total PUFA. Very long chain PUFAs, such as 22:4 and 22:5, were not affected by treatment. Results for mitochondrial membrane fatty acid profile analysis are shown in Table 2. Feeding DDGS decreased ($P<0.05$) concentrations of total monounsaturated fatty acids (MUFA), 16:0, 17:0, and 18:1, and increased ($P<0.05$) concentrations of 18:1T, 18:2, as well as total PUFA in the mitochondria. Long and very long chain PUFAs, such as 20:3, 20:4, 22:4, and 22:5 were unaffected by treatment. Results for the muscle fatty acid profile are shown in Table 3. Feeding DDGS decreased ($P<0.05$) concentrations of 15:0, 16:1, 17:0, and 17:1, increased ($P<0.05$) 18:2 and PUFA, and tended ($P<0.10$) to decrease MUFA. Day 14 mitochondria had higher ($P<0.05$) 18:2 and total PUFA contents compared to day 2 and 8 mitochondria, as shown in Table 4.

The SR membrane phospholipids (phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol) were not affected by diet. Likewise,

mitochondrial membrane phospholipids (phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, and cardiolipin) were not significantly different between the diets.

Results for artificial oxidation of mitochondria are shown in Figure 1. Oxidized mitochondria retained significantly less Ca than non-oxidized ($P<0.05$) mitochondria. Results for aging impact on mitochondrial calcium retention are shown in Figure 2. Day 2 mitochondria retained significantly less Ca than day 8 ($P<0.05$) mitochondria. Results for dietary impact on oxidation are shown in Figure 3 and 4. Overall, mitochondria from cattle finished on corn tended ($P<0.10$) to retain more Ca than mitochondria from cattle finished on DG.

DISCUSSION

When compared, the SR and mitochondrial membrane fatty acid profiles follow the same trend in conjunction with the whole muscle. This supports our hypothesis in using mitochondria as a model system for the SR. Modification of the mitochondrial membrane fatty acid profiles by feeding DDGS likely occurs by the same mechanism that modification of the SR membrane fatty acid profile occurs. Although the majority of unsaturated fatty acids undergo biohydrogenation by microbes in the rumen, DDGS have triple the amount of PUFAs (Ham et al., 1994), which leads to greater deposition of PUFA in the muscle and thus the organelles. In the cell, mitochondria and SR are very close in proximity. The two organelles are connected in bidirectional communication via a mitochondrial associated matrix, and gaps as small as 10 nm and up to 50 nm between the organelles have been documented (Franzini-Armstrong, 2007; Csordás et al., 2006). Not only are these organelles in close proximity to each other, they are physically

tethered to one another by proteins (Boncompagni et al., 2009). This allows for a direct calcium communication as well as the passage of phospholipids from the SR to the mitochondria after biosynthesis, according to (Rusinol et al., 1994). The close association of the mitochondria and the SR make it easy for reactive oxygen species (ROS) to go from the mitochondria and propagate on the PUFA of the SR membrane. Perhaps this is the mechanism by which the SR leaks calcium.

Postmortem, endogenous prevention mechanisms for ROS break down, thus allowing ROS to accumulate and cause rapid oxidation of membrane phospholipids and proteins (Esposti, 2002). The presence of PUFA in a membrane structure predisposes that membrane to a higher risk for oxidation, just by the nature and presence of double bonds and their high reactivity with ROS. Machlin and Bendich (1987) demonstrated that lipid oxidation occurs at an accelerated rate in cell membranes containing a high amount of PUFA.

Oxidation of membranes has many consequences for the integrity and function of that membrane. Some of the major consequences of oxidation on membranes include: increased permeability, decreased fluidity, deactivation of enzymes and proteins that are bound to the membrane, and cross-linking of proteins on the membrane (Stark, 2005). These consequences of oxidation can cause the stability of the membrane to decrease, thus allowing all of the contents bound by the membrane to be released. In the case of the sarcoplasmic reticulum and mitochondria, an oxidized membrane could possibly lead to a spillage of large quantities of calcium (Senaratne, 2012; Chao, 2015).

The reason why the decrease in MUFA in both organelle membranes occurred is unknown. Medium and long chain MUFA are products of biohydrogenation from dietary

PUFA and SFA to MUFA conversion from delta-9-desaturase (Wood et al., 2008). Perhaps, because the corn diet had less available PUFA, steers from the corn diet needed to generate more MUFA from SFA. Or, perhaps the overabundant PUFA concentrations in the DDGS diet suppressed the activity of delta-9-desaturase in the steers fed DDGS since there was readily available PUFA to biohydrogenate.

The aging effects on PUFA in the mitochondria were expected, because during aging of meat, oxidation propagates the formation of additional trans double bonds in other fatty acids, creating more unsaturated fatty acids (Schneider, 2010).

The medium used for artificial oxidation instigated the Fenton reaction. This reaction uses two steps: $2\ Fe^{2+} + 2\ H_2O_2 \rightarrow 2\ Fe^{3+} + 2\ OH^{\bullet} + 2\ OH^{-}$ and $2\ Fe^{3+}$ + Ascorbate $\rightarrow 2\ Fe^{2+}$ + Dehydroascorbate. Looking at these equations, one can see that ferrous iron (Fe^{2+}) reacts with hydrogen peroxide (H_2O_2) to form a hydroxyl radical ($OH^{\bullet}$). Ascorbate functions to reduce ferric iron (Fe^{3+}) into Fe^{2+} to promote further reactions with H_2O_2 to produce more $OH^{\bullet}$. The rapid production of $OH^{\bullet}$ is a model of ROS impact on the membrane of the mitochondria and the SR. Player and Hultin (1978) investigated the effects of lipid peroxidation on the calcium flux in sarcoplasmic reticulum microsomes. Player and Hultin (1978) found that when introducing pro-oxidant effects via the Fenton reaction and ascorbic acid, the release of calcium from the microsomes were increased two-fold.

The oxidation results from this study support our hypothesis. The artificially oxidized mitochondria retained less calcium due to the dramatic oxidative degradation of the membrane via the Fenton reaction. Isaeva et al. (2005) discovered that oxidative insult from the ROS molecules accelerated the onset and frequency of calcium sparks, or

impulsive bursts of calcium, from the sarcoplasmic reticulum. It was reasoned that this occurred due to membrane and IP3 receptor instability. Likewise, Byrd (1992) found that calcium spark was prolonged after fatiguing muscle activity, a process that produces many ROS. Another study by Hidalgo et al. (2005) showed that calcium spark activity is increased under conditions that overwhelm the local redox control mechanism of the mitochondria.

Mitochondria from DDGS fed steers tended to retain less calcium. This can be linked to the greater amount of PUFA deposited in the membranes of DDGS fed cattle, and directly supports our hypothesis.

Previous studies (Chao et al., 2015; Mlekusch et al., 1993) have demonstrated a trend of phosphatidylethanolamine phospholipid increasing and phosphatidylcholine phospholipid decreasing with a diet higher in PUFA. This study did not show any significant differences comparing mitochondrial phospholipids between the diets, however. Therefore, it may be that the actual hydrophobic portion of the membrane is more influential in calcium retention as opposed to the entire phospholipid bilayer. The effect of aging on oxidation of mitochondrial membranes did not support our hypothesis. It is well known and expected that oxidation increases as fats age, and this impacts shelf life and storage of meat (German 1999). Our results show the exact opposite effect in terms of the oxidation of the mitochondrial membranes. The reason for this is unknown. Perhaps measuring lipid oxidation on muscle tissue is not a good indicator of lipid oxidation behavior in the organelle membranes. Also, it may be that as oxidation increases, the fluidity of the organelle's membrane actually increases and thus becomes more resistant to breakage. Tai et al (2010) found that the hydroxyl radical

($OH^{\bullet}$) significantly increases the fluidity of membranes as compared to other radical species, such as H_2O_2. This may explain why we saw this phenomenon, since we employed the Fenton reaction to produce such a large amount of $OH^{\bullet}$. Perhaps because the day 8 mitochondria were subjected to ROS and lipid oxidation conditions for longer than the day 2 samples, they became more fluid and resistant to breakage. More research on the fluidity and holding capacity of membranes should be conducted to clarify this issue.

Although this study focuses on membrane oxidation, there is much evidence that protein oxidation may also have a tremendous influence on calcium flux. For example, specific studies on the proteins of the sarcoplasmic reticulum and the mitochondria by Sharov et al. (2006) show that because the SERCA, RYR1, and IP3 have many cysteine amino acid residues, they are of greater risk for oxidative damage. The most common molecular targets for ROS are the cysteine thiol groups in proteins. These thiol groups will react with ROS and undergo multiple alterations that impact the function of the proteins severely (Hidalgo and Donoso, 2008; Bishop et al., 1988). In the SERCA it has been discovered that extensive exposure to massive quantities of ROS will inhibit the SERCA pump, causing a calcium depletion induced stress on the sarcoplasmic reticulum (Li and Camacho, 2004). Postmortem, this serves as a prevention of calcium flux back into the sarcoplasmic reticulum and allows a great deal of calcium to be free floating in the sarcoplasm. Although the true mechanisms for free calcium release are still unclear, there is evidence for a new perspective on the molecular basis for meat tenderization.

CONCLUSION

Results indicate that greater PUFA content deposited in organelles may affect Ca flux by increased susceptibility to oxidation. A DG diet may influence Ca flux and ultimate tenderness by this mechanism.

Literature Cited

Allan, D. and S. Cockcroft. 1982. A modified procedure for thin-layer chromatography of lipids. J. Lipid Res. 23: 1373-1374.

Baron, C. B., and R. F. Coburn. 1984. Comparison of two copper reagents for detection of saturated and unsaturated neutral lipids by charring densitometry. J. Liq. Chrom. 7: 2793-2801.

Bishop, J. E., T. C. Squier, D. J. Bigelow, and G. Inesi. 1988. (Iodoacetamido) fluorescein labels a pair of proximal cysteines on the Ca2+-ATPase of sarcoplasmic reticulum. J. Biochem. 27:5233-5240.

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.

Boncompagni, S., A. E. Rossi, M. Micaroni, G. V. Beznoussenko, R. S. Polishchuk, R. T. Dirksen, and F. Protasi. 2009. Mitochondria are linked to calcium stores in striated muscle by developmentally regulated tethering structures. J. Mol. Biol. Cell 20:1058-1067.

Byrd, S. K. 1992. Alterations in the sarcoplasmic reticulum: a possible link to exercise-induced muscle damage. J. Med. Sci. Sports Exercise 24:531-536.

Chao, M. 2015. Impact of wet distillers grains plus solubles and antioxidants on a basic mechanism of beef tenderization. Dissertation, University of Nebraska-Lincoln.

Csordás, G., C. Renken, P. Varnai, L. Walter, D. Weaver, K. F. Buttle, T. Balla, C. A. Mannella, and G. Hajnóczky. 2006. Structural and functional features and significance of the physical linkage between ER and mitochondria. J. Cell Biol. 174:915-921.

Depenbusch, B. E., C. M. Coleman, J. J. Higgins, and J. S. Drouillard. 2009. Effects of increasing levels of dried corn distillers grains with solubles on growth performance, carcass characteristics, and meat quality of yearling heifers. J. Anim. Sci. 87: 2653-2663.

Esposti, M. D. 2002. Measuring mitochondrial reactive oxygen species. Cancer research campaign molecular pharmacology group. Methods 26:335-340.

Franzini-Armstrong, C. 2007. ER-mitochondria communication. How privileged? J. Physiol. 22:261-268.

German, J. B. 1999. Food processing and lipid oxidation. Adv. Exp. Med. Biol. 459: 23-50.

Ham, G., R. Stock, T. Klopfenstein, E. Larson, D. Shain, and R. Huffman. 1994. Wet corn distillers byproducts compared with dried corn distillers grains plus solubles as a source of protein and energy for ruminants. J. Anim. Sci. 72: 3246-3257.

Hemmings, S. J. 2001. New methods for the isolation of skeletal muscle sarcolemma and sarcoplasmic reticulum allowing a comparison between the mammalian and amphibian beta(2)-adrenergic receptors and calcium pumps. J. Cell Biochem. 19:133-141.

Hidalgo, C. and P. Donoso. 2008. Crosstalk between calcium and redox signaling: from molecular mechanisms to health implication. Antioxidant Redox Signal 10:1275-1312.

Hidalgo, C., P. Donoso, and M. A. Carrasco. 2005. The ryanodine receptors Ca2+ release channels: cellular redox sensors? IUBMB Life 57:315-322.

Isaeva, E. V., V. M. Shkryl, and N. Shirokova. 2005. Mitochondrial redox state and Ca2+ sparks in permeabilized mammalian skeletal muscle. J. Physiol. 565:855-872.

Klopfenstein, T. J., G. E. Erickson, and V. R. Bremer. 2007. Board-invited interview: Use of distillers byproducts in the beef cattle feeding industry. J. Anim. Sci. 86.5: 1223-1231.

Leray, C., X. Pelletier, S. Hemmendinger, and J. P. Cazenave. 1987. Thin-layer chromatography of human platelet phospholipids with fatty acid analysis. J. Chromoatography 420:411-416.

Li, Y. and P. Camacho. 2004. Ca2+-dependent redox modulation of SERCA 2b by ERp57. J. Cell Biol. 164:35-46.

Machlin, L. J. and A. Bendich. 1987. Free radical tissue damage: protective role of antioxidant nutrients. FASEB J. 1:441-445.

Martonosi, A. and R. Feretos. 1964. Sarcoplasmic reticulum: the uptake of Ca++ by sarcoplasmic reticulum fragments. J. Biol. Chem. 239:648-658.

Medh, J. D. and P. H. Weigel. 1989. Separation of phosphatidylinositols and other phospholipids by two-step one-dimensional thin-layer chromatography. J Lipid Res. 30: 761-764.

Metcalfe, L. D., A. A. Schmitz, and J. R. Pelka. 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Analytical Chem. 38: 514-515.

Miller, M. F., M. A. Carr, C. B. Ramsey, K. L. Crockett, and L.C. Hoover. 2001. Consumer thresholds for establishing the value of beef tenderness. J. Anim. Sci 79: 3062-3068.

Mlekusch, W., C. Celedin, R. C. Aloia, and R. Moller. 1993. Effect of a high fat diet on phospholipid class distribution and fatty acid composition in rat liver. Int. J. Biochem. 25:1539-1547.

Morrison, W. R. and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride – methanol. J. Lipid Res. 5:600-608.

Player, T. J. and H. O. Hultin. 1978. The effect of lipid peroxidation on the calcium-accumulating ability of the microsomal fraction isolated from chicken breast muscle. J. Biochem. 174:17-22.

Rusinol, A. E., Z. Cui, M. H. Chen, and J. E. Vance. 1994. A unique mitochondria-associated membrane fraction from rat liver has a high capacity for lipid synthesis and contains pre-golgi secretory proteins including nascent lipoproteins. J. Biol. Chem. 269:27494-27502.

Schneider, Claus. 2010. An update on products and mechanisms of lipid peroxidation. J. Mol. Nutr. Food Res. 53: 315-321.

Segers, J., R. Stewart Jr., C. Lents, T. Pringle, M. Froetschel, B. Lowe, R. McKeith, and A. Stelzleni. 2011. Effect of long-term corn by-product feeding on beef quality, strip loin fatty acid profiles, and shelf life. J. Anim. Sci. 89: 3792-3802.

Senaratne, L. S. 2012. Mechanism and control of beef toughening during retail display in high oxygen modified atmosphere packages. Dissertation, University of Nebraska-Lincoln.

Sharov, V. S., E. S. Dremina, N. A. Galeva, T. D. Williams, and C. Schoneich. 2006. Quantitative mapping of oxidation-sensitive cysteine residues in SERCA in vivo

and in vitro by HPLC-electrospray-tandem MS: selection protein oxidation during biological aging. J. Biochem. 394:605-615.

Smith, A. L. 1967. Preparation, properties, and conditions for assay of mitochondria: slaughterhouse material, small scale. Methods in Enzymology. 10: 81-86.

Stark, G. 2005. Functional consequences of oxidative membrane damage. J. Mem. Biol. 205:1-6.

Tai, W-Y., Y-C. Yang, H-J. Lin, C-P. Huang, Y-L. Cheng, M-F. Chen, H-L. Yen, and I. Liau. 2010. Interplay between Structure and Fluidity of Model Lipid Membranes under Oxidative Attack. J. Phys. Chem. 114: 15642-15649.

Wood, J. D., M. Enser, A. V. Fisher, G. R. Nute, P. R. Sheard, R. I. Richardson, S. I. Hughes, and F. M. Whittington. 2008. Fat deposition, fatty acid composition and meat quality: A Review. Meat Sci. 78: 343-358.

Table 1. Fatty acid profile of the sarcoplasmic reticulum membrane from strip loins (*Longissimus lumborum*) from steers fed dried distillers grains plus solubles (DDGS) and corn-only finishing diets.

	Dietary treatments			
Fatty Acids, %	50% DDGS	Corn	SEM	P-value
C14:0	1.21	1.51	0.26	0.24
C14:1	0.60	0.67	0.22	0.77
C15:0	1.68	1.31	0.46	0.43
C15:1	1.56	1.42	3.7	0.57
C16:0	16.12	15.54	3.87	0.87
C16:1	1.54	1.72	0.25	0.47
C17:0	0.98	1.41	0.19	0.03
C17:1	2.94	6.12	2.69	0.76
C18:0	9.82	9.34	1.85	0.43
C18:1T	10.55	12.30	4.52	0.70
C18:1	18.21	22.15	4.85	0.51
C18:1V	6.50	5.50	2.08	0.61
C18:2	16.97	7.61	2.11	<0.01
C18:3	1.50	1.08	0.78	0.60
C20:3	1.55	1.04	0.26	0.06
C20:4	3.85	3.01	0.97	0.07
C22:4	3.97	3.78	1.77	0.92
C22:5	0.38	0.67	0.78	0.40
SFA	29.81	29.11	3.64	0.78
UFA	70.12	67.07	3.72	0.97
SFA:UFA	0.43	0.43	0.05	0.40
PUFA	28.22	17.19	3.95	0.09
MUFA	41.90	49.88	4.42	0.55

SFA=saturated fatty acids, UFA=unsaturated fatty acids, PUFA=polyunsaturated fatty acids, and MUFA = monounsaturated fatty acids

Table 2. Fatty acid profile of the mitochondrial membrane from strip loins (*Longissimus lumborum*) from steers fed dried distillers grains plus solubles (DDGS) and corn-only finishing diets.

	Dietary treatments			
Fatty Acids, %	50% DDGS	Corn	SEM	P-value
C14:0	1.34	1.53	0.15	0.18
C14:1	0.48	0.50	0.12	0.85
C15:0	0.93	1.15	0.23	0.35
C15:1	1.69	1.50	0.24	0.39
C16:0	21.50	23.26	0.74	0.02
C16:1	1.73	2.02	0.19	0.13
C17:0	0.95	1.33	0.1	<0.01
C17:1	1.32	1.23	0.28	0.76
C18:0	14.24	13.32	0.58	0.12
C18:1T	3.52	1.80	0.59	<0.01
C18:1	25.33	31.92	1.86	<0.01
C18:1V	2.80	3.37	0.94	0.54
C18:2	15.47	8.94	1.11	<0.01
C18:3	0.90	0.63	0.75	0.72
C20:3	2.01	1.17	0.52	0.11
C20:4	4.00	3.64	0.53	0.11
C22:4	0.92	0.94	0.24	0.95
C22:5	0.88	0.81	0.27	0.79
SFA	38.96	40.59	1.17	0.14
UFA	61.04	58.48	1.23	0.05
SFA:UFA	0.64	0.69	0.03	0.12
PUFA	24.19	16.30	1.99	<0.01
MUFA	36.87	42.34	1.36	<0.01

SFA=saturated fatty acids, UFA=unsaturated fatty acids, PUFA=polyunsaturated fatty acids, and MUFA = monounsaturated fatty acids

Table 3. Fatty acid profile of the muscle from strip loins (*Longissimus lumborum*) from steers fed dried distillers grains plus solubles (DDGS) and corn-only finishing diets.

	Dietary treatments			
Fatty Acids, %	50% DDGS	Corn	SEM	P-value
C14:0	2.46	2.51	0.23	0.83
C14:1	0.62	0.59	0.08	0.78
C15:0	0.41	0.52	0.03	<0.01
C15:1	0.53	0.55	0.06	0.77
C16:0	23.93	24.06	0.69	0.85
C16:1	2.41	2.86	0.18	<0.05
C17:0	1.09	1.66	0.09	<0.01
C17:1	0.76	1.29	0.08	<0.01
C18:0	13.92	13.32	0.79	0.46
C18:1T	2.61	1.77	0.29	<0.01
C18:1	35.44	38.19	0.91	<0.01
C18:1V	7.49	6.78	0.85	0.41
C18:2	5.50	3.02	0.27	<0.01
C18:3	0.45	0.32	0.09	0.16
C20:3	0.26	0.22	0.03	0.17
C20:4	0.80	0.75	0.09	0.57
C22:4	0.16	0.16	0.02	0.93
C22:5	0.15	0.15	0.02	0.72
SFA	41.81	42.08	1.24	0.83
UFA	57.01	56.57	1.26	0.73
SFA:UFA	0.74	0.75	0.04	0.86
PUFA	7.15	4.54	0.39	<0.01
MUFA	49.86	52.03	1.04	0.05

SFA=saturated fatty acids, UFA=unsaturated fatty acids, PUFA=polyunsaturated fatty acids, and MUFA = monounsaturated fatty acids

Table 4. Effect of aging on 18:2 and polyunsaturated fatty acid (PUFA) content in the mitochondrial membrane.

Aging (days)	18:2 (%)	PUFA (%)
2	12[a]	19[a]
8	9[a]	16[a]
14	15[b]	23[b]

Figure 1. Calcium concentrations retained in mitochondria from strip loins (*Longissimus lumborum*) that were non-oxidized versus oxidized.

Figure 2. Calcium concentrations retained in mitochondria from strip loins (*Longissimus lumborum*) aged for 2 or 8 days.

Figure 3. Calcium concentrations retained in mitochondria from strip loins (*Longissimus lumborum*) from steers fed dried distillers grains plus solubles and corn-only finishing diets.

Figure 4. Calcium concentrations retained in mitochondria from strip loins (*Longissimus lumborum*) from steers fed dried distillers grains plus solubles and corn-only finishing diets separated by oxidation treatment.

Figure 1.

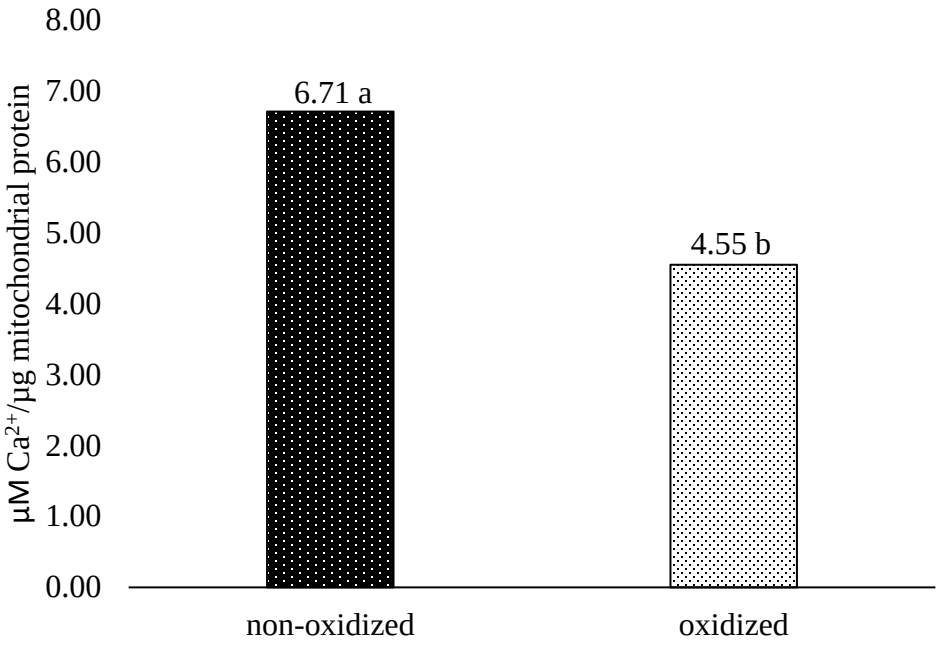

8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
µM Ca2+/µg mitochondrial protein
6.71 a
4.55 b
non-oxidized
oxidized

Figure 2.

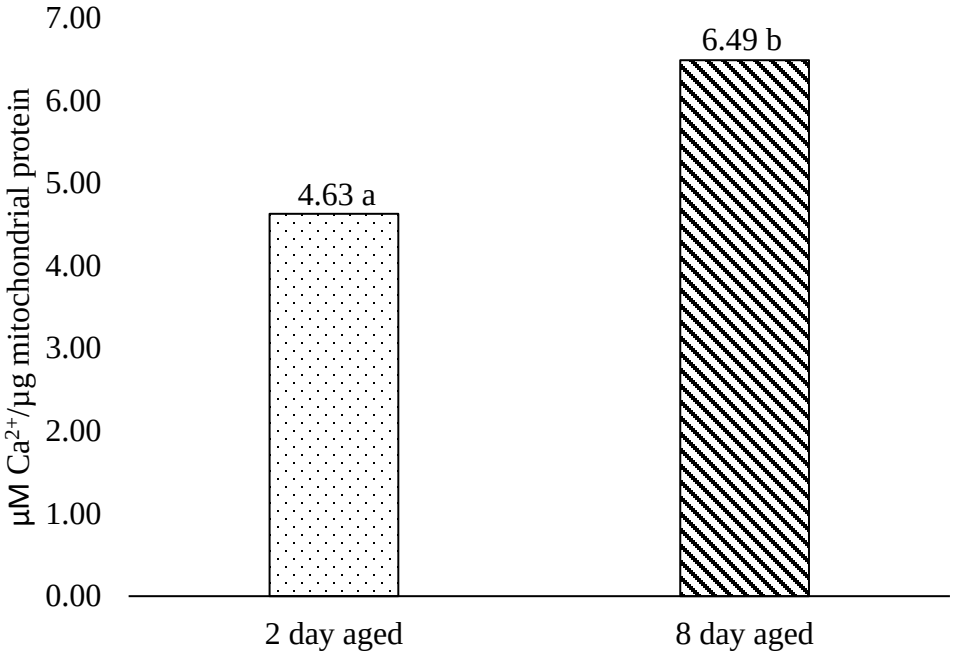

7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
µM Ca2+/µg mitochondrial protein
4.63 a
6.49 b
2 day aged
8 day aged

Figure 3.

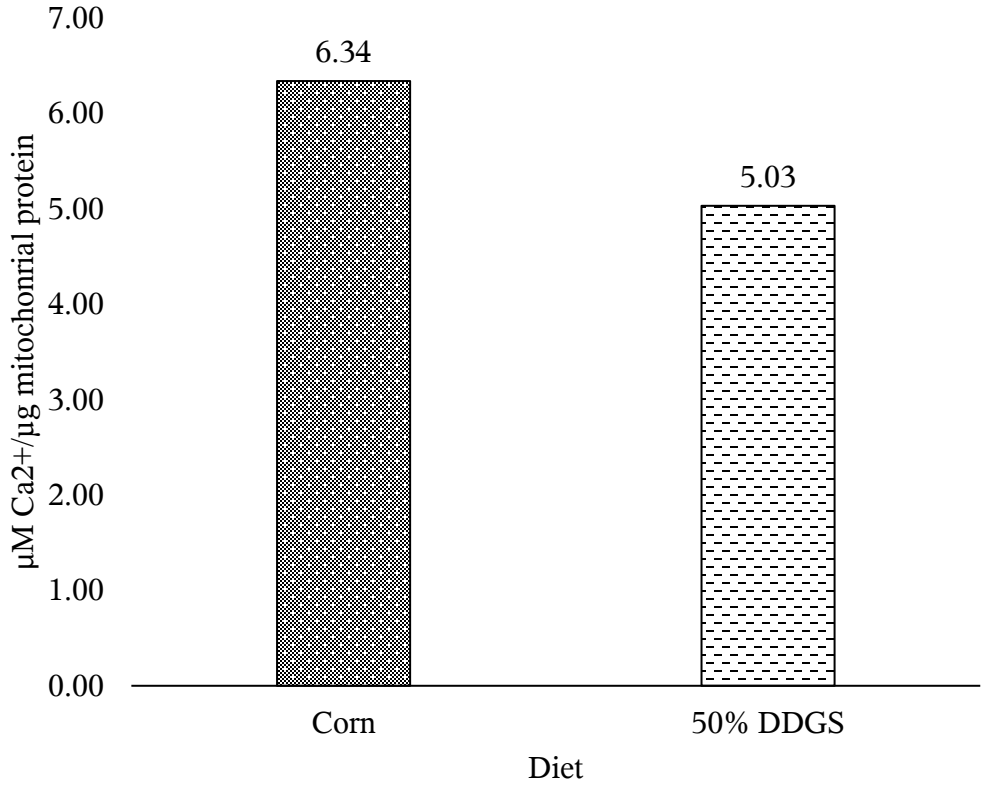
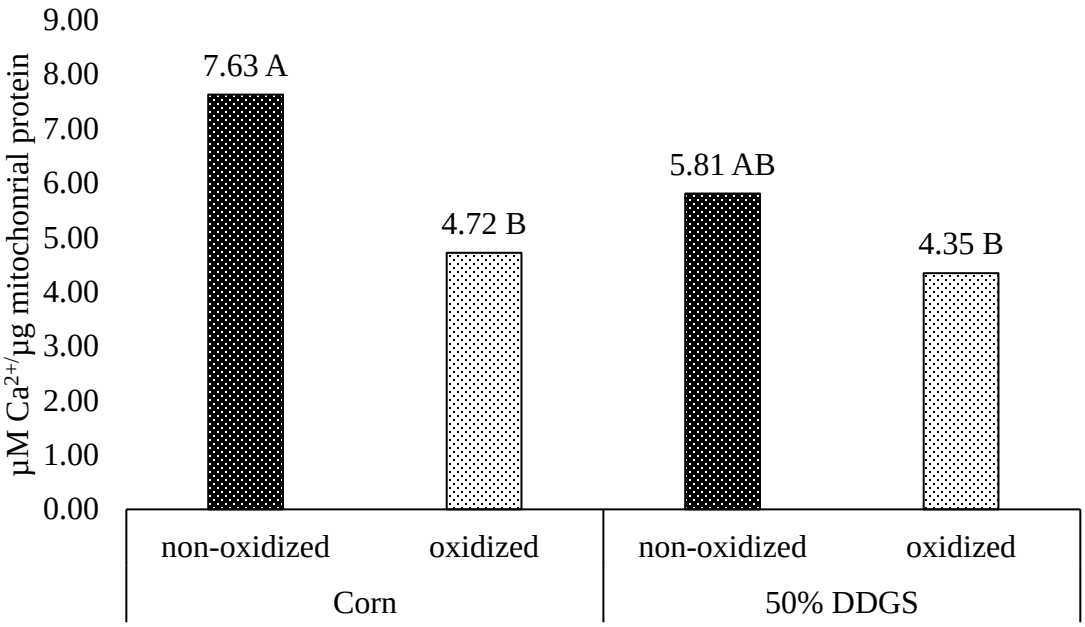

7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
µM Ca2+/µg mitochonrial protein
6.34
5.03
Corn
50% DDGS
Diet

Figure 4.


9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
µM Ca2+/µg mitochonrial protein
7.63 A
4.72 B
5.81 AB
4.35 B
non-oxidized
oxidized
non-oxidized
oxidized
Corn
50% DDGS

RECOMMENDATIONS FOR FUTURE RESEARCH

This research has opened gateways into further research on this topic; the possibilities are endless. I do not believe that oxidation of membranes is the sole culprit for calcium release from the mitochondria postmortem. There are many proteins that regulate the flux of calcium between the mitochondria, sarcoplasm, and sarcoplasmic reticulum in the living muscle cell. These proteins are also subject to oxidation. Furthermore, the proteins involved in ATP manufacture within the mitochondria are subject to oxidation. I think it would be useful to extract mitochondria and observe the metabolic shifts that occur between diets, focusing on protein function and alterations. There is equipment that can monitor the oxygen consumption and metabolic trends in respiring mitochondria. Investigating these trends may help decipher some differences in calcium management between mitochondria that have undergone stress of whatever sort. In addition, measuring the rate of respiration, either by ADP consumption or ATP production would be a good method to observe the functionality of the mitochondria. In order to do this, I believe it would be beneficial to use pre rigor muscle, or muscle biopsies. Studying aged mitochondria does not help us solve the conditions that preceded the aging results. Moreover, fluorometric and radiology methods would be more effective in quantification and looking into the movement behavior of calcium.

Some questions worth exploring are:

How does feeding diets high in PUFA (such as distillers grains) affect protein function of the SR and mitochondria?

How can we correlate protein oxidation or protein function and calcium flux?

How can we correlate genetics in this equation? Mitochondria have their own RNA that is passed along the maternal line. Are there epigenetic differences in dams that can be passed to their offspring that affect meat quality through mitochondrial metabolic processes?

These concepts cannot be explored by the meat science department only. Collaboration with the nutrition, biochemistry, and proteomic departments should be considered.

On another note, I am personally very intrigued with fetal programming and its effects on the performance of offspring. There is increasing evidence that demonstrates that the level of maternal nutrition alters fetal skeletal development, with long-term effects on offspring growth and performance. Not only does maternal nutrition impact skeletal development: it also influences adipogenesis (fat development) and fibrogenesis (connective tissue, i.e. collagen, development). A low plane of maternal nutrition has demonstrated decreased birthweights, smaller sized offspring, and many other growth and development hindrances over the lifetime of the offspring.

The fetal stage is crucial for skeletal development, because no hyperplasia (increasing number of muscle fibers) occurs postpartum, only hypertrophy (enlarging of muscle fibers) occurs after birth. A lower nutritive quality in the cow can decrease the number of muscle fibers available in the offspring, thus decreasing the muscle mass and negatively impacting animal performance later on. It is known that skeletal muscle has less priority in the developing fetus as compared to the fetus' brain, heart, and other organs. Therefore, any source of nutrient deficiency in the dam will render the skeletal

development of the offspring much more susceptible to poor development. Muscle fibers, research shows, are developing in the fetus around mid to late (2-8 months) gestation, therefore nutrient deficiency during this stage will have very severe physiological consequences for offspring. Conversely, adequate nutrition may improve the lean-to-fat ratio of offspring and improve the overall production efficiency of the offspring. Other research has demonstrated that marbling of offspring can be increased by putting cows on good pasture conditions.

Likewise, the fetal stage is crucial for adipogenesis, as it is initiated around mid-gestation in ruminants. Marbling is correlated with the number and size of adipocytes inside the muscle. Increased fetal adipocyte number results in more intramuscular adipocytes, which accumulate fat and form marbling during postnatal growth. Research has shown that steers that were calved by mothers that were fed lower protein supplement had lower marbling scores when compared with steers whose mothers were fed a full protein supplement. Additionally, the steers who were calved by full supplement mothers had less subcutaneous “waste” fat. Thus, these steers were more efficient in the finishing phase of production.

Relative to adipocyte development, fibrogenesis is also impacted during mid-gestation of fetal development. Fibroblasts are the precursors for connective tissue, collagen specifically. It is well known that collagen and connective tissue are the major contributors to the background toughness associated with meat. Therefore, it would be interesting to see how certain planes of nutrition of the cow affect the tenderness of the meat of their offspring, as no research on this topic has been done. Additionally, because adipogenesis and fibrogenesis share similar precursor cells, it provides an opportunity to

favor adipogenesis. Enhancing adipogenesis can increase marbling, thus improving flavor, while decreasing fibrogenesis may reduce connective tissue, thus decreasing background toughness. With both an increased adipogenesis and a decreased fibrogenesis, the eating quality of beef, both by tenderness and flavor may be improved.

It is taught that maternal nutritional management on altering marbling has greatest effects in the fetal stage, followed by the neonatal stage, followed by the early weaning stage, followed by the weaning and older stages.

Focusing on fetal development is likely to prove very beneficial in management to improve the growth efficiency of cattle and the eating quality of beef.

Even though genotype has a tremendous play in efficiency of animals in quality and yield, growth and development of animals is also extremely influenced by epigenetic changes. “Epigenetics” refers to the environmental factors that switch genes on and off and affect how cells read genes, as opposed to just having base pair changes in DNA. Environmental factors have an extreme play in an animal’s potential. These can be defined broadly, but it is known that nutrition is a key epigenetic influence. Environmental factors begin in the womb, considering the nutrients available from the mother for the offspring, and continue when the offspring is growing and developing post-partum. Furthermore, an animal’s body is always adjusting to new changes from its environment.

One of the organelles that is in control of energy partitioning and metabolism, and nutrient partitioning and adjustments in the muscle cell is the mitochondria. It is known that the mitochondria is maternally passed on to offspring. But there are many questions that still remain as to how the lack of nutrients in a mother can alter the mitochondrial

function in the prenatal offspring, as well as in post-partum offspring growth and development. Perhaps these epigenetic influences on the fetus will instigate a mitochondrial shift in the offspring's energy metabolism, thus influencing their ultimate potential in growth and metabolic functions, which will impact marbling and yield of product. Research shows that increased amounts of riboflavin, thiamin, lipoic acid, and other cofactors derived from adequate, balanced diets aids in mitochondrial function, while the opposite hurts mitochondrial efficiency and produces higher amounts of ROS. Perhaps the link between nutritional inadequacy and poor performance is much more involved than simply the poor muscle or fat or connective tissue cell development. Perhaps, the underlying reason why low nutrient availability to mothers who produce offspring with low performance in yield and quality is due to mitochondrial dysfunction and excessive ROS production.

That is the concept behind my design for this fetal programming of tenderness. It relies less on mesenchymal cell focus and more on mitochondrial development and function. We can look at mitochondrial function, ROS production, ATP generation, and mitochondrial RNA differences between mothers and the offspring at different points in their life (weaning, stocker, feedlot) and see how those epigenetic differences affect the mitochondrial function and efficiency of metabolism in the animals. To top things off, we can carry out the experiment one step further than anyone else and see what differences in tenderness show.

Furthermore, I learned about mTORC1, which is the mammalian target of rapamycin complex 1. It is a protein complex that functions as a nutrient and redox

sensor and controls growth and development in mammals. mTORC1 functions when AMPK is not functioning. The mTORC1 pathway is associated with diabetes and cancer in humans and is widely studied in the biomedical field. I think it would be neat to explore this mTORC1 function in association with different diets and genetics with cattle. Keep in mind that the methods to study this pathway are very intricate and require a well-equipped biochemistry lab. Specifically some questions to ask are:

How is mTORC1 capacity affected by increased PUFA in the diet? Does sestrin2 (a molecule upstream of mTORC1) get more oxidized and lose function?

How does mTORC1 activity change with induced stress on the animal (diet, handling, etc)?

How does mTORC1 activity alter meat quality in association to animal diet and stress? How is calcium flux impacted by mTORC1 activity under different diets or stresses?

How does mTORC1 activity differ between ruminants and non-ruminants?

Appendix I

SR Membrane Extraction
(Hemmings, 2011)

1. Weigh 10 g powdered meat sample into a 100 ml plastic beaker.
2. Add 35 ml of 4^{o}C homogenization buffer: 10mM $NaHCO_3$, 2 mM NaN_3, 10 mM Tris-Cl (pH 7.5).
3. Homogenize on ice with Polytron homogenizer on setting 6 for 15 sec, setting 7 for 15 sec, and setting 8 for 15 sec, check for entire homogenization. If not homogenized completely, redo setting 6 for 30 sec.
4. Filter homogenate through two layers of cheesecloth into a 50 ml plastic centrifuge tube.
5. Centrifuge at 2,000 x g for 10 min at 4^{o}C.
6. Collect and retain supernatant.
7. Add 10 ml homogenization buffer and suspend the pellet by vortexing for 5 seconds.
8. Centrifuge at 2,000 x g for 10 min at 4^{o}C.
9. Collect supernatant and combine with retained supernatant in a 70 ml centrifuge tube (Thermo Scientific, 010-1333).
10. Centrifuge at 10,000 x g for 30 min at 4^{o}C.
11. Transfer supernatant to another 70 ml centrifuge tube.
12. Add 3.15 g KCl.
13. Place on shaker for 30 min on ice.
14. Centrifuge at 100,000 x g for 60 min at 4^{o}C (Sorvall F37L-8x100 Thermo Scientific, 096-087056).
15. Discard supernatant. Use a transfer pipette to remove any remaining supernatant.
16. Add 1 ml of 10 mM Tris-Cl and suspend the pellet in solution.
17. Transfer the suspended pellet to a 13x100 mm screw cap glass tube.
18. Store sample at -80^{o}C.

Reference:

Hemmings, S. J. 2001. New methods for the isolation of skeletal muscle sarcolemma and sarcoplasmic reticulum allowing a comparison between the mammalian and amphibian beta(2)-adrenergic receptors and calcium pumps. Cell Biochem. Funct. 19:133-141.

Appendix II

Mitochondria Extraction
(Smith, 1967)

1. Weigh 10 g of powdered meat sample into a 100 ml plastic beaker.
2. Add 50 ml maintenance buffer: 70mM sucrose, 200mM mannitol, 5mM HEPES, and 10mM K_2PO_4.
3. Homogenize with polytron at setting 5 with the large probe for two, 10 sec intervals on ice (if not completely homogenized after two intervals, one more may be used, but be delicate!!)
4. Filter the homogenate using a double layered cheesecloth into two 50 ml centrifuge tubes.
5. Centrifuge at 650xg for 10 mins at 4^{o}C.
6. Strain both supernatants through double layered cheesecloth and put into another two 50 ml centrifuge tubes.
7. Repeat steps 5 and 6 one more time.
8. Collect both retained supernatants and place into a 70 ml PC centrifuge tube (Thermo Scientific, 010-1333).
9. Centrifuge at 7750xg for 25 mins at 4^{o}C using a fixed angle rotor (Sorvall F37L-8x100 Thermo Scientific, 096-087056).
10. Decant the supernatant and keep the pellet.
11. Wash the pellet three times with maintenance buffer to get rid of the partially destroyed mitochondria on the pellet surface.
12. Suspend pellet in 1 ml maintenance buffer.
13. Freeze in -80^{o}C freezer until ready to use.

**If continuing on for artificial oxidation, quantify mitochondrial protein via BCA protein assay. Use 100 µL mitochondria stock from step 12 and add 100 µL maintenance buffer as sample preparation and proceed with kit instructions.

Reference:

Smith, A. L. 1967. Preparation, properties, and conditions for assay of mitochondria: slaughterhouse material, small scale. Methods in Enzymology. 10: 81-86.

Appendix III

SR and Mitochondria Membrane Lipid Extraction
(Bligh and Dyer, 1959)

1. Add 3.75 mL 1:2 chloroform:methanol (v/v) to the 13 x 100 mm glass tube with the mitochondria/SR pellet.
2. Vortex for 5 sec.
3. Keep the mixture at 4^{o}C overnight.
4. Add 1.25 ml chloroform and mix on shaker for 1 min.
5. Add 1.25 ml water and mix on shaker for 1 min.
6. Filter through Whatman #2 filter paper into another 13 x 100 mm screw cap tube.
7. Centrifuge samples at 1,000 x g for 5 min.
8. Aspirate off aqueous (top) layer.
9. Evaporate to dryness under nitrogen at 60^{o}C.
10. Continue on for either fatty acid analysis or lipid profile analysis.

Reference:

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.

Appendix IV

SR and Mitochondrial Fatty Acid Analysis through Gas Chromatography
(Metcalfe et al., 1966; Morrison and Smith, 1964)

1. Add 0.5 mL of 0.5 M NaOH in methanol to each dried SR or mitochondria membrane lipid sample.
2. Vortex for 5 sec.
3. Heat in oven for 5 min at 100^{o}C.
4. Add 0.5 mL of boron triflouride in 14% methanol.
5. Vortex for 5 sec.
6. Heat in oven for 5 min at 100^{o}C.
7. Add 1 mL of a saturated salt solution and 1 mL of hexane.
8. Vortex for 5 sec.
9. Centrifuge samples at 1,000 x g for 5 min.
10. Remove hexane (top) layer, making sure not to disrupt the aqueous (bottom) layer, and place in glass tube.
11. Evaporate the hexane layer to dryness under nitrogen at 60^{o}C in the GC vial.
12. Add 100 µL of hexane and immediately transfer to a 100 µL spring bottom vial insert.
13. Place insert inside the GC vial and purge with nitrogen, cap, and crimp cap.
14. Store at -80^{o}C until the sample is ready to be analyzed by GC machine.

GC Settings:

Column: Chrompack CP-Sil 88 (0.25mm x 100 m)
Injector Temp.: 270^{o}C
Detector Temp.: 300^{o}C
Head Pressure: 40 psi
Flow Rate: 1.0mL/min
Temperature program: Start at 140^{o}C and hold for 10 min. following 10 min, raise temperature 2^{o}C/min until temperature reaches 220^{o}C. Hold for 20 min at 220^{o}C.

References:

Metcalfe, L. D., A. A. Schmitz, and J. R. Pelka. 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem. 38: 514-515.

Morrison, W. R. and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride – methanol. J. Lipid Res. 5:600-608.

Appendix V

SR and Mitochondria Membrane Phospholipid Analysis through 1D Thin Layer Chromatography
(Leray et al., 1987; Baron and Coburn, 1984)

1. Add 30 µL of 2% methanol and 1% distilled deionized water in chloroform to each dried SR or mitochondria membrane lipid sample.
2. Pre-wash TLC plate (Whatman K5, Clifton, NJ) with 1:1 chloroform:methanol (v/v).
3. Air-dry plate completely in fume hood.
4. Thoroughly wet plate with 2.3% boric acid in ethanol.
5. Drain and completely dry plate in fume hood.
6. Dry plate in oven for 15 min at 100^{o}C.
7. Draw reference starting line using a pencil.
8. Spread 20 µL at each designated spot (one per sample) on the reference line (about 8 per plate).
9. Place prepared plate in 100 mL solvent (chloroform/ethanol/water/triethylamine 30:35:7:35 v/v/v/v) in a TLC tank until migration of solvent reaches 1 cm from the top of the plate (about 2.5-3 hours).
10. Dry in fume hood until plate is completely dry.
11. Saturate plate completely with 10% cupric sulfate in 8% phosphoric acid.
12. Dry in fume hood until completely dry.
13. Place plate in oven at 180^{o}C until lipid spots show (about 5-20 mins).
14. Analyze plate using Quantity One 1D image analysis software (Bio-Rad, Hercules, CA).
15. Measure each phospholipid as percentage of lane.

References:

Baron, C. B., and R. F. Coburn. 1984. Comparison of two copper reagents for detection of saturated and unsaturated neutral lipids by charring densitometry. J. Liq. Chrom. 7: 2793-2801.

Leray, C., X. Pelletier, S. Hemmendinger, and J. P. Cazenave. 1987. Thin-layer chromatography of human platelet phospholipids with fatty acid analysis. J. Chrom. 420:411-416.

Appendix VI

Mitochondria Artificial Oxidation
(Martonosi and Feretos, 1964)

1. Transfer isolated mitochondria to a 5 mL ultracentrifuge tube (Thermo Fisher, 03127).
2. Add 2 mL loading buffer: 0.1 M KCl, 5mM HEPES, 0.5 mM $CaCl_2$, 0.2 mM $MgCl_2$, 4 mM ADP.
3. Vortex for 2 sec
4. Let sit for 30 mins
5. To half the samples: add 2 mL oxidation medium: 0.1 M KCl, 5mM HEPES, 0.2 mM $MgCl_2$, 4 mM ADP, 0.1 mM $FeCl_3$, and 0.25 mM ascorbic acid.
6. Vortex for 2 sec
7. Let sit for 5 mins
8. To other half of the samples: add 2 mL loading buffer.
9. Vortex for 2 sec
10. Let sit for 5 mins
11. Centrifuge samples at 10,000xg for 25 mins at 4^{o}C using a swinging bucket rotor (AH-650 Thermo Scientific, 54294).
12. Empty supernatant and keep the pellet
13. Suspend pellet in 2 mL extraction buffer (70mM sucrose, 200mM mannitol, 5mM HEPES, and 10mM K_2PO_4).
14. Sonicate at a 30% amplitude for 30 sec (Branson Digital Sonifier 450, 2004, 50/60Hz 4.0 A)
15. Transfer sonicated sample to a 2 mL eppendorf tube.
16. Ship samples to Ward Lab (Kearny, NE) for ICP-OES Ca++ concentration analysis.

Reference:

Martonosi, A. and R. Feretos. 1964. Sarcoplasmic reticulum: the uptake of Ca++ by sarcoplasmic reticulum fragments. J. Biol. Chem. 239:648-658.

Appendix VII

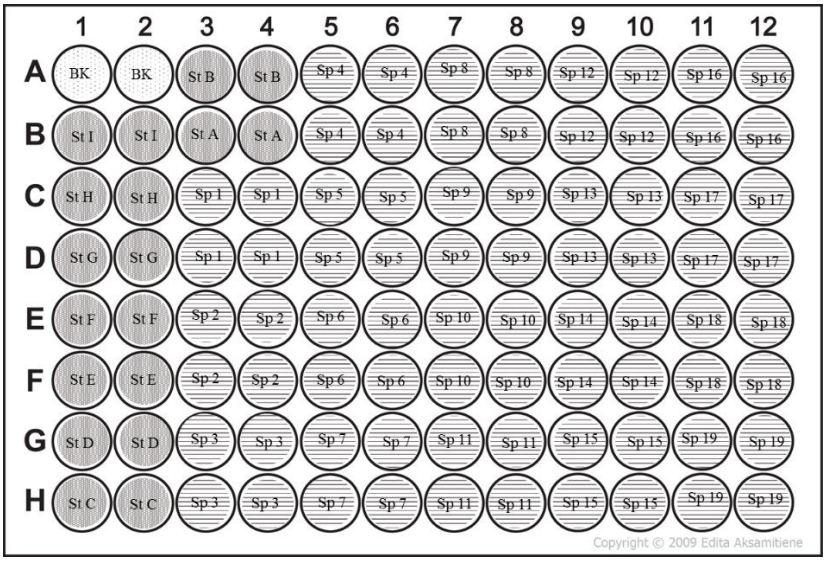
Mitochondria Protein Concentration
(Pierce BCA protein assay kit; Pierce Biotechnology, Rockford, IL)

1. Add 100 µL mitochondria stock to 100 µL maintenance buffer and vortex thoroughly.

2. Prepare concentration series (20 – 2,000 µg/mL) of bovine serum albumin (BSA) in the kit as follows:

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

3. Place 25 µL of BSA standards and the diluted mitochondrial protein samples on a 96 well microplate as follows:



BK = blank
St = standard
Sp = sample

4. Add 200 µL BCA working reagents (50:1, Reagent A: reagent B) to respective wells in the microplate.

5. Mix protein samples and BCA working reagents thoroughly on a plate shaker for 30 seconds.

6. Incubate the microplate at 37°C for 30 minutes and cool to the room temperature.

7. Read absorbance at 562 nm with a microplate spectrophotometer (Model Epoch, Biotek, Winooski, VT).

8. Protein concentrations are expressed as µg/mL.