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Impact of Diet and Quality Grade on Meat Quality Characteristics and Their Relationship to Oxidative Stress

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by

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Impact of Diet and Quality Grade on Meat Quality Characteristics and Their Relationship to Oxidative Stress

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Marbling and tenderness in meat are some of the most impactful factors for the consumer eating experience. The cause of the relationship between marbling and tenderness is still unresolved. This study was conducted to evaluate the effects of feeding different types of processed corn, with or without distiller grains on oxidative stress, and how that relates to tenderness and shelf life of beef steaks of differing quality grades. Steers (n= 240) were finished on dry rolled corn (DRC), DRC + 30% dried distillers grains (DDGS), steam flaked corn (SFC), or SFC + 30% DDGS. Cattle were fed 10 per pen. Only pens with both upper 2/3 Choice and Select-grade carcasses were sampled, with a goal of two (and a minimum of one) of each grade per pen. Three pens per treatment met the selection criterion and 36 carcasses were chosen (21 upper 2/3 Choice and 15 Select). Both strip loins were collected from each carcass, halved, and aged for 2, 9, 16, or 23 d. After aging, steaks were placed under retail display for 7 d. Subjective discoloration and instrumental color (L*, a*, and b*) were determined daily, and Warner-Bratzler shear force (WBSF), slice shear force (SSF), and lipid oxidation (thiobarbituric acid reactive substances; TBARS), were measured after 0 and 7 d of retail display. Fatty acid profile, proximate composition, sarcomere length, sarcoplasmic calcium, pH, proteomic analysis, isoprostane content, and troponin-T (Tn-T) degradation of the lean were obtained. No impacts on sarcomere length, pH, sarcoplasmic calcium, and isoprostane content were found ($P > 0.05$). The

difference in proximate composition was due to fat content between quality grades ($P < 0.05$). As aging continued, increases in tenderness, degradation of Tn-T, percent discoloration, and lipid oxidation were found, along with a decrease of redness values ($P < 0.05$). Increases in objective tenderness, discoloration, and decreases in color stability of retail display occurred from 5 to 7 d ($P < 0.05$). Steaks from cattle fed DRC (compared to SFC) and without DDGS (compared to with DDGS) were statistically lower for discoloration, and C18:2 (linoleic acid) and higher in redness and TBARS values ($P < 0.05$). Proteomic data revealed greater oxidation of proteins related to tenderness in Upper 2/3 Choice-grade steaks than Select-grade steaks and in steaks from cattle fed DRC+DDGS than steaks from cattle fed DRC. Steaks from cattle fed DRC were more tender than steaks from cattle fed SFC and Upper 2/3 Choice-grade steaks were more tender and have greater oxidative damage in the proteins than Select-grade steaks.

ACKNOWLEDGMENTS

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"Strive not to be a success, but rather to be of value."

Albert Einstein

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Introduction

When humans consume high-fat diets, research has indicated that accumulation of free radicals, collectively called reactive oxygen species (ROS), can occur, inducing a phenomenon called oxidative stress (Montuschi, 2004; Savini et al., 2016). When an imbalance between ROS and antioxidant protection favors the former, oxidative stress occurs (Dalle-Donne et al., 2001; Sies, 2015; Savini et al., 2016). This oxidative stress often leads to greater cellular damage in DNA, proteins, and lipids (Halliwell and Gutteridge, 2015), and looking at cattle, there is evidence to support that oxidative stress impacts beef tenderness (Smuder et al., 2010; D'Alessandro et al., 2011; Guillemin et al., 2011; D'Alessandro et al., 2012; Chen et al., 2019; Malheiros et al., 2019). This is consistent with previous research showing high-fat diets, like distillers grains fed to cattle, impact tenderness (Gordon et al., 2002; Depenbusch et al., 2009a; Aldai et al., 2010; Senaratne, 2012; Chao et al., 2018). Distillers grains fed to cattle yield beef with increased levels of polyunsaturated fatty acids (PUFA) and fat deposition (Pethick et al., 2004; Gill et al., 2008; Depenbusch et al., 2009a; Mello et al., 2012; Domenech-Pérez et al., 2017; Chao et al., 2018; Ribeiro et al., 2019). The PUFA are deposited in the phospholipid membrane of cellular structures like the sarco-endoplasmic reticulum (SR) and mitochondria (Betteridge, 2000; Blokhina et al., 2003; Wood et al., 2008). This increased presence of PUFA can be problematic due to their susceptibility to free radical attack, that can target the multiple double bonds, because the double bond weakens the carbon-hydrogen bond at the adjacent carbon atom (Betteridge, 2000). This phenomenon has been proffered as a factor that explains why cattle distillers grains have improved tenderness (Chao et al., 2018). Furthermore, feeding high-fat diets, like distillers grains, in the finishing stage of growth, promotes increased rate of

marbling development (Blanchard et al., 1999; Houben et al., 2000; Pethick et al., 2004; Smith and Johnson, 2016).

Marbling is a highly valuable and recognized factor for meat quality and consumers are willing to pay more for a higher marbled product (Smith and Johnson, 2016; USDA, 2020). Similarly, tenderness has been found to be a major factor of palatability and consumers are willing to pay a premium for guaranteed tender beef (Morgan et al., 1991; Huffman et al., 1996; Boleman et al., 1997; Neely et al., 1998; Feuz et al., 2004; Calkins and Hodgen, 2007; Verbeke et al., 2010). Consequently, several books and research studies recognize the relationship between marbling and tenderness (Morrison, 1937; Blumer, 1963; McBee and Wiles, 1967; Jeremiah et al., 1970; Tatum et al., 1980; Smith et al., 1987; Wheeler et al., 1994; Wood et al., 1999; Kim et al., 2008; D'Alessandro et al., 2012; Emerson et al., 2013). Some hypotheses that attempt to explain the relationship between marbling and tenderness include the lubrication effect and bulk density theory. The lubrication effect is a hypothesis that suggests that lipid from marbling in the meat can serve as a "lubricant" around the myofibrils and the muscle fibers facilitating a perceived more tender and juicier product (Jeremiah et al., 1970). While, bulk density theory describes the increased tenderness to be caused by the increased presence of marbling, decreasing the mass per volume in the meat (Smith and Carpenter, 1976). Despite the several proposed hypotheses for the relationship of marbling to tenderness, none adequately explain this relationship (Smith and Carpenter, 1976). This suggests that not only are multiple factors likely involved, but a major cause has not been elucidated. Given that high-fat diets can increase marbling, perhaps the relationship between marbling and tenderness is related to the occurrence of oxidative stress. The lack of consistency in tenderness hinders profitability of beef (Warner et al., 2005). The causal relationship between marbling and tenderness is still unclear.

Therefore, the objectives of this study were to evaluate the effects of differing high-fat diets and marbling on tenderness/proteolysis and shelf life, the impact of aging across dietary treatments, and the relationship between the degree of marbling and tenderness, proteolysis, and shelf life.

Literature Review

This research was conducted to evaluate the effects of feeding differing high-fat diets and marbling on tenderness/proteolysis and shelf life, the impact of aging across dietary treatments, and the relationship between the degree of marbling and tenderness, proteolysis, and shelf life.

Defining Oxidative Stress

Oxidative stress encompasses many biological pathways in the body. It is an imbalance caused by a greater quantity of reactive oxygen species (ROS) compared to types of antioxidant protection (Betteridge, 2000; Dalle-Donne et al., 2001; Sies, 2015; Savini et al., 2016). Oxidative stress can be created by either a buildup of ROS or a depletion of antioxidants (Halliwell, 2006; Halliwell and Gutteridge, 2015). Such stress can lead to damage to structures, called oxidative damage, caused by ROS 'attacking' susceptible components of cells (Halliwell and Whiteman, 2004).

Initially, ROS were considered a "toxic by-product" by many that recognized the effects oxidative stress had in the body, as ROS were constantly produced by aerobic metabolism (Harman, 1956; Karuppanapandian et al., 2011; Singh et al., 2016). Subsequent research has revealed that lesser amounts of ROS can serve as a signaling molecule utilized by humans, plants, and animals alike (Dalle-Donne et al., 2001; Finkel, 2003; Wrzaczek et al., 2013; Singh et al., 2016). This intermediate signaling, from ROS, can occur in a variety of cellular pathways including response for cell adaptation, growth, gene expression, and protein-protein interactions

(Bigarella et al., 2014; Holmström and Finkel, 2014). The function of the ROS in the body as a signaling molecule along with the associated biological response seems to be related to the concentration of reactive species present (Dalle-Donne et al., 2001; Dröge, 2002; Valko et al., 2007). A common example is nitric oxide (NO). Nitric oxide is a reactive species that in low quantities can function as a signaling molecule involved in pathways like regulating the free Ca^{2+} in the sarcoplasm or mediating vasodilation, while higher concentrations of NO supplies potent oxidants that can induce cellular apoptosis (Liu and Sundqvist, 1997; Durham et al., 2008; Stamler et al., 2008). Hydroperoxide is another example of a ROS that is often considered deleterious (Gardner, 1979). Hydrogen peroxide (H_2O_2) has also been identified to modulate apoptosis, along with growth and development (Gechev et al., 2006). Moreover, macrophages can stimulate ROS generation to combat bacterial infection (Grimsrud et al., 2008). Indeed, the actions of ROS and ROS-mediated processes can contradict the negative perception of ROS-induced oxidative stress (Valko et al., 2007).

In humans and animals, the process of growth and adaptation from extracellular stimuli produces ROS, the effects of which can be inhibited by antioxidant defenses (Schreck et al., 1991; Lo and Cruz, 1995; Sundaresan et al., 1995). Aerobic respiration is a major process antemortem that can allow for a buildup of ROS from the mitochondria or byproducts of metabolism, in the mitochondria, allowing oxidative stress to occur (De Zwart et al., 1999; Zhang et al., 2008; Bigarella et al., 2014). The mitochondria is considered a major generator of ROS during aerobic metabolism because of oxygen's ability to be an effective electron acceptor (Bigarella et al., 2014; Huang et al., 2016). Common ROS produced by the aerobic respiratory pathway includes the free radical superoxide, hydroperoxide, and a hydroxyl radical (see Figure 1) (Bigarella et al., 2014). Superoxide is the initial ROS produced from complex I and III's

NADH dehydrogenase and NADPH oxidase during energy metabolism in the electron transport chain (Valko et al., 2007; Marchi et al., 2012; Bigarella et al., 2014; Steffens, 2014).

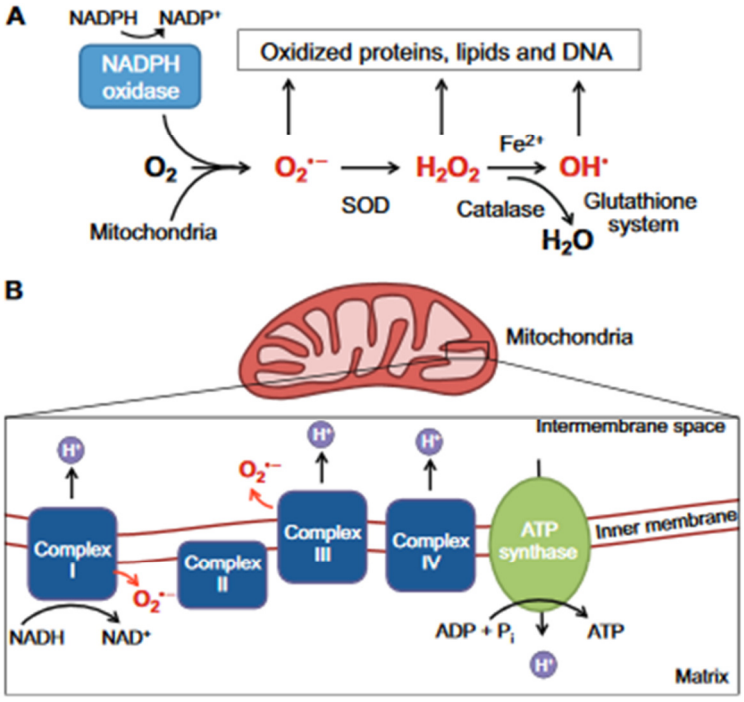

Figure 1. Visualizing ROS generation that can take place in the mitochondria during aerobic respiration. (A) Reactive oxygen species (ROS) include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($OH^{\bullet}$) (shown in red). $O_2^{\bullet-}$ can be generated from complexes I and III (shown in B) or through the oxidation of NADPH by NADPH oxidases. Subsequent reduction to H_2O_2 is catalyzed by superoxide dismutase (SOD). H_2O_2 can be further reduced to water (H_2O) by catalase or can spontaneously oxidize iron (Fe^{2+}) to form the highly reactive $OH^{\bullet}$. Under conditions of oxidative stress, when ROS generation outpaces the ROS scavenging system, accumulating levels of ROS oxidize and damage various cellular components. (B) The electron transport chain complexes I-IV harness electrons from NADH in a series of redox reactions, which are coupled to pumping protons (H^+) into the mitochondrial intermembrane space. The proton motive force, a combination of the membrane potential (charge) and the concentration gradient (pH), powers ATP synthase (complex V). Normally, O_2 acts as the final electron acceptor at complex IV, but an aberrant reduction of O_2 can occur at complexes I and III (red arrows), leading to the generation of $O_2^{\bullet-}$ (red)." (Source from Bigarella et al., 2014)

A free radical molecule contains one or more unpaired electrons (Halliwell, 2006), and can be a unit of ROS (Betteridge, 2000; Montuschi, 2004). Because of this unpaired electron, the

radical is unstable and reactive to other structures, mostly non-radical, in donating or accepting electrons to become energetically stable (De Zwart et al., 1999; Betteridge, 2000). This phenomenon can become cyclic (Kanner, 1994; Morrissey et al., 1998). An accumulation of radicals can spread throughout a localized region, causing damage to the DNA, lipids, and proteins, by radicalizing and oxidizing susceptible structures in an organism (Dalle-Donne et al., 2001; Dröge, 2002; Montuschi, 2004; Halliwell, 2006; Bekhit et al., 2013). During oxidative stress, ROS is capable of damaging a variety of cells, including muscle tissue, and inducing apoptosis (Hüttemann et al., 2012; Lana and Zolla, 2015). Common sites targeted by ROS include oxidation of methionine and cysteine's thiol groups, protein carbonyls, and polyunsaturated fatty acids (PUFA) like arachidonic acid (Grimsrud et al., 2008; Bigarella et al., 2014). Previous research has recognized oxidative stress as a major trigger for apoptotic-inducing factors (AIF) (Chen et al., 2019). Halliwell et al. (1993) described initial oxidative stress damage to cells commonly causing the injury of proteins and DNA with subsequent damage of the lipid, via lipid peroxidation. The location of oxidative stress can vary depending on the cell, type of stress, nearness of ROS to a susceptible substrate, and the degree of stress imposed (Dalle-Donne et al., 2001). In vivo, this balance is maintained in healthy organisms through the body's production of factors like antioxidants and heat shock proteins that will function to help mitigate the harm that excessive ROS can cause (Dalle-Donne et al., 2001; Halliwell and Gutteridge, 2015). The antioxidant enzymes that are involved, in resolving free radical production and subsequent forms of autooxidation include glutathione peroxidase, superoxide dismutase, and catalase (Montuschi, 2004; Gill and Tuteja, 2010). Though oxidative stress can induce apoptotic pathways, the production of antioxidant defenses and heat shock proteins can protect, stabilize, and restore damaged cells in an attempt to maintain homeostasis

(Dalle-Donne et al., 2001; Kültz, 2003; Beere, 2005; Ouali et al., 2013; Wrzaczek et al., 2013). While structures like actin can be susceptible to ROS, endothelial cells are resistant to the constant presence of ROS in the bloodstream because heat shock proteins like HSP27 can stabilize the cell (Huot et al., 1996; Huot et al., 1997; Huot et al., 1998). When oxidative stress occurs and overwhelms the antioxidant defense and heat shock proteins, a variety of negative effects can occur including insulin resistance, immune dysfunction, inflammation, cancer, and other diseases or abnormalities (Gutteridge and Professor Halliwell, 1993; Dröge, 2002; Escobedo et al., 2004; Halliwell, 2006; Valko et al., 2007; Grimsrud et al., 2008). These types of oxidative stress damage are seen in the form of free-thiol oxidation, breaking of DNA strands, depletion of ATP pools, increased lipid peroxidation and permeability in the plasma membrane, and elevation of free Ca^{2+} that can accumulate in the body (Bigarella et al., 2014; Halliwell and Gutteridge, 2015). Ways to measure oxidative stress encompasses many methods from trying to detect ROS in the blood or urine, downstream consequences of lipid peroxidation in the form of isoprostanes, protein carbonylation, genomics, transcriptomics, or proteomics (Montuschi, 2004; Grimsrud et al., 2008; Celi, 2011; D'Alessandro et al., 2012; Iverson et al., 2013). Though these methods have been recognized to vary in the degree of specificity and sensitivity (Milne et al., 2007). Understanding this, one can discern a pattern that as the degree of oxidative stress occurs, there is a change in cellular response from oxidative damage, to a buildup of antioxidant defenses, then eventual development of deleterious health concerns or diseases (Figure 2) (Halliwell, 2006). Therefore, oxidative stress can also be subclassified to distinguish metabolic, environmental, drug-dependent, or nutritional forms of stress (Sies et al., 2005; Savini et al., 2016).

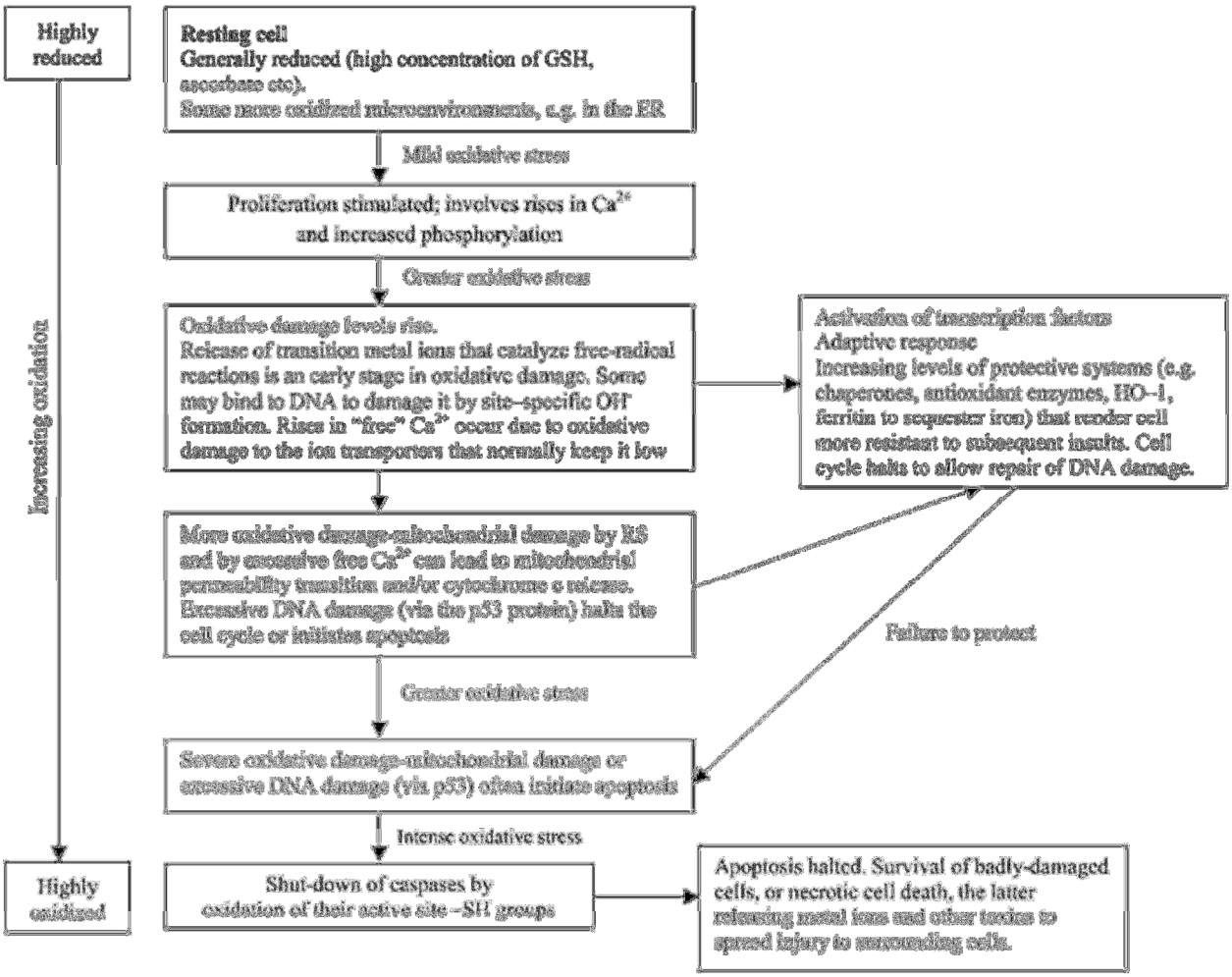

Figure 2: Cellular changes as oxidative stress increases. (Source: Halliwell, 2006)

Besides the effects oxidative stress can have in the body, for good or for ill, oxidative stress seems to be involved in meat quality (Bekhit et al., 2013; Li et al., 2014; Malheiros et al., 2019). For meat postmortem, the balance is shifted entirely in favor of excess ROS and oxidative products that can further induce the damage of oxidative stress on a system with limited total antioxidant capacity (Renerre et al., 1996; Descalzo and Sancho, 2008; Chen et al., 2019). Even in postmortem cattle muscle, the increased oxidative stress from processes from aging of meat is considered inevitable (Chen et al., 2019). This has also been found to occur antemortem where

oxidative stress accelerated rates of aging with the indication being related to proteolysis or atrophy in the muscle due to calpain and caspase activity (Harman, 1972; Miyoshi et al., 1996; Smuder et al., 2010). This gives credence to the idea that oxidative stress effects antemortem can carry through to postmortem muscle, but aspects of oxidation can propagate in the meat product. Because oxidative stress with meat is a relatively new source of investigation and is generally studied indirectly antemortem, the degree of impact it can have on the product is unclear and is worthy of further investigation.

Factors that Relate to Oxidative Stress

Genetics

Although there is minimal literature discussing the genetic variation on oxidative stress, animal selection has been occurring for more than a century. This typically includes selecting for improved growth-related traits antemortem, as well as meat quality factors postmortem (Renand et al., 1995; Hopkins et al., 2005). In nature, there is great genetic diversity within species of animals. Even so, animals' genes adapt to environmental stress and those that adapt will represent a greater portion of the species (Nevo, 1995; Nevo, 2000; Nevo, 2001). Depending on the regional environment, cattle and breed can be selected based on resilience to potential stress factors like temperature that impact the growth and ultimate meat quality of the beef. Dairy cattle under heat stress conditions can develop biological responses and alterations of geno- and pheno-types (Holter et al., 1997; Cai et al., 2005; Wheelock et al., 2010). Heat shock protein accumulation may occur at a greater rate in cattle more sensitive to heat stress, impacting the milk yield and quality (Valtorta and Gallardo, 2004; Cai et al., 2005). Shimizu et al. (1996) reported increased heat shock response in Brahman implanted embryos compared to Holstein

embryos, indicating genetic-based resistance to heat stress. Multiple studies have recognized the genetic variation between the more heat stress resistant *Bos indicus* compared to *Bos taurus* impacting growth and meat quality (McDowell et al., 1953; Malayer and Hansen, 1990; O'Connor et al., 1997; Hansen, 2004; O'Brien et al., 2010; Sodhi et al., 2013).

Metabolic stress can take place and is often not recognized until health concerns develop. This can include diseases like milk fever, ketosis, and displaced abomasum (Chesnais et al., 2016). In response, selecting for genes to increase resistance for concerns like ketosis has lowered the chance of severe incidences of metabolic stress (Cole et al., 2013; Koeck et al., 2015; Jamrozik et al., 2016). Although it is uncommon to see such differences to stress response within breed compared to between breeds, growth and product quality always varies in studies since animals respond differently to a variety of factors because each has unique genetics. Stress is one of the major factors involved. Research from de Oliveira et al. (2014) found differences in genetic variation within Nellore cattle that impacts feed efficiency. If stressors were present, the animals' ability to digest and utilize the feed for growth can be impacted (Luiting et al., 1994; Richardson et al., 2004; Herd and Arthur, 2009). These stressors can impact meat quality. For instance, it is well recognized different types of stress can impact meat color stability (Mancini, 2009). Other research has recognized the impact of lamb genotypic variation on meat color stability (Hopkins and Fogarty, 1998; Hopkins et al., 2007b; Hopkins et al., 2007a; Ponnampalam et al., 2007; Warner et al., 2007). Looking at possible indicators for oxidative stress, genotypic effects have been reported for traits like meat pH, intramuscular fat, amount of unsaturated fatty acids (UFA) and polyunsaturated fatty acids (PUFA), iron, and zinc; traits that are related to oxidative events or pathways (Hopkins et al., 2011; Cesar et al., 2014). Based on the current literature of genotypic effects of growth traits and product quality factors that are

altered by types of stress, it seems reasonable to conclude that genetic variation may influence the degree of oxidative stress the animal encounters and impact meat quality. Oxidative stress ability to alter DNA and thus phenotype will be discussed later within this section.

Lipid oxidation & Stability of the Sarcoplasmic Reticulum (SR)

For living systems, oxidative damage is caused when there is an imbalance between oxidizing sources like ROS and antioxidant factors (Morrissey et al., 1998). Lipids being a major target of oxidation, ROS target susceptible regions like the polyunsaturated fatty acids (PUFA) found in abundance in the SR and phospholipid membrane (Betteridge, 2000; Blokhina et al., 2003; Wood et al., 2008). Polyunsaturated fatty acids are particularly susceptible to free radical attack because the presence of a double bond weakens the carbon-hydrogen bond at the adjacent carbon atom (Betteridge, 2000). With phospholipid membranes containing a high concentration of PUFA's, free radicals like superoxide and hydroxyl can target the hydrogen proton near the PUFA double bond and thus initiate lipid oxidation (Esterbauer et al., 1991; Götz et al., 1994; Bekhit et al., 2013). Lipid oxidation is typically described as a cyclic or auto-oxidizing process following 3 steps: Initiation, Propagation, and Termination (Kanner, 1994). A process where highly reactive free radicals like ROS (Bekhit et al., 2013), often formed from the interaction of a prooxidant or consequence of a biochemical pathway, initiates lipid oxidation by radicalizing a carbon along with the lipid structure (Figure 3). The radical will react with oxygen, forming a peroxy- radical. Another lipid will react with the peroxy- radical producing a peroxide and a lipid radical that can reenter the cycle. This is called propagation since a radical lipid structure reenters this step of autooxidation along with radical products from initiation. During the termination step, radicals can react with another radical forming a non-radical product (Morrissey et al., 1998). This process occurs because free radicals exist with valence electrons in

a thermodynamically unfavorable state and thus try to become more stable by reacting with other molecules, donating or accepting an electron (Halliwell and Gutteridge, 2015). These series of electron exchanges among molecules can lead to lipid oxidation in the localized region (Bekhit et al., 2013). The lipid hydroperoxides formed degrade into secondary products like aldehydes, ketones, alcohols, hydrocarbons (Ross and Smith, 2006).

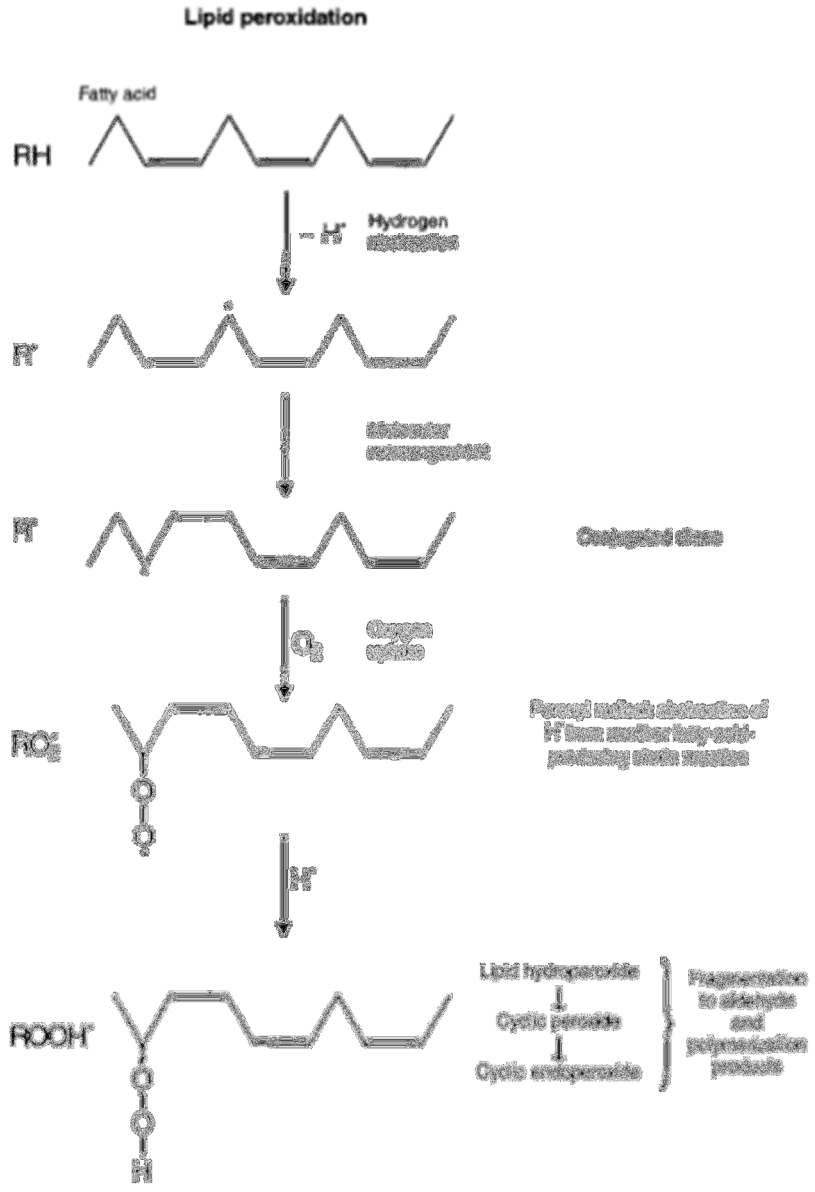

Figure 3. ROS induced lipid peroxidation (Source from (Gutteridge, 1995)).

Isoprostanes are compounds formed antemortem by the free radical-catalyzed peroxidation of arachidonic acid, independent of cyclooxygenase, forming prostaglandin F_2- like structures (Morrow et al., 1990; Montuschi, 2004). Since ROS-induced oxidative stress has been reported to target arachidonic acid, measurement of F_2- isoprostanes are considered a more reliable approach for assessing oxidative stress in the living system (Lawson et al., 1999; Montuschi, 2004; Morrow, 2005; Montuschi et al., 2007). While quantifying an increased free radical activity, isoprostanes were measured and found to be higher in a variety of health concerns like patients with hepatorenal syndrome and acute paracetamol intoxication, smokers, systemic sclerosis, lung disease, and cystic fibrosis compared to healthy patients (Morrow et al., 1992; Morrow et al., 1995; Stein et al., 1996; Montuschi et al., 1998; Kharitonov et al., 2000). These disease states presumably induce oxidative stress conditions. This indicates how isoprostanes, as a measure of increased lipid oxidation in the body, can be used to measure oxidative stress via free radicals activity in vivo (Frankel, 1984; Halliwell et al., 1993).

Gray and Pearson (1994) recognized several factors that impact lipid oxidation, including ones already discussed in addition to diet, antioxidants, and lipid composition. Because free radicals readily attack lipids, preferentially PUFAs, peroxides and volatile secondary products are produced (Morrissey et al., 1998; De Zwart et al., 1999; Roberts and Milne, 2009; Ponnampalam et al., 2012a). Additionally, molecules that typically function for signaling, like nitric oxide, can react with another radical, superoxide, and initiate lipid oxidation (Ladikos and Lougovois, 1990). Research recognizes it is common, under oxidative stress, for intracellular free Ca^{2+} to increase (Halliwell, 2006; Halliwell and Gutteridge, 2015). This can be caused by damaging the SR's calcium-regulating systems leading to calcium leakage, and subsequent activation of calpains and lipoxygenase, which can impact meat quality (Bekhit et al., 2013).

Diet

Feeding high-fat diets can lead to oxidative stress and a greater risk of oxidative damage to cells that exaggerate this imbalance. Researchers looking at long term consequences of high-fat diets for humans have correlated products of lipid oxidation and oxidative stress to issues like obesity, cardiovascular disease, the progression of insulin resistance, and atherosclerosis in diabetics (Inoguchi et al., 2000; Evans et al., 2002; Olusi, 2002; Keaney et al., 2003; Furukawa et al., 2004; Tran et al., 2012; Warolin et al., 2014). Other issues from oxidative stress can also include cell cytotoxicity, inflammation, and aging in the human body(Angeli et al., 2011; Sottero et al., 2019). Recognizing high-fat diets as one of the more notable precursors of obesity highlights the effects long term oxidative stress can have on the body (Figure 4) (Savini et al., 2016; Mirmiran et al., 2018).

Distillers grains are an ingredient in cattle diets that contain a higher nutritional value than corn with up to three times the concentration of protein, fat, and fiber. (Klopfenstein et al., 2008). Distillers grains are a popular feed source because of distillers grains' ability to improve cattle's average daily gain, gain to feed ratio, and other growth traits compared to corn (Buttrey et al., 2012). Dried distillers grains with solubles (DDGS) has 130% higher energy value than that of corn (Klopfenstein, 1996). Because of the high energy and fat available, fat deposition increases and fat accumulate in the muscle when dried distillers grains are fed (Rice University, 2013). The addition of DDGS, as a high energy cattle feed, increases the absorption of PUFAs (Klopfenstein, 1996; Klopfenstein et al., 2008; Vander Pol et al., 2009; Mello et al., 2012; Chao et al., 2017; Domenech-Pérez et al., 2017; Ribeiro et al., 2018), possibly increasing the oxidative potential in the system.

Looking at other sources of increased PUFA content, Ponnampalam et al. (2002) found the addition of 7% fish oil increased PUFA content in lamb. This is consistent with Najafi et al. (2012) who found a 3% addition of fish oil increased long-chain omega-3 fatty acids in goats, compared to those fed palm or soybean oil. This increase intake of PUFAs absorbed, like by cattle fed on DDGS, can aggravate the imbalance of ROS and increase oxidative damage in lipids like those that encompass cells (Esterbauer et al., 1991; Morrissey et al., 1998; Enser et al., 2000; Dannenberger et al., 2007). With the increased concentration of PUFA present in membranes, oxidative stress-related lipid peroxidation can occur, producing factors that can be later measured, like isoprostanes and thiobarbituric acid reactive substances called thiobarbituric acid reactive substance (TBARS) (Montuschi, 2004; Birben et al., 2012).

Oxidation from oxidative stress may be suppressed with the addition of antioxidants to the diet (Fang et al., 2002; Halliwell, 2009). Multiple studies have shown the decrease of generated F_2-isoprostanes with high amounts of vitamins C and E added to human diets (Reilly et al., 1996; Davi et al., 1997; Praticò et al., 1998). Supplementing antioxidants like ascorbate and tocopherols are effective, despite animals already being able to produce ascorbate (Halliwell and Gutteridge, 2015). This is because the synthesis of ascorbate also generates the ROS H_2O_2, making supplementation with antioxidants preferable in many cases (Puskás et al., 1998). Animals fed vitamin E (α-tocopherol) have more vitamin E in meat, making it a useful antioxidant (Faustman et al., 1989; Rowe et al., 2004a; Chao et al., 2017).

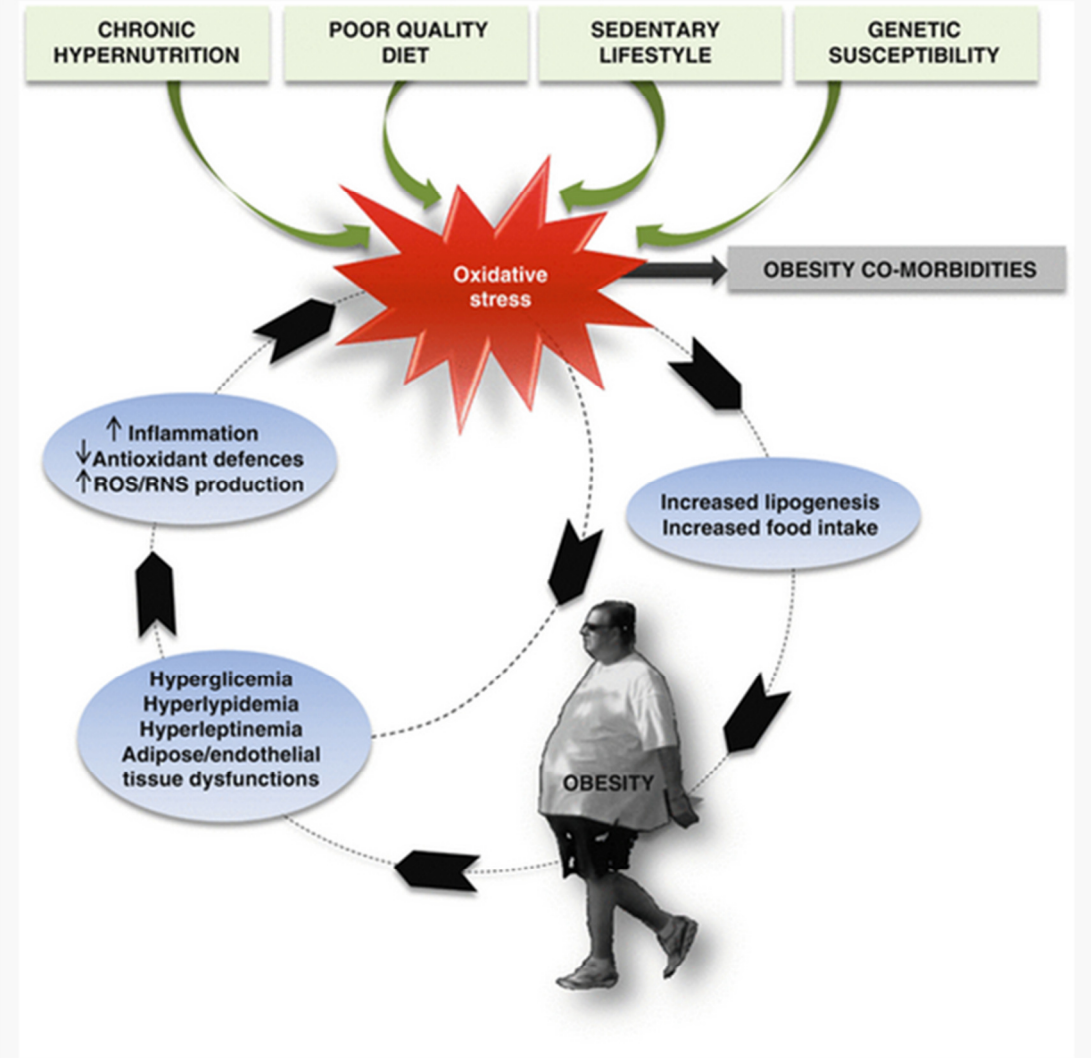

Fig. 6.1
Relationship among oxidative stress, obesity and obesity-associated diseases. Excessive caloric intake, low-quality diet and sedentary lifestyle, even before weight gain, are suggested to be primary triggers of systemic oxidative stress and inflammation; genetic variants are involved as well. A vicious circle is established: by stimulating white adipose tissue deposition and altering food intake, oxidative stress contributes to the onset and progression of obesity, as well as to development of obesity-associated diseases. Both obesity and oxidative stress trigger inflammatory conditions that, in turn, lead inexorably to a worsening of the situation (see Text for further details)

Figure 4. Visualizing the relationship between the causes of oxidative stress and negative health impacts. (Source from (Savini et al. 2016))

Furthermore, vitamin E's ability, when imbedded in membranes, to prevent mitochondrial instability makes the vitamin a valuable supplement in meat (Brigelius-Flohé and Traber, 1999;

Betteridge, 2000). A deficiency of vitamin E in the diet has been found to reduce catalase and glutathione peroxidase activity (Chow et al., 1969). Since tocopherols are only available in plants, consumption is necessary to make use of these antioxidant variants in the body (Halliwell, 2006). Meat from grass-fed animals was found to have increased β-carotene and α-tocopherol (Daley et al., 2010; Bekhit et al., 2013). Multiple studies have recognized grass-fed cattle or lamb have increases vitamin E and PUFA content in the muscle, postmortem (Descalzo and Sancho, 2008; Warren et al., 2008; Maughan et al., 2012). This is consistent with Santé-Lhoutellier et al. (2008) who found higher PUFA and more vitamin E (6.42 mg/kg tissue compared with 1.61 mg/kg tissue) in meat from lambs fed on pasture versus concentrate, respectively. In contrast, while Ponnampalam et al. (2012b) found increase PUFA, they found less vitamin E in cattle fed grain compared to cattle fed on pasture. Petron et al. (2007) found lamb fed leguminosa compared to ryegrass or a botanically diverse pasture had more glutathione peroxidase activity in the *Longissimus thoracis*. Researchers have reported higher activity of the antioxidant enzyme catalase in meat from pasture-fed cattle vs. grain-fed (Descalzo et al., 2005; Descalzo et al., 2007). Other researchers found increased total antioxidant status in the blood of lambs grazing annual pastures with flaxseed or flax meal compared to lambs fed on annual pastures with oat grain (Burnett et al., 2012).

It is reasonable to propose that high-fat diets can still cause oxidative stress and oxidative damage in cattle to a differing degree, as it has been demonstrated in humans. The quality of the diet consumed by humans and animals plays a key role in the biochemical molecules and oxidation pathways in muscles (Ames et al., 1993; Fang et al., 2002). This may lead to the further formation of radicals and subsequent byproducts of oxidation in antemortem and postmortem muscle (Dabbagh et al., 1994; Stephens et al., 1996). Considering the dietary impact

on oxidative stress helps give context to how oxidative damage may impact animals and the resulting meat.

DNA Alteration

In vivo, ROS can serve as signaling molecules that can lead to gene expression or transcription in response to oxidative changes (Dröge, 2002; Seifried et al., 2007). Excessive generation of free radicals and ROS can target and cause oxidative damage to DNA (Ames and Gold, 1991; Halliwell, 2002). With cysteine residues present in DNA binding sites, ROS can modulate activity in the DNA (Birben et al., 2012). This can cause the expression of genes involved in signal transduction (Poli et al., 2004; Valko et al., 2006). The impact can be noted in a variety of processes, like the functioning of the antioxidant enzyme glutathione peroxidase (Birben et al., 2012). By impacting systems like glutathione peroxidase, ROS can diminish the methods of curtailing ROS generation.

Oxidative stress has been reported to cause depletion of the pyridine nucleotide and break DNA strands (Dizdaroglu et al., 2002; Halliwell and Gutteridge, 2015). Multiple studies recognize that oxidative damage via ROS, like hydroxyl radicals, have deleterious effects like the modification of purines and pyrimidine bases, deoxyribose backbone and crosslinking of DNA-related molecules (De Zwart et al., 1999; Dizdaroglu et al., 2002; Valko et al., 2006; Bekhit et al., 2013). This can lead to mutations of the DNA, crosslinking with protein, and subsequent damaged RNA and proteins if the body fails to prevent, repair, or remove these damaged structures (Jones and Wolffe, 1999; Halliwell, 2002; Caldecott, 2003; Birben et al., 2012). This oxidative stress can break double-stranded DNA, possibly leading to apoptosis, while methylation of the DNA can cause gene silencing (modulating expression), chromatin expression, and DNA repair (Valko et al., 2006; Birben et al., 2012). Despite the body's ability

to repair DNA (De Zwart et al., 1999), eventually, the ROS-induced oxidative stress can cause permanent damage that would presumably be deleterious for the living system. Permanent modifications of DNA can lead to mutations, causing increased oxidative DNA lesions as found in tumors, carcinogenesis, and aging in humans (Valko et al., 2006). With the generation of ROS prominently occurring in the mitochondria, it makes sense that when oxidative stress occurs, mitochondrial DNA is more susceptible to oxidation compared to nuclear DNA. Oxidized mitochondrial DNA can cause mitochondrial dysfunction and modifications of the mitochondrial DNA, RNA, and proteins, possibly leading to apoptosis of the organelle (Cadenas and Davies, 2000; Inoue et al., 2005). To prevent cancerous processes, the damaged mitochondria will undergo apoptosis. In the presence of chloride ions, H_2O_2 is converted to hypochlorous acid (Bekhit et al., 2013). Hypochlorous acid is a potent ROS that can react with DNA (Birben et al., 2012). This causes covalent cross-linkages between DNA and protein, forming oxidized products of pyrimidine bases, and modifying the DNA by adding chloride to the bases (Whiteman et al., 1997; Kulcharyk and Heinecke, 2001). Also, free iron can function as a prooxidant and react with ROS to create free radicals that are capable of attacking DNA (Halliwell, 2006). The ability to stabilize iron to protect the DNA against reactions like the Fenton reaction is crucial in preventing damage that may lead to the mutations of parts of the DNA and subsequent RNA to protein (Figure 5) (Wiedenheft et al., 2005). Besides ROS attacking DNA sites, research has found malondialdehyde (MDA), a secondary product of lipid peroxidation, also can attack DNA bases, causing mutations and subsequent deleterious effects like DNA-DNA and DNA-protein crosslinking (Chaudhary et al., 1994; Burcham, 1998; Nair et al., 1998; Marnett, 1999; Bartsch and Nair, 2004; Valko et al., 2006). This is notable because it supports the idea that lipid membranes, containing PUFA, are attacked by free radicals and create downstream effects on

DNA functionality. This idea provides supporting evidence of the importance of regulating the oxidative processes that impact cell functionality, viability, and proliferation, encompassing regional function due to DNA alteration.

$$Fe^{2+} + H_2O_2 \rightarrow \text{intermediate complexes} \rightarrow Fe(III) + OH^{\cdot} + OH^{-}$$

Figure 5: Fenton Reaction (Source from (Halliwell, 2006)).

Stability of the Mitochondria and Apoptosis

Mitochondria, the "powerhouse of the cell," is regarded as the main source of free radical generation in aerobic respiring animals (Cadenas and Davies, 2000; Valko et al., 2006; Zhang et al., 2008; Marchi et al., 2012). Lipid peroxidation can impact the functions of the mitochondria (Bekhit et al., 2013) and be initiated in PUFAs found in the membrane bilayer of the organelle. Products of lipid oxidation, the lipid peroxides, can modify functions like respiration, uptake of calcium, and the general integrity of the mitochondrial membranes (Orrenius et al., 2007). The inability to retain calcium can impact the oxygen consumption rate OCR and ultimately impact meat quality factors like color and tenderness (Bekhit et al., 2013). Similar to ROS, oxidized metals can function as prooxidants, attacking the PUFA of the mitochondrial membrane, causing lipid peroxidation, and subsequently destabilizing the mitochondria (Bacon et al., 1983; Bucher et al., 1983; Esterbauer et al., 1991; Marnett, 1999). Mitochondria often address growth and damage to the organelle by either undergoing fusion or fission (Youle and Karbowski, 2005). While fusion is merging mitochondrial parts, particularly the DNA, to override the partial

damage caused by oxidation reactions, fission creates two new mitochondria. Undergoing fission means separating the mitochondria into a functional/healthy mitochondria and a damaged one (Youle et al., 2012). Should that damaged mitochondria be beyond repair due to something like oxidative stress, structures like lysosomes can releases molecules like cathepsins to amplify the signaling for programmed cell death of the mitochondria (Cadenas and Davies, 2000; Suen et al., 2008; Repnik et al., 2012; Youle et al., 2012). This indication suggests mitochondrial fission is related to apoptosis (Youle and Karbowski, 2005; Orrenius et al., 2007; Youle et al., 2012).

Apoptosis, also termed programmed cell death, is believed to be the beginning of converting muscle to meat (Sentandreu et al., 2002; Ouali et al., 2006). During the slaughter process, cells become increasingly deprived of nutrients and oxygen, therefore initiating apoptosis on a large scale (Ouali et al., 2006). In vivo, the function of apoptosis is to destroy excess, damaged, or potentially hazardous cells to maintain the normal function of the system (Kerr et al., 1972; Fidziańska et al., 1991; Hengartner, 2000). This apoptotic function can be instigated by the downstream effects of ROS-induced oxidative stress (Fiers et al., 1999). If the oxidative stress is relatively high, it can induce apoptosis, however in a lesser amount it can cause a biological response to protect the cell (Ouali et al., 2006). If apoptotic pathways do not occur, high ROS can cause uncontrolled cell death, called necrosis, releasing the internal components like ROS that can damage other cells (Valko et al., 2006). Cytokines, active during immune responses, can generate ROS, a secondary messenger to signal for apoptosis of damaged cells (Valko et al., 2006). This indicates that ROS-induced apoptosis can be for the benefit of the body, however, ROS-induced oxidative stress is distinct by also creating the need for the cell to be destroyed. Some of the common mechanisms of apoptosis include the release of cytochrome c and the activation of caspase 3 (Green and Reed, 1998; Ow et al., 2008; Kemp and Parr, 2012).

Cytochrome c can escape the inner mitochondrial membrane through ROS- triggered permeabilization of the membrane, beginning apoptosis (Lassus et al., 2002; Orrenius et al., 2007). Found in the mitochondria, cytochrome c helps modulate apoptotic pathways that can lead to lipid peroxidation and increased susceptibility for cell death (Kagan et al., 2004; Wang et al., 2018). This supports cytochrome c as another factor that relates to oxidative stress. Mitochondrial breakdown happens earlier in the apoptotic cell death (Youle and Karbowski, 2005; Suen et al., 2008), leading to the timely release of cytochrome c and other factors. Reports indicate that the calcium released from the cytosol are moved to the mitochondria triggering apoptosis in the structure (Mattson and Chan, 2003; Orrenius et al., 2003). There are many apoptotic inducing factors (AIF), but oxidative stress is considered the most notable (Chen et al., 2019). The mitochondria are considered to be a major regulator in apoptosis by releasing AIF, endonuclease, and amplify caspase activity (Lassus et al., 2002). Recognizing apoptosis can be modulated by a multitude of pathways related to mitochondria emphasizes the many ways oxidative stress can induce cell damage and how mitochondria play a central role in oxidative stress and apoptosis.

Antioxidants and Heat Shock Proteins

Heat-shock proteins (HSP) are defined as chaperone proteins that are meant to stabilize or protect the cell after the damage that may otherwise result in cell death (Kültz, 2003; Beere, 2005). Heat-shock proteins inhibit or prevent apoptosis by binding to cytochrome c, preventing caspase 3 activation, and stabilizing pathways from cell survival (Beere, 2005; Van Ba et al., 2015). Therefore, it is aptly named anti-apoptotic in activity (Cramer et al., 2018). It is relevant to note that cytochrome c can also interact with ROS and function as an antioxidant (Hüttemann et al., 2011). Heat shock proteins are shown to have increased expression as a result of various

stressors like increased temperature, electrical shock, lower internal pH, and oxidative stress from ROS (Welsh and Gaestel, 1998; Escobedo et al., 2004; Beere, 2005; Ouali et al., 2006; Lomiwes et al., 2014a). Heat shock proteins are capable of delaying the activity of cytochrome c, as well as interacting with caspase to prevent the subsequent proteolytic pathways (Paul et al., 2002; Voss et al., 2007). Heat shock proteins also can restore the structure and functionality of proteins damaged (Ouali et al., 2013). Heat-shock protein 27 is a notable anti-apoptotic protein due to its involvement in regulating and stabilizing myofibrillar proteins showing resistance to oxidative damage (Huot et al., 1996; Paul et al., 2002; Escobedo et al., 2004). The ratio of pro- and anti-apoptotic factors being released from locations like the mitochondria, at death, seems to be the defining aspects that influence the subsequent pathways that potentially impact tenderization (Ouali et al., 2013). These results seem to indicate HSP as another part of the living system's defense against oxidative stress-induced damage.

Similar to HSP's opposition to factors of oxidative stress, the antioxidant defense can function to curtail peroxidation and serves as a counterbalance to reactive species induced oxidative stress (Dalle-Donne et al., 2001). Gutteridge (1995) defined antioxidants as any compound in low relative quantities, compared to the substrate, that delays or prevents oxidation of the substrate. Antioxidants in homeostasis can minimize the effects of ROS while still allowing their presence for biological functions (Halliwell, 2006; Halliwell and Gutteridge, 2015). There also seems to be a relationship between high levels of ROS and altered activities of antioxidants and antioxidant enzymes in tumor cells (Valko et al., 2006). Common antioxidants present in the body, including some within the cell membrane, include ascorbate, tocopherols, glutathione, coenzyme Q, and β-carotene (Gerard-Monnier and Chaudiere, 1996; Betteridge, 2000; Halliwell and Gutteridge, 2015). These substances step into processes like lipid oxidation,

described above, to terminate the oxidative process (Betteridge, 2000). Coenzyme Q is found in lipoproteins protecting the cell membranes from oxidation. Similarly, β-carotene protects the oxidation of the lipid (Bekhit et al., 2013). Beta-carotene can work with vitamin E, a derivative of tocopherols, to scavenge superoxides and peroxyl radicals (Burton and Ingold, 1984). Ponnampalamm et al. (2012a) recognized vitamin E as a key factor in determining the degree of lipid oxidation in meat. This is because vitamin E in the phospholipid bilayer is an effective scavenger of peroxyl radicals and therefore interrupts lipid peroxidation (Traber and Kayden, 1989; Betteridge, 2000). That is why vitamin E is considered a chain-breaking antioxidant (Burton et al., 1982; Brigelius-Flohé and Traber, 1999). Moreover, vitamin E can work with glutathione peroxidase and prevent peroxide formation and thus the breakdown of arachidonic acid, a fatty acid precursor of isoprostanes (Montuschi, 2004; Bekhit et al., 2013). Vitamin C, ascorbate, can scavenge ROS, protects against cell death, and helps reduce the radicalized tocopherol (Betteridge, 2000; You, 2000; Valko et al., 2006). However, vitamin C can act as a pro-oxidant in the presence of ferric iron (Fe^{3+}) or copper (Bekhit et al., 2013). Therefore, it is important to note antioxidants in high quantities may not help reduce oxidants, but in fact can further promote oxidation through ROS generation (Bowry et al., 1992; Lee, 2001; Valko et al., 2006; Foyer and Noctor, 2016; Singh et al., 2016).

The body is described to have a total antioxidant capacity (TAC) that also includes antioxidant enzymes like catalase, glutathione peroxidase, and superoxide dismutase (Renerre et al., 1996; Betteridge, 2000). Renerre et al. (1996) described these enzymes as free radical scavengers that break down hydrogen peroxides. Hydrogen peroxides are a primary product of lipid oxidation that can also be catalyzed from superoxide dismutase and have different levels of activity between muscles. In the mitochondrial matrix, superoxide dismutase facilitates the

reaction between two superoxides ($O_2^{\bullet}$) and 2 hydrogen protons (H^+) to produce H_2O_2 and oxygen (Betteridge, 2000; Halliwell, 2006; Bekhit et al., 2013). Doing so can stop further radical production and create a more stable ROS (H_2O_2). However, in high concentrations, hydrogen peroxide can still cause deleterious effects due to its ability to permeate through the membrane, combine to form the highly reactive hydroxyl radical, and break down into volatile secondary products (Halliwell and Gutteridge, 1984; Betteridge, 2000; Ross and Smith, 2006; Birben et al., 2012; Bekhit et al., 2013). Catalase provides minimal aid of removing H_2O_2 in the mitochondria since the enzyme which breaks down H_2O_2 to water, which are nearly all kept in the peroxisome (Schrader and Fahimi, 2004; Halliwell and Gutteridge, 2015). Nevertheless, glutathione peroxidase, an enzyme that oxidizes glutathione, converts H_2O_2 to 2 H_2O, is present in multiple locations, including the mitochondria, and controls H_2O_2 when reduced glutathione are also present (Halliwell, 2006; Birben et al., 2012). It has been reported that under elevated oxidative stress conditions, there are increases of oxidized glutathione (Valko et al., 2006), indicating this antioxidant, and presumable glutathione peroxidase, has increased activity in response to the stress. Peroxiredoxin is another notable H_2O_2 reducing enzyme (Rhee et al., 2005). Although peroxiredoxin catalyzes H_2O_2 slower than glutathione peroxidase, the enzyme comes in large quantities and can be found in the cytosol and all subcellular organelles (Halliwell, 2006). The presence of these multiple enzymes, that address H_2O_2, suggests the need to regulate hydrogen peroxide in the body (Marchi et al., 2012). Also, it underscores the importance of preventing the formation of the highly reactive hydroxyl radical that can damage cells or initiate lipid peroxidation by transforming PUFAs into lipid hydroperoxides (Birben et al., 2012).

These key enzymes are significant in maintaining the system's redox homeostasis (Li and Liu, 2012). Despite this, the antioxidant defense is not flawless as oxidative damage still occurs

in DNA, proteins, and lipids, possibly leading to damage of structures, aging of tissues, and development of diseases in animals (Ames, 1983; Betteridge, 2000; Halliwell, 2002). Often when events of oxidative stress occur, cells will adapt by upregulating antioxidant defenses or HSP to stabilize and repair the structures (Halliwell, 2006). This can be recognized in differentiated skeletal myoblasts that respond to oxidative stress with increased expression of heat shock protein (HSP25), glutathione, and glutathione peroxidase activity to mitigate the potential oxidative damage (Escobedo et al., 2004). Entering postmortem, the antioxidant defenses deteriorate, while the byproducts of oxidative stress can continue to accumulate unencumbered, thus, impacting meat quality (Bekhit et al., 2013). Heat shock proteins and antioxidant defenses are factors impacting the effects of oxidative stress that will be relevant in the meat, postmortem.

Like humans, livestock have a variety of factors, like genes, that can predispose them to stress responses. Stress can impact and be modulated by a variety of factors like diet, oxidation of lipid and DNA molecules, and several responding pathways like apoptosis, antioxidant defenses, and heat shock proteins. The involvement of oxidants is a common trend in all the events described. Any alteration of these factors and pathways can induce ROS-related oxidative stress and damage lipids, DNA, or proteins. Antioxidant defenses and heat shock proteins respond to this ROS buildup to prevent oxidative stress. When this system fails, membranes can lose stability, cell damage can occur possibly leading to apoptosis, and in severe cases, it can cause severe health consequences. Recognizing the multitude of ways oxidative stress can be involved and the potential responses, accounting for oxidative stress in livestock and its impact on the meat can be valuable.

Impact of Oxidative Stress on Meat Quality

Although it is difficult to measure ROS directly, the downstream benefits to meat quality are notable. These include meat quality factors like increased susceptibility of lipid oxidation, color stability, and tenderness.

Lipid Oxidation

When evaluating factors impacting shelf life, oxidation damage of lipids is considered a major factor (Descalzo et al., 2005; Min et al., 2010). In recognizing the relationship of high-fat diets, deposition of PUFA, and oxidative stress, it is postulated that some of the major initiators of lipid oxidation are reactive oxygen species (ROS) and peroxides. If so, this would be particularly true for cattle that deposit enough fat to be classified as high marbled (Choice or Prime quality grade). Increased marbling or fat deposition in the muscles (intramuscular fat) would indicate the cattle did not need the energy source immediately, so it was stored and accumulated (Rice University, 2013). When fed a high-fat diet as found with the addition of DDGS, not only can the high energy feed itself increase the oxidative stress in the body, but so can the increased absorption of PUFAs (Klopfenstein, 1996; Klopfenstein et al., 2008; Vander Pol et al., 2009; Mello et al., 2012; Chao et al., 2017; Domenech-Pérez et al., 2017; Ribeiro et al., 2018). Postmortem, while the amount of total antioxidant capacity (TAC) is set, prooxidant factors can increase oxidation along with ROS to impact meat quality (Descalzo and Sancho, 2008). Detection of lipid oxidation, particularly in meat, is done by measuring the primary and secondary oxidation products of lipid autooxidation as described above (Morrow and Roberts, 2002; Bekhit et al., 2013). Some methods allow the measurement of lipid oxidation based on the primary product, hydroperoxides, via a lipid hydroperoxide protocol and secondary products like malonaldehyde via Thiobarbituric Acid Reactive Substances (TBARS) protocol (Ahn and Kim,

1998; Kiliç et al., 2014). To accurately get a measurement of total lipid oxidation, both primary and secondary products of lipid oxidation should be measured in the food product. Generally, many only measure the secondary products of lipid oxidation in meat via TBARS since it has found to be strongly correlated to sensory in fresh meat (Fernández et al., 1997). Okolie et al. (2009) linked one of the secondary products of lipid oxidation, malonaldehyde, with intracellular oxidative stress. Montuschi (2004) described secondary products of lipid oxidation, like aldehydes, as highly reactive substances that may worsen and magnify cell damage by covalently modifying critical biomolecules. Red meat products are generally recognized as susceptible to oxidation in the lipids because the myoglobin containing heme can serve as a catalyst (Gutteridge, 1986; Prasad et al., 1989; Ahn and Kim, 1998; Min et al., 2010). These factors are further highlighted in the notable relationship of lipids oxidizing and producing radicals causing subsequent protein oxidation, further increasing susceptibility to oxidative stress, and consequential oxidative damage in the meat (Gardner, 1979; Mercier et al., 1995; Mercier et al., 1998; Estévez et al., 2007). This, in part, is due to the phospholipids of cell membranes containing high amounts of PUFA that are necessary to maintain membrane fluidity (Wood et al., 2008). This is further confirmed in the strong correlation of measuring protein oxidation, via carbonyls, and measuring lipid oxidation, via TBARS, in differing oxygen concentration conditions (Zakrys et al., 2008). Grimsrud et al. (2008) concluded that oxidative stress-induced lipid peroxidation leads to the production of aldehydes that can react with amino acid residues like cysteine and modify protein function, referred to as protein carbonylation. Oxidized proteins have been purported to serve as indicators of oxidative stress ante- and post- mortem (Malheiros et al., 2019). When compared to other meat sources, beef is more prone to oxidize in the lipid due to the higher heme content (Min et al., 2010). Consequently, oxidative stress and oxidation

of proteins can more effectively increase of lipid oxidation and the subsequent decrease in meat quality.

Although not a commonly used method of measurement in meat science, isoprostanes can provide a novel measurement to detect the effect of oxidative stress on lipids. As a measurement of arachidonic acid oxidation, isoprostanes are considered an effective method to measure lipid oxidation from ROS induced-oxidative stress (Frankel, 1984; Halliwell et al., 1993). Furthermore, the assay distinguishes isoprostanes from other measures of lipid oxidation due to its improved sensitivity and reliability (Morrow and Roberts, 1997; Roberts and Morrow, 2000). When compared to the widely used TBARS method, isoprostanes are more ideal because not only is malonaldehyde not a specific product of lipid peroxidation, the TBARS method is not specific to malonaldehyde (Halliwell, 2000). Thiobarbituric acid reactive substances is a simple assay and although not as reliable when evaluating a specific component of lipid peroxidation, is reliable in correlating to sensory quality in meat. When performing both analyses, that blood isoprostanes concentration is positively correlated to lipid oxidation of meat and n-6 PUFAs for lamb from sheep fed commercial feedlot pellets versus grass-fed diets (Ponnampalam et al., 2017).

In animal production, beef with high levels of PUFA are more prone to lipid peroxidation (Scollan et al., 2006), and this is important since F_2- isoprostanes come from oxidation of PUFAs with three or more double bonds, specifically, arachidonic acid (Yin et al., 2003). The isoprostanes assay should be a useful measurement in meat samples since research has indicated F_2- isoprostanes are stable in biological samples (Kadiiska et al., 2005; Morrow, 2005; Roberts and Milne, 2009). Based on the studies discussed, there's a strong indication that free radicals and ROS are major factors that initiate lipid oxidation, and oxidation of myoglobin's heme can

further increase free radicals in meat (Li and Liu, 2012). Understanding the lipid oxidation that occurs in a living animal by using isoprostanes can help discern the causes of lipid stability in the meat. By measuring lipid oxidation, particularly isoprostanes, researchers can better grasp the impact oxidative stress has on lipid oxidation in the meat. Furthermore, oxidative stress occurring and targeting susceptible membranes can lead to a decrease in color stability and impact tenderness (Faustman et al., 2010; Kunze et al., 2017).

Color Stability

Oxidative damage in cell structures for protein and lipids has been found to negatively impact the color stability of meat (Faustman and Cassens, 1990; Zakrys et al., 2008; Li and Liu, 2012; Bekhit et al., 2013). It's well known that high concentrations of PUFA can increase lipid and myoglobin oxidation in meat (Faustman et al., 2010). This increased oxidation of PUFAs leads to a loss of color stability (Wood et al., 2004; Bekhit et al., 2013). An example of this can be found in beef from cattle fed distillers grains. Addition of distillers grains have been found to have detrimental effects on color stability, in beef, under retail display (Roeber et al., 2005; Nute et al., 2007; Leupp et al., 2009; Segers et al., 2011; Mello et al., 2012; Domenech-Pérez et al., 2017; Chao et al., 2018; Ribeiro et al., 2018). As prooxidants and ROS accumulate in meat and damage lipids and proteins, the ability of beef to maintain the desired cherry red color diminishes (Li and Liu, 2012). This is due to the oxidation of the heme iron (Fe^{2+}) into the oxidized state (Fe^{3+}) making the meat color turn brown and will produce more ROS and products of oxidation (Gutteridge, 1986; Min et al., 2010). Furthermore, the long-term functionality of the metmyoglobin reducing activity (MRA) and oxygen consumption rate will continue to decrease as sources of oxidation convert the heme iron to the ferric state and myoglobin becomes metmyoglobin (Madhavi and Carpenter, 1993). This is believed to be the case, postmortem,

because of the lack of substrates like NADH limiting the sustainability of MRA (Bekhit et al., 2003; McKenna et al., 2005). Based on the research discussed, the persistent cellular damage caused by factors of oxidative stress decreased over time, with depleted antioxidants and enzymes found postmortem, it is reasonable that this will ultimately lead to repeated oxidation of the heme iron, overwhelming the limited reducing capacity. The cellular damage caused by ROS-induced oxidative stress found by Chen et al. (2019) can also have a potential impact. As more areas in meat become increasingly susceptible to oxidation, the loss of function or depletion of remaining antioxidant enzymes and reducing capacity will become apparent in oxidized meat products (Bekhit et al., 2013).

Tenderness

Meat tenderness is considered both one of the most important factors in meat quality and one of the most inconsistent (Kemp and Parr, 2012). There are a variety of pathways that have an impact on meat tenderness including the degree of contraction of the sarcomere, amount and quality of connective tissue, oxidative stress, apoptosis, heat shock proteins, and proteolysis (Huff-Lonergan et al., 1995; D'Alessandro et al., 2012; Picard et al., 2014; Baldassini et al., 2015; Picard et al., 2015; Picard and Gagaoua, 2017; Malheiros et al., 2019). This section will focus on how oxidative stress impacts tenderness through the lens of apoptosis, heat shock proteins, and proteolysis with a pre-existing susceptibility to lipid oxidation due to consumption and deposition of PUFAs. Furthermore, the section will discuss "omics" work that looks closely at proteins and how regulation seems to change.

Proteolysis of specific myofibrillar proteins is considered one of the important influencers of improved tenderness in meat (Huff-Lonergan et al., 1996; Melody et al., 2004). Enzymes recognized as important in the proteolysis include the calpain family, the caspase

system, and cathepsins (Sentandreu et al., 2002; Ouali et al., 2006; Ouali et al., 2013). When discussing tenderization caused by proteolysis, the calpains are recognized as the main enzymes that account for the majority of proteolysis (Koohmaraie, 1992). Of the calcium-dependent cysteine proteases, μ-calpain and m-calpain are recognized for their capacity to breakdown certain cytoskeletal proteins. Calpastatin, however, functions to inhibit these proteases and thus functions as an anti-apoptotic enzyme (Koohmaraie, 1992; Boehm et al., 1998; Goll et al., 2003; Van Ba et al., 2015).

In living animals, sarcoplasmic reticulum (SR) is a structure that 'houses' calcium ions that are crucial for muscle contraction (Gordon et al., 2000). Postmortem, muscle contraction is no longer a factor to influence tenderness, following the completion of the actomyosin crossbridge at rigor mortis. Still, residual calcium that remains in the cytosol or has leaked out of the SR can be used to activate calpains to breakdown protein structures in the sarcomere (Koohmaraie, 1992). A study found increased free calcium in the sarcoplasm in postmortem muscle when cattle were fed a high-fat diet containing distillers grains (Senaratne, 2012). Chao et al. (2018) observed that cattle fed a high-fat diet had increased deposition of PUFAs in the membrane of the sarcoplasmic reticulum, which may increase Ca^{2+} leakage. This would help explain the improved tenderness found early postmortem for meat from cattle fed distillers grains. Other research has speculated that increased PUFA content in the organelles, in beef from cattle fed distillers grains, increased susceptibility of oxidation damage and may alter calcium flux, thus increasing tenderness (Kunze et al., 2017). The addition of more PUFA in membranes can lead to increased lipid oxidation and can cause damage to calcium regulatory structures, resulting in the release of calcium and activation of calpains and lipoxygenase, which could lead to improved tenderness (Bekhit et al., 2013). From an animal nutrition perspective, this is

important since feeding distillers grains results in an increase deposition of PUFA in meat (Klopfenstein, 1996; Klopfenstein et al., 2008; Vander Pol et al., 2009; Mello et al., 2012; Domenech-Pérez et al., 2017; Chao et al., 2018; Ribeiro et al., 2018). Gordon et al. (2002) found that a sensory panel could determine a linear increase in myofibrillar tenderness and overall tenderness on beef steaks from cattle fed DDGS in an increasing inclusion of 0% to 75%. Similar results found beef from cattle fed distillers grains was more tender than beef from cattle fed corn (Depenbusch et al., 2009a; Segers et al., 2011; Senaratne, 2012). Beef from cattle supplemented with dietary antioxidants was found to be less tender compared to when antioxidants were not fed (Senaratne, 2012; Chao et al., 2018). Collectively, these results suggest that feeding high distillers grains increases PUFA in the membranes, increasing the oxidative potential, and calcium release that can impact meat quality, though this is negated when feeding cattle antioxidants.

This can also impact the meat quality like tenderness, for instance, through affecting the proteolysis of myofibrillar proteins. Oxidative stress can increase the availability of calcium, activating proteolytic enzymes to function, and thus cause cytoskeletal protein degradation (Dalle-Donne et al., 2001; Mehlhase and Grune, 2002) and seemingly improve tenderization early postmortem. Baldassini et al. (2015) found increased calcium present, within muscle tissue, in tender experimental groups of Nellore cattle compared to tough groups. This may support the hypothesis that oxidative stress-induced ROS modulates tenderization through proteolysis (D'Alessandro et al., 2011; Guillemin et al., 2011).

According to Xiong (2000), meat tenderness may be increased by oxidative changes in myofibrillar proteins during aging due to contributions of post-mortem proteolysis of meat, as the peptide bonds of oxidized proteins become easily accessible by enzymes. Oxidative stress

has also been reported to increase calpain and caspase-related degradation in rat myofibrils, with an increase in stress relating to increased proteolysis (Smuder et al., 2010). Looking at proteomic work, D'Alessandro et al. (2012) observed a higher level of oxidative stress in the tender-Maremmana, Italian cattle breed, beef, along with indicators of disruption of calcium homeostasis. Furthermore, the study found higher/more active levels of glycolytic enzymes and lactate with tender meat, suggesting less shortening of the sarcomere compared to tough meat (D'Alessandro et al., 2012). Similar results have since been found with higher levels of glycolytic enzymes in tender beef compared to tough (Baldassini et al., 2015). Elevated levels of glycolytic metabolism are related to type II muscle fibers and increased postmortem meat tenderness (Picard and Cassar-Malek, 2009). Malheiros et al. (2019) further found increased oxidative damage in structural proteins, oxidative stress of antioxidant enzymes, and heat shock proteins associated with tender meat, which was associated with lower oxidative damage in metabolism-related enzymes. This trend of oxidatively damaged proteins in tender meat is consistent with previous research (Guillemin et al., 2011; Picard et al., 2014; Picard et al., 2015).

Some studies have concluded there are negative consequences of oxidative stress. Oxidation of myoglobin generates a superoxide anion which rapidly breaks down to hydroperoxide (H_2O_2) resulting in further myoglobin and lipid (with PUFAs) oxidation (Gotoh and Shikama, 1976; Faustman et al., 2010). Rowe et al. (2004) found that increased oxidation of muscle proteins early post-mortem could have negative effects on meat tenderness. Since calpains require reducing conditions to be active, oxidizing conditions influence the activity of μ-calpain as the cysteine in the active site of the calpain is easily oxidized (Guttmann et al., 1997). Based on the calpain's functionality being dependent on oxidative processes in early

postmortem meat, it seems reasonable to state the degree of oxidative stress, as a whole, can have an influence on calpains (Zakrys-Waliwander et al., 2012). A different study by Rowe et al. (2004b) induced high oxidation in meat, through irradiation, and found decreased μ-calpain activity and autolysis, decreasing the extent of meat tenderization. The study also found that oxidizing conditions did not impact m-calpain or rate of inactivation of calpastatin. Similarly, research has shown low levels of oxidation can be preferable for flavor and proteolysis (Estévez, 2011). These results highlight that high levels of oxidation, that can be caused by oxidative stress, ultimately can have negative impacts, specifically on tenderization. However, other studies found improved tenderness from oxidative stress. The data seem to indicate the degree of oxidative stress may be the critical factor in whether improved tenderness or greater toughness occurs.

Proteolysis of proteins like troponin- T, desmin, titin, and nebulin allow for decreased tension in the muscle and improved tenderization (Taylor et al., 1995; Boyer-Berri and Greaser, 1998; Huff-Lonergan et al., 2010; Zakrys-Waliwander et al., 2012). As already noted in the previous sections, ROS have been found to have both positive and negative consequences caused by a variety of factors. Nitric oxide (NO), a well-known ROS, is involved in regulating the activity of sarcoplasmic reticulum Ca^{2+}- ATPase (SERCA) pump and the ryanodine receptor (RyR) (Bellinger et al., 2008; Durham et al., 2008; Stamler et al., 2008). The SERCA pump and RyR control the calcium concentration available for processes like muscle contraction in the sarcomere and contain cysteine residues that can be oxidized by NO (Xu et al., 1997). Those systems can have a key role in the tenderization postmortem since the resulting available Ca^{2+} can be used by calpains during proteolysis (Hare, 2003). Previously, Cook et al. (1998) concluded that NO could also have an impact on NOS inhibitor decreasing and NO

donor/enhancer improving tenderness of hot-boned longissimus thoracis muscle at 2 h postmortem. In contrast, Zhang et al. (2018) evaluated how NO impacts μ-calpain activity and proteolysis in semimembranosus muscle. It was found that NO donor (GSNO) decreased μ-calpain autolysis at 1 d postmortem and degradation of desmin and troponin-T at 7 d postmortem. The nitric oxide synthase (NOS) inhibitor (L-NAME), however, increased μ-calpain autolysis at 1 d postmortem and degradation of desmin and troponin-T at 7 d postmortem (Zhang et al., 2018). Similar results indicate the addition of NO inhibitor improves tenderness in *Longissimus thoracis* and *lumborum* (Ponnampalam et al., 2005; Suster et al., 2005; Cottrell et al., 2008). It is interesting to note that Ponnampalam et al. (2005) reported that semimembranosus muscle toughened with additional NO inhibitor. Nitric oxide donors have been shown to cause the inhibition of calpains (Michetti et al., 1995; Forsythe and Befus, 2003). Inhibition of Ca^{2+} pick up by the SERCA pump and/or preventing NO presence could oxidize thiol residues on calpains and thus impact (reduce) proteolytic activity (Warner et al., 2005). This gives credence to the idea that oxidative stress, exemplified with a radical in NO, can impact biological processes both pre- and post- mortem that modulate proteolysis (Li et al., 2014; Liu et al., 2015; Liu et al., 2016).

Regarding other proteolytic enzymes like the caspases and cathepsins, less is known. While many cathepsins are classified as aspartic or serine peptidases, there are those of the cysteine variety (Sentandreu et al., 2002). Cathepsins are typically involved in protein degradation and turnover, but require a reducing and slightly acidic environment for optimal functionality (Turk et al., 2012). They are also generally recognized for the ability to influence apoptosis of cells (Leist and Jäättelä, 2001; Chwieralski et al., 2006; Conus and Simon, 2008; Repnik et al., 2012). Cysteine cathepsins are considered significant in context to lysosomal

enzymes (Turk et al., 2012). When oxidative stress occurs, the cysteine is targeted by ROS, likely modulating the functionality of the cysteine cathepsins. This is because of cathepsin inhibitors, cystatin, work similarly by targeting cysteine peptidases, implying they may target the cathepsin cysteine residue (Barrett, 1987; Shackelford et al., 1991). In vivo, cysteine cathepsins are upregulated in cancers and initiate caspase-dependent cell death (Mohamed and Sloane, 2006; Droga-Mazovec et al., 2008). Cancers and other forms of the disease are closely related to extreme oxidative stress in the body. It is postulated that the oxidative stress that causes excess ROS and cancer may also lead to the release of caspases due to increased lysosomal membrane permeability (Blomgran et al., 2007). It is unclear, though likely, that the cysteine cathepsins are inactivated by the ROS. Typically, cathepsins are trapped in the lysosome. The impact of cathepsins on postmortem proteolysis is debated (Nowak, 2011). There is evidence to support cathepsins' ability to escape the lysosome in fast glycolytic muscles with the low pH postmortem causing increased proteolytic activity (O'Halloran et al., 1997). The low pH is significant since their sensitivity to neutral pH can cause cathepsins to become irreversibly inactivated (Turk et al., 1995). Despite the conclusion cathepsins have minimal influence on tenderization, multiple reports have recognized cathepsins ability to influence signaling of apoptosis and therefore may indirectly cause the release of factors related to proteolysis and the decrease of muscle structural integrity (Leist and Jäättelä, 2001; Chwieralski et al., 2006; Conus and Simon, 2008; Repnik et al., 2012). Other researchers have hypothesized that oxidative stress activates apoptosis and can impact the tenderization process (Zhang et al., 2013; Wang et al., 2018). The role of cathepsins in tenderization was further elevated by Wang et al. (2014). In dry-cured duck, some cathepsins were found to be involved in tenderization based on myofibrillar proteome analysis and enzymatic activity (Wang et al., 2014). Despite the role of cathepsins in tenderization not being

well understood and controversial, evidence indicates it may still function as a marker of tenderness like heat shock proteins and other metabolic enzymes related to meat tenderization (Lomiwes et al., 2014a; Lana and Zolla, 2016).

Like calpains and some cathepsins, caspases are cysteine (and -aspartate) specific proteases that can lead to apoptosis (Kemp and Parr, 2012; Lana and Zolla, 2016). By initiating apoptotic signals, caspase can also cause the breakdown of damaged mitochondria. This is important since the damaged mitochondria are generating ROS but are now impaired in its ability to regulate excess production. With caspase signaling apoptosis for the damaged mitochondria, caspase can prevent further ROS accumulation and oxidative stress (Repnik et al., 2012). As stated before, however, the cysteine can be targeted by ROS which would inactivate the caspases. In human prostate cancer, it has been found that caspase function and a degree of apoptotic control are lost as cancer progress (Winter et al., 2001). This seems to indicate that cancer can grow partially unencumbered because oxidative stress-induced ROS can oxidize cysteine, inactivating caspase, and thus limit the apoptotic function that could regulate the cancerous cell growth.

Previous research has supported the relationship between caspases and tenderness early postmortem (Kemp et al., 2006). Caspase 3 is a specific caspase that may have a role in proteolysis, indirectly at least, by working with other proteasomes (Du et al., 2004; Herrera-Mendez et al., 2006). The gene encoding caspase 3 is upregulated in high-quality meat of 15-month-old cattle (Bernard et al., 2007). Furthermore, caspase 3 has been found to break down calpastatin, the inhibitor of calpains (Isabella Pörn-Ares et al., 1998; Kemp et al., 2009; Mohrhauser et al., 2013; Huang et al., 2014). Therefore, caspases can indirectly improve tenderness by degrading the inhibitor that impacts the central proteolytic enzyme system,

calpains (Wang, 2000). Although not fully understood, there are indications that caspases are involved in the tenderization process, and that oxidative stress helps modulate their activity (Earnshaw et al., 1999; Nakagawa et al., 2000; Lassus et al., 2002; Morishima et al., 2002). Increased understanding of how ROS impact these proteolytic enzymes could expand our knowledge of oxidative stress and tenderness.

Factors that are involved in making the structure more resistant to oxidative stress, like heat shock proteins can, therefore, make meat tougher (Ouali et al., 2006). While apoptosis is considered a factor that impacts the tenderization of meat (Sentandreu et al., 2002; Ouali et al., 2006; Cramer et al., 2018). This has led to the hypothesis that apoptosis can be involved in meat tenderness (Sentandreu et al., 2002; Lana and Zolla, 2016). Research has indicated increased mitochondria membrane degradation, caused by apoptosis, leads to caspase activation in tender meat (Laville et al., 2009; Ouali et al., 2013). Moreover, Chen et al. (2019) demonstrated ROS-induced oxidative stress increased mitochondrial membrane permeability, allowing the release of apoptotic-inducing factors and leading to improved tenderness.

Even postmortem, heat shock proteins have been shown to stabilize and resist proteolytic activity (Lomiwes et al., 2014b). Small heat shock proteins (sHSP), like HSP27, were found to be in higher concentration in tougher meat indicating a possible relationship with myofibrillar degradation (Lomiwes et al., 2013; Ouali et al., 2013; Balan et al., 2014; Lomiwes et al., 2014a). Heat shock protein 27 has a negative correlation with Warner Bratzler Shear Force (WBSF) (Kim et al., 2008). Cramer et al. (2018) found a correlation between sHSP, particularly HSP27 degradation, and myofibrillar proteins and proteolytic enzymes. Furthermore, HSP27 degradation and troponin-T degradation product were positively related ($R=0.72$; $P< 0.0001$) (Cramer et al., 2018). This is consistent with other reports that found HSP27 was downregulated

in tender meat (Bernard et al., 2007; Morzel et al., 2008; Guillemin et al., 2011). Another report indicated HSP27's value as a biomarker with increased expression in tough, low marbled Hanwoo beef compared to high marbled, Hanwoo tender beef (Kim et al., 2008). In contrast, some research has associated the upregulation of sHSP with tenderness in certain muscles like longissimus thoracis (Picard et al., 2014). These studies emphasize the importance of HSP and how they can relate to oxidative stress and overall meat tenderness. Further proteomic work is warranted to look at these related factors of apoptosis and heat shock proteins, as it relates to oxidative stress, on differing levels of tenderness and marbling.

By understanding some of the consequences of oxidative stress and oxidative damage, perhaps we can discern how oxidative stress caused by high-fat diets could relate to the high marbling and improved tenderness that is commonly found. Although the ability of antemortem ROS to react with proteins and lipids contributes to aging, senescence, and cell death is considered deleterious in living organisms (Warner et al., 2005), it may be positive for tenderization postmortem. Previous research indicates a potential-positive relationship between tenderness and marbling (Blumer, 1963; McBee and Wiles, 1967; Tatum et al., 1980; Smith et al., 1987; Wheeler et al., 1994; Kim et al., 2008; D'Alessandro et al., 2012; Emerson et al., 2013), and there is evidence to suggest they may be connected by oxidative stress. Despite these indicators, the cause(s) of high marbled meat and increased tenderness remain unclear. Evaluating the effects of oxidative stress on meat quality can help direct future strategies to balance the negative consequences on lipid stability while increasing the potential positive effects on tenderness.

Impact of Distillers Grains on Marbling

The U.S. has long recognized the value of high marbled meat distinguished by the increased price of the higher beef quality grades (USDA, 2020). This increased price for high marbled meat is fueled by consumer demand for the product (Smith and Johnson, 2016). Diet is considered one of the major factors that alter the intramuscular fat content of the meat (Houben et al., 2000). It is generally recognized that grain-finishing diets increasing marbling along with deposition of subcutaneous and intermuscular fat, compared to pasture finished beef (Pethick et al., 2004). Smith and Johnson (2016) recognized that grain-based diets are crucial to promoting marbling development. Compared to low energy feeds like forage diets, high energy feeds like corn are more effective at improving marbling (Williams et al., 1983; Gunter et al., 1996; Bindon, 2004; Chung et al., 2007; Wood et al., 2008; Arnett et al., 2012; Costa et al., 2013). This is because feeding high-fat diets causes fattening (Dolezal et al., 1982; Pethick et al., 2004). This is logical, since it is well known excess dietary energy, that are not used as an immediate energy source, can be converted to fat, stored, and accumulated (Rice University, 2013). Along with diet, synthesis of triglycerides in the body is important for increased deposition of intramuscular fat (Pethick et al., 2004; Park et al., 2018). Synthesis of triglycerides involves acetate or glucose as substrates (Hanson and Ballard, 1967). Rhoades et al. (2007) suggests that feeding corn-based diets increases glucose uptake compare to a forage-based hay diet and thus improved marbling. This could also be applied to distillers grains, a product of corn processing. Compared to corn, distillers grains has a higher nutritional value with up to three times the concentration of fat, protein, and fiber, making it a high energy feed (Klopfenstein, 1996; Klopfenstein et al., 2008). Multiple studies have demonstrated feeding distillers grains can alter fat content, compared to corn alone (Klopfenstein, 1996; Klopfenstein et al., 2008; Vander Pol et al., 2009; Mello et al.,

2012; Chao et al., 2017; Domenech-Pérez et al., 2017; Ribeiro et al., 2018). Despite this, numerous studies have revealed no detrimental effects on marbling from adding levels of different types of distillers grain, compared to corn (Klopfenstein et al., 2008; Corrigan et al., 2009; Depenbusch et al., 2009b; Leupp et al., 2009; Vander Pol et al., 2009). This indicates that the addition of distillers grains is similar to corn and thus a grain-based diet that promotes marbling. By providing high energy, distillers grain diets can increase marbling faster than forage-based diets (Dolezal et al., 1982; Pethick et al., 2004). Schoonmaker et al. (2010) found the addition of 20% distillers grains inclusion to a high forage diet improved marbling score in beef compared to 0% distillers grains inclusion, indicating distillers grain diets can improve marbling, like other high-fat diets, in a shorter time than forage-based diets. Recognizing the ability of distillers grains to provide marbling in meat helps contextualize the potential relationship that a high-fat diet, like distillers grains, can have on this aspect of meat quality.

Relationship between Marbling and Tenderness

Consumer acceptance of a meat product is crucial to success and is typically influenced by palatability and a combination of flavor (often related to marbling), juiciness, and tenderness (Simone et al., 1958; Jeremiah et al., 1970). It has been long taught that tenderness and overall palatability steak is improved “when accompanied by considerable fat” (Armsby, 1908). For over a century, multiple sources have recognized the relationship of high marbling and meat tenderness, both anecdotally and with data (Morrison, 1937; Blumer, 1963; McBee and Wiles, 1967; Jeremiah et al., 1970; Tatum et al., 1980; Smith et al., 1987; Wheeler et al., 1994; Wood et al., 1999; Kim et al., 2008; D’Alessandro et al., 2012; Emerson et al., 2013). Most scientists agree that a more marbled product provides a more tender steak and a positive eating experience

(Feuz et al., 2004). Over the decades, scientists have tried to determine the causes of this relationship. The "marbling effect" has been termed the lubrication effect or the bulk density effect. Discerning a relationship that can be used to create a consistently high-marbled and tender product is important economically since consumers are willing to pay extra for these products (Armsby, 1917; Feuz et al., 2004).

Diet

Diet is one of the major factors involved in the marbling-tenderness relationship. Diet is a major factor impacting fat composition (Dolezal et al., 1982). Feeding high-fat diets in the finishing stage of growth increases marbling of the meat (Blanchard et al., 1999; Houben et al., 2000; Pethick et al., 2004; Smith and Johnson, 2016). Diet can also have an impact on the tenderness of the meat (Blanchard et al., 1999; Roeber et al., 2005; Gill et al., 2008; Leupp et al., 2009; Koger et al., 2010; Segers et al., 2011; Mello et al., 2012). By providing a high-fat diet, an animal can get fattened and that fat can separate muscle bundles, making the meat more tender (Henry and Morrison, 1916; Morrison, 1937; Dolezal et al., 1982; Pethick et al., 2004). This capacity to directly impact marbling and tenderness, though less consistent in producing consistently tender beef, signifies the importance of feeding cattle on meat quality.

Marbling

Lubrication Effect

The lubrication effect is the name of a hypothesis that suggests that marbling in the meat can serve as a "lubricant" around the myofibrils and the muscle fibers facilitating the perception of a tender and juicy product. Tenderness and juiciness are readily associated aspects of palatability (Smith and Carpenter, 1976). According to Jeremiah et al. (1970), the marbled fat

that melts during cooking becomes a part of the meat juices that “lubricates” during chewing, improving juiciness. As juices are released from the marbling that surrounds the muscle, the perception of tenderness increases. This is supported by Berry et al. (1974) that found fat content impacted the expressible area of juice, a measurement related to the lubrication effect and bulk density, and increased expressible area of juice improved tenderness. As the marbling is deposited within and throughout the muscle, unlike seam fat, the fat supplies the “lubrication” during eating to create a perceivably more tender product (Briskey and Kauffman, 1971). Therefore, marbling contributes indirectly to improve tenderness by directly increasing juiciness.

Bulk Density

The term bulk density describes the increased tenderness caused by the increased presence of marbling, decreasing the mass per volume in the meat (Smith and Carpenter, 1976). Doing so decreases the bulk density, making the meat seem more tender. This is because the intramuscular fat requires less force to shear than the protein that would otherwise take up space in the meat, impacting the tenderness of the product. An early explanation of this theory was the deposition of marbling in muscle bundles creates the space between bundles that improves the tenderness (Henry and Morrison, 1916). A later perspective described the potential cause to be due to the marbling taking up space, leading to a decrease of connective tissue (Jeremiah et al., 1970). This could be supported by Nelson et al. (1930), who found a decrease in shear force from fattened animals compared to thinly finished animals, supporting that perceived difference in tenderness may be caused by fat being present where protein would be otherwise. Berry et al. (1974) found increased fat percent and lower moisture percentage to be positively associated with tenderness. Regardless of the cause- the separation of muscle or filling the space used by

connective tissue- marbling seems to improve tenderness by lowering the bulk density and therefore fewer muscle fibers have to be sheared per bite during consumption.

Clearly, multiple factors impact the subsequent tenderness of the product making it a variable difficult to control in creating a consistently tender meat product. Marbling is a major factor that relates to tenderness. Despite all of the explanations that relate marbling to tenderness, none have proven sufficient (Smith and Carpenter, 1976), suggesting multiple factors may be related and that the main cause perhaps has not been determined. As discussed in the previous sections, oxidative stress relates to many factors and there is evidence it can have a downstream impact on meat quality. Taking this into consideration, perhaps oxidative stress can be a link between marbling and tenderness that has yet to be investigated.

Impact of Diet of Beef Quality Characteristics and Shelf Life

Color

Color is a crucial factor in consumers purchasing meat products (Forbes et al., 1974; Savell et al., 1989). The shelf life of beef, during retail display, is a major factor that impacts how long the meat can be presented before discounting due to discoloration (Warren et al., 2008). Stolzenbach et al. (2009) defined shelf life as the length of time in which a beef product remains acceptable despite the development of off-odors/flavors and appearance. When purchasing beef products, consumers in the US desire bright-cherry red color meat, and may avoid meat that is noticeably discolored (Kropf, 1980; Faustman et al., 1989). Typically, the retail shelf life of high dollar cuts like tenderloins and strip loins are limited to 1 day or 3 days case life, respectively (Smith et al., 1996). If those muscles are discolored, before the end of the case life, will be discounted, leading to economic loss (Faustman et al., 1989; Smith et al., 1996).

According to Williams et al. (1992), an estimated 5.4% of fresh meats show a value loss. In 2000, an estimated 15% of meat was discounted due to discoloration, resulting in a 1 billion dollar loss/annually (Smith et al., 2000).

When evaluating color in meat, protein pigments contribute to consumers' visual perception of wholesomeness in the meat product. This is due to the proteins having heme connected to the protein structure. Multiple pigments like hemoglobin, cytochrome enzymes, and catalase play a role in perceived color, but myoglobin is as it contributes 80-90% of the total pigments (Aberle et al., 2012). Myoglobin is called a pigment-protein due to the attached heme prosthetic group with a globin moiety (Suman and Joseph, 2013). The heme ring contains an iron (Fe) that can exist in a reduced (Ferrous) or oxidized state (Ferric). The Fe state and what is bound to the 6^{th} ligand of the Fe are what ultimately impacts the color (Mancini and Hunt, 2005; Suman and Joseph, 2013). When Fe is in the reduced state, as ferrous iron, with an unbound 6^{th} ligand, it forms a purple pigment called deoxymyoglobin (Suman and Joseph, 2013). In an oxygen-rich atmosphere, the meat will “bloom” as oxygen binds to the 6^{th} ligand of the ferrous iron, producing a bright cherry red color. This process forms a more stable pigment form called oxymyoglobin (Lanari et al., 1995; Neethling et al., 2017). When the iron in the heme ring is oxidized into ferric iron, the oxygen is displaced, allowing water to bind to the 6^{th} ligand (Faustman et al., 2010). This produces a brown color in the myoglobin pigment and is associated with discoloration in meat (Mancini and Hunt, 2005; Suman and Joseph, 2013). Reducing factors like NADH and other metmyoglobin reductants facilitate the reduction of metmyoglobin back to deoxymyoglobin. However, with the limited reducing ability and a buildup in oxidation factors, the meat will continue to cycle back to metmyoglobin until it is 100% discolored. Once meat approaches 20% metmyoglobin on the surface, the meat acceptance and sales decline, leading to

product discounts in price (Faustman et al., 2010). A variety of factors that can impact ultimate meat color and stability and an understanding of these factors can be helpful in minimizing this economic loss.

Color related in Distillers Grain

The negative effects of feeding distillers grains to cattle on the shelf life of beef steaks have been well established (Gordon et al., 2002; Roeber et al., 2005; Gill et al., 2008; Depenbusch et al., 2009a; Leupp et al., 2009; Aldai et al., 2010; Segers et al., 2011; Mello et al., 2012; Buttrey et al., 2013). On a dry matter basis, beef from cattle fed 15% corn distillers grains had higher L* (lightness) but lower a* (redness) compared to sorghum distillers grains when either feed was added to a steam flaked corn (SFC) diet (Gill et al., 2008). In contrast, Depenbusch et al. (2009a) found that an increasing percentage of DDGS fed to cattle resulted in a linear decrease in brightness (L*) at 0 d of retail display. Furthermore, researchers found decreased redness (a*) with increasing inclusion of DDGS after 7 d of retail display (Depenbusch et al., 2009a). Segers et al. (2011) found that a 25% inclusion of DDGS increased discoloration and increased the rate of redness loss under the retail display. The addition of DDGS in heifer finishing diets also led to lower a* and b* values (Gordon et al., 2002). Similarly, the inclusion of 30% DDGS found reduced L*, a*, and b*(yellowness) compared to a dry rolled corn (DRC) diet during the growing or finishing phase of production (Leupp et al., 2009). Even at 15% DG, beef samples had more lightness but less redness than cattle fed SFC (Gill et al., 2008). However, Aldai et al. (2010) found contradicting results that beef from cattle fed 40% DDGS had higher oxymyoglobin content and L* than 20% DDGS. This was explained to be in part due to darker lean color and yellowish marbling color in the 20% DDGS (Aldai et al., 2010).

Research has found the addition of wet distillers grains with solubles to the diet seems to reduce color, but the effect varies by inclusion percentage (Roeber et al., 2005; Mello et al., 2012). Lower redness values (a*) under retail display were found from beef steaks from cattle fed 30% WDGS for strip loins, tenderloins, and top blade steaks, compared to beef steaks from cattle not fed WDGS (Mello et al., 2012). Regardless of DRC- or SFC- based diets, the addition of 35% WDGS decreased redness value during retail display (Buttrey et al., 2013). Alternatively, (Roeber et al., 2005) did not find negative impacts of feeding WDGS to cattle until over 40% of the dietary DM was WDGS, with minimal impact, otherwise. Overall, it seems the addition of WDGS has a detrimental impact on meat color under retail display.

Lipid oxidation

As previously stated, the perceived color of fresh meat, particularly beef, is dependent on the state of myoglobin: deoxymyoglobin, oxymyoglobin, or metmyoglobin (Hood and Riordan, 1973; O'Keeffe and Hood, 1982). Faustman et al. (2010) recognized that lipid oxidation is a key precursor to oxidation of oxymyoglobin to metmyoglobin. Often called autooxidation, oxidation of unsaturated fatty acids (UFAs) and proteins, like myoglobin, produce a radical in the molecule that leads to the subsequent creation of hydrogen peroxide. With the increase of peroxides, this process becomes cyclic as peroxides other susceptible lipids or proteins become oxidized. The peroxides can further degrade into measurable secondary products like aldehydes, ketones, and alcohols (Faustman et al., 2010). Excess secondary products cause negative factors like increased rancidity. Malondialdehyde, a secondary product of lipid oxidation, is measurable through TBARS (Thiobarbituric Acid Reactive Substances) method (Ahn et al., 1998). In evaluating meat samples, Zakrys et al. (2008) found a relationship with TBARS and changing a* and oxymyoglobin values caused by the propagation of lipid oxidation. The likelihood of lipid

oxidation is greatly increased in the meat when animals are fed a diet that results in greater deposition of polyunsaturated fatty acids (PUFAs). This increased susceptibility to lipid oxidation leads to increased amounts of discoloration (Nute et al., 2007). Similarly, Grimsrud et al. (2008) reported that the accumulation of products from lipid oxidation increased the formation of metmyoglobin. As a major precursor of accelerated discoloration in fresh meat, methods to minimize lipid oxidation are often sought to prevent or delay discounting or loss of meat.

Lipid oxidation related to DG

Additions of distillers grain in cattle feed has been shown to increase deposition of PUFA in the beef (Gill et al., 2008; Depenbusch et al., 2009a; Mello et al., 2012; Domenech-Pérez et al., 2017; Chao et al., 2018; Ribeiro et al., 2019). With a higher concentration of PUFAs, there is an increased likelihood of lipid oxidation in beef (Holman and Elmer, 1947). This can lead to subsequent protein/ myoglobin oxidation and overall detrimental effects on shelf life when cattle are fed distillers grains, particularly at a higher level (Roeber et al., 2005; Depenbusch et al., 2009a; Leupp et al., 2009; Segers et al., 2011; Mello et al., 2012; Domenech-Pérez et al., 2017; Chao et al., 2018; Ribeiro et al., 2019). Chao et al. (2018) found that the addition of DG to the diet resulted in an increase of PUFA that was deposited in the membrane of the sarcoplasmic reticulum. This created a greater susceptibility for oxidative damage and subsequent leakage of calcium (Chao et al., 2018). Kunze et al. (2017) evaluated feeding DG diet, compared to corn-based diets, on membrane stability of the mitochondria. They reported a higher PUFA content and a tendency (P= 0.08) for greater calcium leakage than the corn-based diet (Kunze et al., 2017). In both instances, this oxidative damage in the membranes allowed for a flux of calcium in the sarcoplasm. This available calcium can be used by calpain-dependent proteolytic enzymes

like calpains to increase tenderness in the meat (Koohmaraie, 1992). Overall, research suggests the increase of DG leads to increase deposition of PUFAs in cell membranes. Although this generally has negative impacts on color and shelf life, other research seems to support the idea tenderness might improve through disrupting cells and increasing calcium availability for proteolytic enzymes, like calpains.

Tenderness related to diet or other postmortem factors

During the conversion of muscle to meat, muscle stiffening is observed when key myofibrillar proteins, actin and myosin, form cross-bridges in the sarcomere (Swartz et al., 2009). The process is given the name rigor mortis or "stiffness in death." In a living system, ATP can break the cross-bridge that is formed during contraction in the muscle. After death, the rapid depletion of oxygen and ATP force the body to use more anaerobic pathways to produce energy (Bate-Smith, 1948). These pathways lead to the decrease in pH, with the accumulation of lactic acid and hydrogen ions, due to the failure of the circulatory system, after exsanguination (Bate-Smith, 1948). Bate-Smith, (1948) describes Lundsgaard (1930) research into this phenomenon as the body is scavenging to find sources of energy early postmortem. Even creatine phosphate is a source used to provide limited energy to put the muscle back into a relaxed state (Lundsgaard, 1930; Bate-Smith, 1948). Following the onset phase of rigor, the completion phase is described by the depletion of ATP and the shortening of sarcomeres (Jeacocke, 1984). This results in the complete formation of the actomyosin cross-bridge (Jeacocke, 1984; Koohmaraie et al., 1996). Entering the resolution phase of rigor, muscle tension is decreased as proteolytic enzymes degrade the structural proteins in the sarcomere. Although not breaking down actin and myosin, proteolysis of proteins like troponin- T, desmin, titin, and nebulin allow for the decreased tension in the muscle (Taylor et al., 1995; Boyer-Berri and Greaser, 1998). Also associated with

tenderization, the calpain family is recognized as the main enzymes that account for the majority of proteolysis (Koohmaraie, 1992). The calpain family includes calpain-dependent enzymes, μ-calpain and m-calpain, and the less understood calpain III. Furthermore, calpastatin is a calcium-dependent molecule that can inhibit calpain function in proteolysis (Boehm et al., 1998). This implies the reduced function of calpains or calpastatin, due to oxidative related stress damage, can impact tenderness.

For repeated purchase, palatability defined as tenderness, juiciness, and flavor is a key aspect of meat quality (Killinger et al., 2004; Dikeman et al., 2005). Over time, tenderness is a major factor, if not the most important sensory attribute, to the consumers eating experience (Morgan et al., 1991; Huffman et al., 1996; Boleman et al., 1997; Neely et al., 1998; Calkins and Hodgen, 2007; Grobbel et al., 2008; Verbeke et al., 2010). The impact of distillers grains on tenderness has been evaluated in previous research. However, there are also cases where there was no impact on tenderness with the addition of distillers grains in the diet (Roeber et al., 2005; Gill et al., 2008; Leupp et al., 2009; Koger et al., 2010; Segers et al., 2011; Mello et al., 2012). It has been reported that beef from cattle fed distillers grains, compared to a corn-based diet, have improved tenderness measured with Warner-Bratzler Shear Force (WBSF) and sensory panels (Gordon et al., 2002; Depenbusch et al., 2009a; Aldai et al., 2010; Senaratne, 2012; Chao et al., 2018). In Depenbusch et al. (2009), it was hypothesized that the increased percentage of fed distillers grains was associated with a linear decrease of connective tissue present in the meat. This was concluded because of the linear increase in perceived tenderness, evaluated by a trained panel, paralleled with the increase in percentage fed distillers grains. Given that the increase in PUFA content, from feeding distillers grains, can cause a greater likelihood of oxidative damage in cell membranes postmortem, it is possible that the addition of distillers grains can improve

tenderization in a variety of ways. Recognizing there are positive impacts of feeding DG on animal growth, feeding DG could also improve tenderness, possibly because of oxidative damage. This can increase the value of adding distillers grains to the diet.

Relationship of Postmortem Aging and Retail Display on Beef Quality Characteristics and Shelf Life

Lipid Oxidation

Regardless of the aging method and retail display, lipid oxidation increases in meat (Epley, 1992). This is particularly true for meat under retail display since it's kept purposefully under light, a known prooxidant (Hood, 1980). Aged meat followed by retail display also showed an increase in lipid oxidation (English et al., 2016). This is consistent with Vitale et al. (2014) who found 14-day aged meat subjected to retail display also had increased lipid oxidation compared to reduced aging times. With extended periods of aging and retail display, lipid oxidation continued to increase, negatively impacting the meat (Mello et al., 2012; Domenech-Pérez et al., 2017; Ribeiro et al., 2018). This is because lipid oxidation has a strong relationship with meat color (Faustman et al., 2010). This increased lipid oxidation during aging and retail display can impact the color stability and shelf life of the meat (Morrissey et al., 1994; Ladeira et al., 2014).

Color Stability

Despite many studies involving postmortem aging, the amount of time involved that causes an apparent difference in color is unclear (Ledward, 1970; Ledward, 1971; Ledward et al., 1986; Vitale et al., 2014). No differences in muscle color were found from 3 and 72 h,

postmortem (Ledward et al., 1986), while Ledward (1970) found no difference in metmyoglobin concentration 5 to 14 d postmortem and Ledward (1971) found no difference from 2 to 4 weeks. Despite this lack of clarity, it is clear postmortem aging and retail display does impact color stability. As the meat is aged or under the retail display, shelf life and color stability continually decrease, leading to discoloration of the product (Epley, 1992). At the outset, however, when internal lean is exposed to the oxygen in the environment, "blooming" takes place, oxygenating the myoglobin and converting the pigment to a bright cherry red color (Pirko and Ayres, 1957). Initially, the color intensity is improved with storage time by allowing greater access of oxygen to myoglobin, compared to the mitochondria, resulting in a higher bloom capacity (MacDougall, 1982; Bekhit and Faustman, 2005). As meat is subjected to retail display and oxygen, the color will cycle repeatedly, using up the meat's blooming capacity at a greater rate than non-aged meat (Mancini and Hunt, 2005). Over time, aging will lead to a depletion of compounds need to maintain color (Hood, 1980; MacDougall, 1982; Mancini and Ramanathan, 2014; Vitale et al., 2014; English et al., 2016). The deterioration of color stability, particularly L*(lightness), was hypothesized to be caused by protein structural alterations during postmortem aging (MacDougall, 1982). Since high rates of oxygen consumption favor deoxymyoglobin, compared to oxymyoglobin, in fresh meat, myoglobin can revert to metmyoglobin as the heme iron is oxidized (O'Keeffe and Hood, 1982). As metmyoglobin is the pigment associated with discoloration, it is an undesirable color for consumers (Hood, 1980; Mancini and Hunt, 2005). It has been reported that aging decreased MRA and color stability by the end of the retail display (English et al., 2016). Vitale et al. (2014) found 3-day aged meat had higher levels of L*, a*(redness), b*(yellowness) at the beginning of retail display, compared to unaged meat. However, 14-day aged meat had lower a* values and color stability after 3 days of retail display

(Vitale et al., 2014). Meat, following aging, discolors more quickly under retail display compared to fresh meat (Hood, 1980; Mello et al., 2012; Domenech-Pérez et al., 2017; Ribeiro et al., 2018). Therefore, postmortem aging and retail display are considered deleterious for color stability as meat becomes discolored with decreased MRA activity and mitochondrial activity.

Tenderness

During the conversion of muscle to meat, actomyosin cross-bridges are formed in the sarcomeres, leading to rigor and muscle tension (Jeacocke, 1984; Swatland, 1997). Regardless of the aging method, postmortem aging improves tenderness (Epley, 1992; Dransfield, 1994). Postmortem aging's impact on tenderness is due to the increased activity of proteolytic enzymes (Huff-Lonergan et al., 1996; Melody et al., 2004). This typically includes the calpain family, the caspase system, and cathepsins, though calpains are regarded as the main enzymes that account for tenderization (Sentandreu et al., 2002; Purslow, 2004; Ouali et al., 2006; Ouali et al., 2013). The decrease in muscle tension improves tenderness as a result of the proteolytic enzymes, mostly calpains, targeting structural proteins (Koohmaraie, 1992; Taylor et al., 1995). Seemingly unable to target the actin and myosin, and therefore the site of the muscle tension, degradation of proteins like troponin-t, desmin, nebulin, and titin are the major sources of improved tenderness (Iversen et al., 1995; Taylor et al., 1995; Boyer-Berri and Greaser, 1998; Huff-Lonergan et al., 2010; Zakrys-Waliwander et al., 2012). Calpastatin is an inhibitory protein of the calpains, and the activity can potentially impact the degree of tenderization that takes place (Koohmaraie, 1992; Boehm et al., 1998; Goll et al., 2003). As aging is extended, a continued proteolytic activity can occur improving the tenderness of the product (Mello et al., 2012; Domenech-Pérez et al., 2017; Ribeiro et al., 2019). The lipid oxidation that also takes place can cause membrane

SR damage allowing for calcium leakage that can be used by the calpains (Koohmaraie, 1992; Chao et al., 2018).

Overall, postmortem aging and retail display can have a significant impact on meat quality factors and the shelf life of the product. Because of the economic potential of these factors, considerations whether to age and, to what extent, place under the retail display is warranted (Faustman et al., 1989; Smith et al., 1996; Killinger et al., 2004; Dikeman et al., 2005; Grimsrud et al., 2008).

Conclusion

Based on previous research, it seems plausible to hypothesize that high-fat diets create levels of oxidative stress that affect meat quality. Reactive oxygen species-induced oxidative stress has a variety of impacts on cattle that are related to the subsequent meat quality. Though research indicates potential negative impacts of oxidative stress on lipid oxidation and color, tenderness improvements may occur. Incidental oxidative stress appears to occur in high-fat diets. Furthermore, there has not been conclusive evidence to connect high marbling to the improved tenderness, though they have been recognized to be related for over a century. The hypothesis is that high-fat diets cause oxidative stress which may be positively linked to improved quality grades and tenderness, providing a meaningful reason why high marbled meat is also more tender than low-marbled meat.

MATERIALS AND METHODS

Impact of Diet and Quality Grade on Meat Quality Characteristics and Their Relationship to Oxidative Stress

Cattle and dietary treatments

A total of 240 crossbred steers (288 ± 0.54 kg) were blocked into three body weights and randomly assigned to one of 24 pens, (n=10/pen), then fed (University of Nebraska feedlot at Ithica, NE) for 202 days. Four dietary treatments were divided among the pens (6 pens/treatment). Cattle were fed either dry rolled corn (DRC), steam flaked corn (SFC), dry rolled corn plus 30% dried distillers grains with solubles (DRC+ DDGS), or steam flake corn plus 30% dried distillers grain with solubles (SFC+ DDGS). Inclusion of distillers grains was calculated on a dry matter basis. All diets contained 8% sorghum silage, 5% supplement (1.393% fine ground corn, 1.69% limestone, 0.125% tallow, 1.4% tallow, 0.3% salt, 0.05% beef trace mineral, 0.015% Vitamin A-D-E, 0.0165% Rumensin-90, and 0.011% Tylan-40). All dietary treatments are provided in Appendix I.

Sample collection

All steers were transported on June 19, 2018, to Greater Omaha Packing Co. (Omaha, NE) and held 12 h before slaughter. After slaughter, carcasses were chilled 48 hours and tagged with a university number corresponding to the pen within which the cattle were fed within. Subsequent USDA quality grading took place, and the intention was to select two upper 2/3 Choice and Select carcasses per pen; at least one of each quality grade had to be selected for a pen to be used. In total, 21 Upper 2/3 Choice and 15 Select carcasses were selected (n=36) from 2-4 pens/ treatment. From each of the selected carcasses, both sides of the strip loins were

marked with food-grade carcass crayons (Industrial Markers Dixon No. 1530R Joseph Dixon Crucible Co., New Jersey, N.J.) and a plastic carcass push pin (Beef brands, KMC-KK1075-064, Butchers and Packers Supplies, Edmonton, AB) with a tag referencing a University of Nebraska-Lincoln (UNL) meat lab number ranging from 1 to 36. Selected loins were subsequently vacuum packaged with sterilized, laminated, and numbered ID tags and transported to the Loeffel Meat Laboratory at the University of Nebraska-Lincoln.

Fabrication

Upon arrival to Loeffel Meat Laboratory, the 2 d aged beef loins were divided in half to be randomly assigned to one of the four aging treatments (2, 9, 16, 23 days). After each aging period, the beef loins were fabricated into steaks for subsequent analysis. The loins were consistently sliced from anterior to posterior, following the fabrication map (Appendix II). Four 2.54 cm steaks were fabricated after each aging period (one steak used for Warner-Bratzler shear force (WBSF) measurement at 0 d of retail display (RD), one steak used for Warner-Bratzler shear force measurement, visual discoloration, and objective color for 7 d of RD, one steak trimmed of all subcutaneous fat for proximate composition, sarcomere length, fatty acid analysis, free calcium concentration, pH analysis, proteomics, Troponin-T degradation, and isoprostanes at 0 d of RD, and an extra steak for possible-future analyses). One 1.9cm thick steak was cut in half and trimmed of all subcutaneous fat to measure lipid oxidation after 0 and 7 d of RD for each aging period. Steaks for WBSF at 0 d RD were set aside to perform slice shear force (SSF) before carrying out WBSF. All loins aged for longer than 2 days were vacuum packaged (MULTIVAC 500, Multivac, Inc., Kansas City, MO) in Prime Source Vacuum pouches (3 mil STD barrier, Prime Sources, St. Louis, MO) and aged in dark storage (2°C). Steaks for laboratory analysis and lipid oxidation were frozen for further analysis (-80°C). Steaks for 7 days

of RD were placed on foam trays (21.6 x 15.9 x 2.1 cm, Styro-Tech, Denver, CO) and overwrapped with oxygen-permeable film (Prime Source PSM 18 #75003815, Bunzl Processors Division, North Kansas City, MO) to be placed under retail display (RD) for 7 d at 3°C. All steaks subsequently used for laboratory analyses were diced into small portions before freezing in liquid nitrogen before powdering in a metal blender cup (Model 51BL32, Waring Commercial, Torrington, CT) on August 6-8, 2018 and kept frozen (-80°C) for the following analyses.

Tenderness determination via Shear Force

Steaks 2.54 cm thick, never frozen, were measured for tenderness via Slice Shear Force (SSF) and Warner-Bratzler Shear Force (WBSF). Before SSF, raw-internal steak temperatures were taken in Celsius (C), in the geometric center of the steaks, using a T-type thermocouple (TMQSS-062U-6, OMEGA Engineering, Inc., Stamford, CT) connected to a portable thermometer (OMEGA 450-ATT, OMEGA Engineering, Inc., Stamford, CT). Raw steak weights were measured with a scale set in grams and recorded. Afterward, all steaks were cooked to an internal temperature of 70°C on a belt grill (TBG60-V3 MagiGril, MagiKitch'n Inc., Quakertown, PA). After cooking, internal steak temperatures and weights were recorded before slice shear force evaluation was conducted using a Slice Shear force blade attached to a Food Texture Analyzer (TMS- Pro, Food Technology Corp., Sterling, VA.). The rest of the steak was bagged (PB-90-C, 0.85 mil., 6 x 3 x 15 in., Get Reddi, Inteplast Group, Livingston, NJ) and stored overnight at 2°C. The next day, six cores (1.27 cm diameter) were cored out with a drill press going parallel to the muscle fibers and sheared using the Food Texture Analyzer with a triangular Warner-Bratzler blade. An average of the six cores was calculated for each steak for statistical analysis.

Instrumental color and visual discoloration

Steaks (2.54 cm) were placed on Styrofoam trays (21.6 x 15.9 x 2.1 cm, Styro-Tech, Denver, CO) and overwrapped with oxygen-permeable film (Prime Source PSM 18 #75003815, Bunzl Processors Division, North Kansas City, MO). The steaks were subsequently placed under the retail display and repositioned daily to minimize location effects (3°C under white fluorescence lighting at 1000 to 1800 lux, Lamp Type: F32T8/TL741 700 Series 32 watt, Phillips, Inc. New Jersey). Instrumental color measurements were taken daily during the 7-day simulated retail display for each aging period. Commission International de l'éclairage (CIE) L*, a*, and b* values were obtained using a Minolta CR-400 colorimeter (Minolta, Osaka, Japan). Parameters for the colorimeter were set with a D65 illuminant, 2° observer, with an 8 mm diameter measurement area. The colorimeter was calibrated daily with a white ceramic tile (Calibration Plate, Serial No. 14933058, Konica Minolta, Japan). Lightness (L*) is measured with a range from 0 (black) to 100 (white), a* is a measurement of redness with a range between red (positive values) and green (negative values), and b* is a measure of yellowness with yellow (positive) to blue (negative). See Appendix III for further details and tips for using the Minolta described. Six measurements were recorded per steak and the mean was calculated for each steak for statistical analysis. Color readings were taken at the same time each day.

The visual discoloration was assessed daily during the retail display period, across aging periods, with 5 trained panelists comprised of graduate students from the University of Nebraska-Lincoln. Panelists were trained using a standardized discoloration guide (Appendix IV). Discoloration % was approximated from 0% to 100% with 0% being no discoloration on the surface and 100% being a fully discolored steak.

Lipid oxidation (TBARS)

Lipid oxidation of secondary products was determined using thiobarbituric acid reactive substances (TBARS). This specifically determines the amount of malonaldehyde present in the sample. This was done for all aging period samples at 0 and 7 d RD following the protocol described by Ahn et al. (1998; see Appendix V). Five g of powdered meat were placed into a 50 mL conical tube and immersed with 14 mL of distilled deionized water and 1 mL of butylated hydroxyanisole (BHA) solution (10% BHA; 90% ethanol). Samples were homogenized using a Polytron (Kinmatica AG, PT-10-35 GT) for 15 seconds at medium to high speed. The samples were centrifuged (2,000 x g for 5 min at 10°C) and one mL of supernatant was transferred into a 15 mL tube. Two mL of thiobarbituric acid/ trichloroacetic acid (TBA/TCA) solution (15% TCA and 20 mM TBA in deionized distilled water) were added and the samples were placed in a water bath at 70°C for 30 min. Subsequently, samples were cooled in a 20 °C water bath for 10 min then centrifuged (2,000 x g for 5 min). After centrifugation, 200 µL of the samples were transferred to a 96 well plate in duplicates along with pre-made standards. The plate was read using a microplate spectrophotometer (Model Epoch, Biotek, Winooski, VT) at an absorbance of 540 nm. The measured results were expressed in mg of malonaldehyde per kg of tissue**.**

Proximate composition

Fat, moisture, and ash (%) were determined from powdered-raw meat samples from all aging periods. Moisture and ash (%) were measured in duplicate using a Thermogravimetric Analyzer (Model 604- 100-400, LECO Corporation, St. Joseph, MI). Total fat (%) was done in triplicate through the Soxhlet procedure (~48 h) of ether extraction after taking the difference of the already quantified moisture percentage (AOAC, 1990) (See Appendix VI) with protein calculated by overall difference.

Sarcomere length

Sarcomere length was determined following the method described by Cross et al. (1981) and Dolazza and Lorenzen (2014) using helium-neon laser diffraction. A pinch of powdered meat sample was placed on a clear-glass microscope slide. A single drop of 0.25 M sucrose solution was added to the sample and topped with a glass coverslip. The distance between the base of the laser and the top of the slide was 100 mm. A piece of paper was placed underneath the stand to mark the three diffraction bands. Five single sarcomeres per sample were determined and sarcomere length (µm) was calculated by the equation provided by Cross et al. (1981):

Sarcomere length (µm)=

$$\frac{0.6328 \times D\sqrt{\left(\left(\frac{T}{D}\right)^2 + 1\right)}}{T}$$

D= distance from specimen to diffraction pattern screen (100 mm)

T= spacing between diffraction bands (mm)

Fatty acid analysis

The extraction of the total lipids was completed with the chloroform-methanol procedure described by Folch et al. (1957). Following the extraction process, lipids were reduced to fatty acid methylated esters according to Morrison and Smith (1964) and Metcalfe et al. (1966). One gram of powdered sample was homogenized with 5 mL of 2:1 chloroform: methanol and kept at room temperature (23°C) for 1 hr. Afterward, the homogenate was filtered through Whatman #2 paper into a screw cap tube and brought to a final volume of 10 mL with the 2:1 chloroform: methanol. Samples were vortexed for 5 s with 2 mL 0.74% KCl solution and centrifuged for 5

minutes at 1,000 x g, and the top layer was aspirated off. After aspirating, the samples were placed in a heating block at 60°C and continually flushed with nitrogen until dried. Once dry, 1 mL of 0.5 NaOH in methanol was added, vortexed for 5 s, and heated for 10 min at 100°C. Subsequently, one mL of 14% boron trifluoride in methanol was added, vortexed for 5 s, and the samples were heated again at 100°C for 5 min. Following the heating period, 2 mL of saturated salt solution and 2 mL of hexane were added to the samples and vortexed (5 s). Samples were then centrifuged at 1,000 x g for 5 min and the hexane layer (top layer) was removed to be analyzed using gas chromatography (TRACE 1310 Gas Chromatography; ThermoFisher Scientific, Waltham, MA). Separation of the fatty acids was done using a capillary column (Chrompack CP-Sil 88- 0.25 mm x 100 m; Inlet temp: 260°C, Oven: 140°C hold for 5 min, increase at 4°C/min to 240°C and hold for 15 min. FID temp: 280°C. Injected at 30:1 ratio) and identified based on their retention times compared to known commercial standards (NU- Check Prep, Inc., Elysiam, MN; # GLC-68D, GLC-79, GLC-87, GLC-455, and GLC-458, see Appendix VII). Determination of fatty acids percentage was done with the peak areas in the chromatograph and value were converted to mg/100 g tissue:

Fatty acid mg/100 g tissue = (% of fatty acid peak area * fat content of samples) * 1000

Free calcium concentration

Free calcium was quantified using the procedure described by Parrish et al. (1981), with modifications. Three g of powdered sample were placed in a thick-wall, polyallomer ultracentrifuge tube (13 x 55 mm; Beckman Coluter, Brea, CA) and centrifuged at 196,000 x g (Beckman Optima XPN-90 Ultracentrifuge, Type 50.2 Ti rotor, Beckman Coulter, Brea, CA) at 4°C for 30 min. Seven hundred µL of the supernatant were collected and treated with 0.1 mL of 27.5% trichloroacetic acid (TCA), then vortexed until the TCA was thoroughly mixed. Samples

were centrifuged at 6,000 x g (accuSpin Micro 17R, ThermoFisher Scientific, Waltham, MA) at 4°C for 10 min. Four hundred µL of supernatant were transferred to a syringe, and the volume was brought to 4 mL with deionized, distilled water (ddH_2O). The diluted calcium sample was then filtered through a 13 mm diameter Millex-LG 0.20 µL syringe filter (Millipore, Bedford, MA). Calcium concentration of samples was quantified at Ward Laboratories (Kearney, NE) using an inductively- coupled plasma emission spectrometer (iCAP 6500 Radial; Thermo Electron, Cambridge, UK) with appropriate calcium concentration standards.

pH analysis

Powdered steak samples from 2 d aging period with 0 RD were weighed into 5 g duplicates with 45 mL of distilled deionized water in 250 mL plastic beakers. A magnetic stir bar was added to each beaker to ensure constant mixing. The pH was measured using a pH meter (Orion 410A plus: ThermoFisher Scientific; Waltham, MA) that was calibrated using 4.0, 7.0, and 10.0 pH standards. The analysis was conducted from the mean of the measured duplicate samples. The pH probe was rinsed with distilled, deionized water and dried with a Kimwipe between every measurement.

Proteomics

Samples were evaluated by Dr. Camila Braga and Dr. Jiri Adamec's lab at UNL's Department of Biochemistry. The protocol follows Malheiros et al., (2019) with modifications.

Protein extraction and derivatization procedure

Steaks, 2d aged, utilized for proteomic analysis were diced into small portions before freezing in liquid nitrogen, powdering in a metal blender cup (Model 51BL32, Waring Commercial, Torrington, CT), and storing frozen (-80°C) for the following analysis. About 50

mg of powdered meat samples were utilized to measuring protein concentrations via the bicinchoninic acid (BCA) method with bovine serum albumin as the standard (Thermo Scientific, BCA Protein Assay Kit, Waltham, MA). Following, electrophoretic runs with derivatized muscle samples were performed for the treatment groups.

Following procedures described in Tamarit et al. (2012) and Boone et al. (2016) with minor modifications, two cyanine dyes coupled with hydrazides, Cy3-Hz and Cy5-Hz, were used in the derivatization of carbonylated proteins. Cyanine dyes were obtained from Lumiprobe (Hunt Valley, Maryland) and reconstituted to 50 mM in dimethyl sulfoxide (DMSO) and then diluted with a derivatization buffer (1:10, 0.1 M sodium acetate pH 5, 1 mM EDTA and 1% SDS). To reach a final concentration of 1 mg mL^{-1}, derivatization solution (cyanine dyes and derivatization buffer) was added to the protein extracts (62.5 µg of protein) and incubated for 2 hours on a gyratory shaker, in the absence of light at 4°C. Following incubation, 34.5 µL of 2 M Tris and 9.5 µL of 0.2 M $NaCNBH_4$ were added to each tube containing protein extracts with samples being left, in the dark, at room temperature for 15 min to quench the reaction.

2D-DIGE - Electrophoretic runs

Samples were labeled with Cy5-Hz and subsequently precipitated with acetone (100%, 1:4 v/v, cold acetone, -20°C, for 2 hours) before centrifugation at 16,000 x *g* for 15 min. To maintain the solubilized proteins, pellets were resuspended in 125 µL of rehydration buffer with minimal agitation. A buffer mixture of 7 M urea, 2 M thiourea, 2% (w/v) CHAPS (sulfate 3-[(3-cloroaminopropil)-dimethylammonio]-1-propane), 0.5% (v/v) ampholytes at pH ranging from 3 to 10 with 1% 1,4 dithiothreitol (DTT) was used for the electrophoretic separations. The combined samples, labeled with fluorophores, were added to a 7-cm IEF strip (BioRad, Hercules, California) for 12 h passive rehydration. Strips were placed gel-side down onto the

sample and overlaid with mineral oil. After the 12 h, the rehydrated strip was isoelectrically focused to resolve the first dimension (IEF, Step 1: 250 V for 15 min, Step 2: 10,000 V for 3 hours, Step 3: 10,000 V to 50,000 V for 4 h).

The strip was equilibrated, following separation in the first dimension, in two steps. Initially, strips were incubated in a solution containing 6 M urea, 2% (w/v) SDS, 30% glycerol (v/v), 50 mM Tris-HCl (pH 8.8), and 10 mg mL^{-1} (w/v) DTT, and then the strips were incubated using the same solution with 25 mg mL^{-1} (w/v) iodoacetamide (IAA) in place of the DTT. Each step lasted for 15 min and was performed at room temperature, under low agitation. To resolve the second dimension, the strips were applied to 12% polyacrylamide gels. The strips were sealed to the gels with an agarose solution (0.5% w/v) and run for approximately 1.5 h at 125 V. After the 1.5 h, the proteins were fixed using a destain solution containing 10% acetic acid (v/v) and 40% (v/v) ethanol. The labeled proteins were visualized by fluorescence using a Typhoon FLA-9500 imager (GE Healthcare, Uppsala, Sweden) with the 532 nm laser voltage set at 530 and a 570 BP 20 filter for Cy3, and the 635 nm laser voltage set at 700 and a 650 LPR filter for Cy5.

Images were aligned, normalized, and analyzed using Progenesis SameSpots version 4.5. Intergel matching of the Cy3-Hz pool was performed across all gels, to discern statistical significance between the groups, for comparative cross gel statistical analysis of all spots based on normalized spot volumes, enabling the detection of differentially oxidized spots between groups ($P < 0.05$). The gels were stained with Coomassie Blue G-250, and spots of interest were manually excised for protein identification.

Protein identification and pathway enrichment analysis

Treating in 15 min cycles, excised gel fragments were chemical washed and incubated with acetonitrile (ACN) and 50 mM ammonium bicarbonate (ABC) solution (40:60% v/v) at room temperature. The process was repeated until all of the stains were completely removed, and the gel fragments were opaque. Afterward, the gel slice was shrunk in 100% (ACN). Trypsin working solution (10 µg mL^1 in 50 mM ABC buffer), was then added to the gel fragment and incubated overnight at 37°C, in the dark, to digest the proteins. The following day, the supernatant was transferred to new 1.5 mL Eppendorf tubes (Hamburg, Germany), and subjected to sonication after the addition of 60% ACN and 5% trifluoroacetic acid (TFA), using a volume sufficient to cover the gel fragment. This was done so the tryptic peptides would be further extracted from the gel. The supernatant was combined with the previous supernatant and the process was repeated two additional times. The pooled supernatants were placed in a SpeedVac (Labconco, Kansas City, MO) to be dried, and the tryptic peptides were resuspended in 25 µL of 0.1% formic acid (FA).

Peptide analysis was performed on a LTQ XL™ Linear Ion Trap mass spectrometer (ThermoFisher Scientific, Waltham, MA) coupled to a dual pump Dionex 3000 RSLCnano LC (ThermoFisher Scientific, Waltham, MA) with mobile phases composed of Solvent A (0.1% formic acid) and Solvent B (0.1% formic acid in ACN). From the resuspended peptides, 5 µL were loaded onto a C18 PepMap100 µ-precolumn (300 µm i.d. x 5 mm; ThermoFisher Scientific, Waltham, MA) at a flow rate of 40 µL/min with 3% Solvent B for 4 minutes. Elution of the peptides was at 400 nL/min from a 15 cm PicoFrit Reprosil-PUR analytical column (New Objective, Woburn, MA) with the following gradient: held in 3% B for 3 min, increased to 15% B over 7 min, increased to 28% B over 30 min, then increased to 99% B in 6 minutes and held

for 1 min, followed by returning to 3% B over 1 min, and held for 22 min at 3% B to allow for column re-equilibration.

In a data-dependent mode, Spectra (m/z 400 to 2000) were acquired with the top 3 ions selected for Collision-induced dissociation (CID) fragmentation at 35% normalized collision energy and a dynamic exclusion of 30 s. Nano spray voltage was set at 1.0 kV with a heated capillary temperature of 250°C. Conversion of the raw data files (.raw) were done by MSConvert (Palo Alto, CA) to .mgf format. This process allowed for protein identification with an 'in house' Mascot database (SwissProt 51.6; Uniprot, Geneva, Switzerland). Mascot parameters were set as follows: 1 missed cleavage, variable methionine oxidation, and both peptide and fragment ion mass tolerance at 0.8 Da.

Troponin-T degradation

Powdered steak samples were evaluated for the degree of Troponin-T degradation according to Chao et al. (2018) with modifications described by Ribeiro et al. (2019).

Myofibrillar protein isolation

Myofibrillar proteins were isolated according to Pietrzak et al. (1997) with modifications. Three g of powdered meat were weighed into a 50 mL conical tube and suspended in 15 mL rigor buffer (0.1 M KCl, 2mM $MgCl_2$, 1 mM EDTA, and 10 mM K_2HPO_4; pH 7.4). Samples were homogenized using a polytron (Kinimatica CH-6010, Switzerland) at medium speed (setting 6) for 10-sec bursts until fully mixed. The homogenized sample was filtered through double-layer cheesecloth into a 50 mL conical tube. Filtered sample was pipetted (1.4 mL) into an Eppendorf tube (2 mL safe-lock tubes; 02236352, Eppendorf AG, Hamburg, Germany) and centrifuged at 4,000 x g for 5 min. The supernatant was decanted, and the pellet was resuspended

in 1 mL rigor buffer. Then the pellet was vortexed until thoroughly mixed into the solution and centrifuged for 5 min at 4,000 x g. This process was repeated three times ending with decanting the supernatant thoroughly. One mL of suspension buffer (0.1 M Tris-base, 1.25 mM EDTA, 5% SDS; pH 8) was added to the pellet and vortexed until mixed into the solution. The sample was centrifuged for 5 min at 4,000 x g, and the supernatant was pipetted into a new 2 mL Eppendorf tube for determination of protein concentration. Samples were kept at -80°F for future protein concentration determination.

Protein Concentration

A concentration series (20-2000 µg/mL) of bovine serum albumin (BSA) standards was prepared using suspension buffer as the diluent. Twenty-five µL of BSA standards and diluted myofibrillar protein samples into a designated well on a 96 wells microplate (Microtest III sterile 96-well flat-bottomed microplate; Becton Dickinson & Company, Franklin Lakes, NJ). Two hundred µL of BCA working reagents (50:1 of Reagent A: Reagent B; Pierce Biotechnology, Rockford, IL) were added to each designated well on the microplate and incubated at 37°C for 40 min and then cooled to room temperature. After cooling to room temperature (23°C), absorbance was read at 562 nm, and protein concentrations were expressed as µg/mL. If needed, myofibrillar protein samples were diluted to 2 µg/µL with ddH_2O. Sample dilution was completed with 2 µg/µL 2x Laemmli sample buffer (65.8 mM Tris-HCl, 2.1% SDS, 26.3% glycerol, 0.01% bromophenol blue) with 5% β-mercaptoethanol to finish with a 50:1 protein ratio. All samples were vortexed then heated for 5 min at 95°C.

Gel electrophoresis

In duplicate, 10 µL of Bio-Rad Kaleidoscope Pre-stained standard and samples were loaded separately to a well in the 12 well, 4-20% Mini-PROTEAN TGX™ precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) using a Bio-Rad Mini-PROTEIN 2 Cells system (Bio-Rad Laboratories, Hercules, CA) immersed in 1x Tris/Glycine/SDS #161-0732 running buffer (25 mM Tris-base, 192 mM, and 0.1% SDS; pH 8.3). The system was run at the constant 200 voltage (V) for 60 min.

Western Blotting

Standard and samples in the gel were blotted on to polyvinylidene difluoride (PVDF) membranes (0.45 µm; Immobilon-FL transfer membrane; Millipore) using a Bio-Rad Mini-Transfer-Blot Electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA) for 60 min at a constant amperage of 180 mA with transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol, 0.1% SDS at pH 9.2). Membranes were placed in a container with 15 mL of Odyssey Blocking Buffer (LI-COR, Lincoln, NE) for 2 h on a rocking platform and incubated in primary anti-troponin-T (JLT-12; Sigma-Aldrich, St. Louis, MO) antibody at a 1:10,000 dilution in Odyssey blocking buffer containing 0.2% Tween-20 for 1 h. Then the membranes were incubated overnight with the diluted primary antibody at 4°C while rocking. Membranes were washed four times on a platform with 15 mL of Tris Buffered Saline containing 0.2% Tween-20 for 10 minutes each, and incubated in IRDye 680LT Conjugated Goat Anti-Mouse IgG1 (LI-COR, Lincoln, NE) secondary antibody diluted 1:10,000 in Odyssey blocking buffer with 0.2% Tween-20 for 1 h on a rocking platform in darkness. Membranes were washed four times for 10 minutes each with Tris Buffered Saline containing 0.2% Tween-20 while rocking. Then the

membranes were washed once with 15 mL of Tris Buffered Saline for 30 minutes to remove Tween20 residue. Membranes were then analyzed using Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) at 700 nm. All intact troponin-T and degraded troponin-T were quantified via band intensities (k. pixels) using Odyssey application software Image Studio Lite Version 5.2. (LI-COR, Lincoln, NE) Bans 1 and 2 (38 and 35 kD, respectively) relate to intact troponin-T while bands 3 and 4 (30 and 28 kD, respectively) relate to degraded troponin-T. Percent troponin-T degraded was measured by band intensities of degraded bands divided by total band intensities of the designated lane.

Isoprostanes

Samples were measured following the guidelines and solutions provided in the OxiSelect 8-iso-Prostaglandin F2α ELISA Kit (Cell Biolabs, San Diego, CA). The kit is a competitive enzyme-linked immunoassay meant to determine levels of 8-iso-PGF2α in biological samples.

Kit Components Provided:

1. Goat Anti-Rabbit Antibody Coated Plate (Part No. 250001): One 96-well strip plate.

2. Anti-8-iso-PGF2α Antibody (Part No. 233701): One 20 µL tube of anti-8-iso-PGF2α rabbit IgG, contains 0.05% sodium azide.

3. Sample Diluent (Part No. 233702): One 50 mL bottle.

4. Neutralization Solution (Part No. 233705): One 20 mL bottle.

5. 10X Wash Buffer (Part No. 310806): One 100 mL bottle, contains 0.2% thimerosal.

6. Substrate Solution (Part No. 310807): One 12 mL amber bottle.

7. Stop Solution (Part. No. 310808): One 12 mL bottle, contains 0.5 N sulfuric acid.

8. 8-iso-PGF2α Standard (Part No. 233703): One 25 µL tube of 200 µg/mL 8-iso-PGF2α in dimethyl sulfoxide (DMSO).

9. 8-iso-PGF2α-HRP Conjugate (Part No. 233704): One 70 µL tube of 8-iso-PGF2α-HRP conjugate.

Sample Preparation

After removing powdered meat from -80 °C storage, 30 mg samples were weighed into 5 mL Eppendorf tubes. Two mL of 2N NaOH was added and the sample was homogenized using a Micropolytron (Pro Scientific Bio Gen Series PRO200) for 20 secs, cleaning between samples, then vortexed for 20 seconds. Samples were placed in a 45 °C water bath for 2 hours to ensure hydrolysis. Subsequently, the samples were for 20 minutes to reach room temperature, then neutralized with 2 mL of 2N HCL and vortexed for 20 seconds. Two mL were allocated into two 2 mL tubes per sample and centrifuged (10,000 x g for 15 min at 4 °C). After centrifugation, 100 µL of the sample were added to 1.5 mL tube and was mixed with 100 µL of Neutralization Solution to ensure the pH was adjusted to a range of 6-8.

Assay Protocol

Dilution of Anti-8-iso- PGF2α Antibody with sample diluent in a 1:1000 ratio. Once diluted and vortexed, add 100 µL of the diluted Anti-8-iso-PGF2α to the Goat Anti-Rabbit Antibody Coated Plate and incubate at 25 °C on an oscillation shaker for 1 hour. During incubation, prepare 100 µL of 1x Wash Buffer by diluting 10x Wash Buffer Concentrate with deionized water. Remove substrate solution from a cooler, and place in room temperature conditions. Fresh standards were prepared and adjusted to fit the appropriate concentration range of the powdered meat samples (see appendix VIII). After incubation, remove the antibody

solution from the wells. Wash the wells 5 times with 300 µL 1x Wash Buffer per well. Ensure excess solution is removed at the last wash by taping the upside-down microwell plate on an absorbent pad. In preparation, dilute 8-iso-PGF2α-HRP conjugate solution with sample diluent in a 1:80 ratio. Combine 55 µL of the 8-iso-PGF2α standard or hydrolyzed sample and 55 µL 8-iso-PGF2α-HRP conjugate in a 0.6 mL microtube and vortex until thoroughly mixed. Transfer 100 µL of the combined solution to each well. Sample Diluent is used as a control. Incubate the coated plate at 25 °C on an oscillation shaker for 1 hour. After incubation, remove solutions from the wells. Wash 5 times with 300 µL of 1x Wash Buffer per well. Ensure excess solution is removed at the last wash by taping the upside-down microwell plate on an absorbent pad. Add 100 µL of Substrate Solution to each well and incubate at room temperature for 30 minutes on an oscillation shaker. Afterward, add 100 µL of Stop Solution to each well, to stop the enzyme reaction, and read absorbance immediately on a microplate reader at 450 nm wavelength.

Statistical analysis

Color data were analyzed as a 2x2x2 factorial with a split plot design with a repeated measure. The processing method of corn, presence or absence of DDGS, and quality grade served as the main plot factors and the aging period was the split-plot factor. The day of retail display served as the repeated measure. Fatty acid profile, free calcium concentration, isoprostanes, pH, proximate composition, and sarcomere length were analyzed as a 2x2x2 factorial. The TBARS, tenderness determination, and Troponin-T degradation data were analyzed as a 2x2x2 split-split plot design. The first split plot was aging period, and the second split plot was the day of retail display with data sliced with the day of aging.. Data were analyzed using PROC GLIMMIX program of SAS with LSMEANS statement. SEM was calculated using

the method described in Levenson et al. (2000). Statistical significance was determined at $P < 0.05$.

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Impact of Diet and Quality Grade on Beef Tenderness, Oxidative Stress, and Shelf Life

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Abstract

The unresolved causal relationship between marbling and tenderness emphasizes the need to better understand the potential underlining mechanism that may be leveraged in producing consistently tender meat. This study evaluated the effects of feeding different types of processed corn, with or without distiller grains on oxidative stress and how that relates to tenderness and shelf life of beef steaks of differing quality grades. Steers (n= 240) were finished on dry rolled corn (DRC), DRC + 30% dried distillers grains (DDGS), steam flaked corn (SFC), or SFC + 30% DDGS. Cattle were fed 10 per pen. Only pens with both upper 2/3 Choice and Select-grade carcasses were sampled, with a goal of two (and a minimum of one) of each grade per pen. Three pens per treatment met the selection criterion and 36 carcasses were chosen (21 upper 2/3 Choice and 15 Select). Both strip loins were collected from each carcass, halved, and aged for 2, 9, 16, or 23 d. After aging, steaks were placed under the retail display for 7 d. Subjective discoloration and instrumental color (L*, a*, and b*) were determined daily, and Warner-Bratzler shear force, slice shear force, and lipid oxidation (thiobarbituric acid reactive substances; TBARS), was measured after 0 and 7 d of retail display. Fatty acid composition, proximate composition, sarcomere length, sarcoplasmic calcium, pH, proteomic analysis, sarcoplasmic reticulum lipidomics, isoprostanes, and troponin-T (Tn-T) degradation of the lean was obtained. No differences for sarcomere length, pH, calcium, or isoprostanes were found ($P > 0.05$). The difference in proximate composition was due to increased fat content for upper 2/3 Choice compares to Select ($P < 0.05$). As aging continued, increases in tenderness, degradation of Tn-T, percent discoloration, and lipid oxidation were found, along with a decrease of redness values ($P < 0.05$). Increases in objective tenderness, discoloration, and decreases in color stability of retail display occurred from 5 to 7 d ($P < 0.05$). Steaks from cattle fed DRC (compared to SFC)

and without DDGS (compared to with DDGS) were statistically lower for discoloration, and C18:2 (linoleic acid) and higher in redness and TBARS values ($P < 0.05$). These data suggest that steaks from cattle fed SFC or with DDGS oxidize, discolor, and lose redness more quickly under retail display than steaks from cattle fed DRC or without DDGS, likely due to changes in fatty acid content, and steaks from cattle fed DRC are more tender than steaks from cattle fed SFC and Upper 2/3 Choice-grade steaks are more tender than Select-grade steaks.

Keywords: Beef tenderness, Oxidative stress, Beef color, Distillers grains, Cattle diet

Introduction

Tenderness is generally recognized as a major factor that contributes to the eating experience of the consumer (Morgan et al., 1991; Huffman et al., 1996; Boleman et al., 1997; Neely et al., 1998; Calkins and Hodgen, 2007; Grobbel et al., 2008; Verbeke et al., 2010). Unsurprisingly, consumers are willing to pay a premium for guaranteed tender beef (Boleman et al., 1997; Feuz et al., 2004). Marbling is already a commonly recognized factor influencing meat quality, as consumers are willing to pay a premium for a higher marbled product (Smith and Johnson, 2016; USDA, 2020). Several books and research studies have been published recognizing the relationship between marbling and tenderness (Morrison, 1937; Jeremiah et al., 1970; Smith et al., 1987; D'Alessandro et al., 2012a; Emerson et al., 2013). Despite the potential explanations for this relationship, none seem to fully explain this consistent occurrence (Smith and Carpenter, 1976). This suggests that not only are multiple factors likely involved, but a major cause has not been elucidated.

Feeding high-fat diets, like distillers grains, in the finishing stage of growth promotes increased rate of marbling development (Blanchard et al., 1999; Houben et al., 2000; Pethick et al., 2004; Smith and Johnson, 2016). There is also evidence to support distillers grains impact on tenderness (Gordon et al., 2002; Depenbusch et al., 2009; Aldai et al., 2010; Senaratne, 2012; Chao et al., 2018). Distillers grains fed to cattle increased the polyunsaturated fatty acids (PUFA) and fat deposition in beef (Pethick et al., 2004; Depenbusch et al., 2009; Mello et al., 2012; Ribeiro et al., 2019). The PUFA can be deposited in the phospholipid membrane of cellular structures like the sarco-endoplasmic reticulum ATPase (SR) and mitochondria (Betteridge, 2000; Blokhina et al., 2003; Wood et al., 2008). This increased presence of PUFA can be problematic due to their susceptibility to free radical attack, that can target the multiple double

bonds, because the double bond weakens the carbon-hydrogen bond at the adjacent carbon atom (Betteridge, 2000). These free radicals can be grouped as reactive oxygen species (ROS) molecules (Montuschi, 2004). When an imbalance between ROS and antioxidant protection favors the former, oxidative stress occurs (Dalle-Donne et al., 2001; Sies, 2015; Savini et al., 2016). This oxidative stress can lead to greater cellular damage to DNA, protein, and lipids (Halliwell et al., 1993; Morrissey et al., 1998; Halliwell, 2002; Halliwell and Gutteridge, 2015). Oxidative stress, induced by ROS, is a recognized consequence of feeding high-fat diets to humans (Savini et al., 2016). Similarly, degrees of oxidative stress appear to impact tenderness in beef (Smuder et al., 2010; D'Alessandro et al., 2011; Guillemin et al., 2011; D'Alessandro et al., 2012b; Chen et al., 2019; Malheiros et al., 2019). Recognizing high-fat diets' ability to provide increased marbling (Pethick et al., 2004; Smith and Johnson, 2016), perhaps the relationship between marbling and tenderness is related to oxidative stress and subsequent protein damage. There is an inconsistency in tenderness of beef that hinders profitability (Warner et al., 2005). The causal relationship between marbling and tenderness is still unclear. Therefore, the objectives of the study were to evaluate the effects of diet and marbling on tenderness, proteolysis, and shelf life, the impact of aging across dietary treatments, and the relationship between the degree of marbling and tenderness/proteolysis.

Materials and Methods

Cattle and dietary treatments

A total of 240 crossbred steers (288 ± 0.54 kg) were blocked into three body weights and randomly assigned to one of 24 pens, (n=10/pen), then fed (University of Nebraska feedlot at Ithica, NE) for 202 days. Four dietary treatments were divided among the pens (6 pens/treatment). Cattle were fed either dry rolled corn (DRC), steam flaked corn (SFC), dry rolled corn plus 30% dried distillers grains with solubles (DRC+ DDGS), or steam flake corn plus 30% dried distillers grain with solubles (SFC+ DDGS). Inclusion of distillers grains was calculated on a dry matter basis. All diets contained 8% sorghum silage, 5% supplement (1.393% fine ground corn, 1.69% limestone, 0.125% tallow, 1.4% tallow, 0.3% salt, 0.05% beef trace mineral, 0.015% Vitamin A-D-E, 0.0165% Rumensin-90, and 0.011% Tylan-40).

Sample collection

Thirty-six USDA Upper 2/3 Choice and Select strip loins (21 Upper 2/3 Choice and 15 Select) were vacuum packaged and obtained from Greater Omaha Packing, Omaha, NE before subsequently being transported to the Loeffel Meat Laboratory at the University of Nebraska-Lincoln. Prior to fabrication, loins kept at (2 ± 5 °C) in dark storage.

Sample Fabrication

The 2 d aged beef loins were divided in half, making four halves (two loins per animal), to be randomly assigned to one of the four aging treatments (2, 9, 16, 23 days). After the assigned aging period, the half beef loins were fabricated into steaks, beginning at the anterior end, for subsequent analysis. Four, 2.54 cm steaks were fabricated; Steak one was used for Warner-Bratzler shear force (WBSF) and slice shear force (SSF) measurement at 0 d of retail

display (RD). Steak two used for Warner-Bratzler shear force, slice shear force measurement, visual discoloration, and objective color for 7 d of RD. Steak three was trimmed of all subcutaneous fat and used for proximate composition, sarcomere length, fatty acid analysis, free calcium concentration, pH analysis, proteomics, Troponin-T degradation, and isoprostanes. Steak four was an extra sample for possible-future analyses. Then, one 1.9cm thick steak was cut in half and trimmed of all subcutaneous fat to measure lipid oxidation after 0 and 7 d of RD for the aging period. Steaks for WBSF at 0 d RD were set aside to perform slice shear force (SSF) before carrying out WBSF. All half loins aged for longer than 2 days were vacuum packaged (MULTIVAC 500, Multivac, Inc., Kansas City, MO) in Prime Source Vacuum pouches (3 mil STD barrier, Prime Sources, St. Louis, MO) and aged in dark storage (2°C). Steaks for laboratory analysis were frozen for further analysis (-80°C). Steaks for 7 days of RD were placed on foam trays (21.6 x 15.9 x 2.1 cm, Styro-Tech, Denver, CO) and overwrapped with oxygen-permeable film (Prime Source PSM 18 #75003815, Bunzl Processors Division, North Kansas City, MO) to be placed under retail display (RD) for 7 d at 3°C. Forty-five days later, all steaks subsequently used for laboratory analyses were diced into small portions, after sufficiently thawing, before freezing in liquid nitrogen before powdering in a metal blender cup (Model 51BL32, Waring Commercial, Torrington, CT) and kept frozen (-80°C) for the following analyses.

Tenderness determination via Shear Force

Steaks 2.54 cm thick, never frozen, were measured for tenderness via Slice Shear Force (SSF) and Warner-Bratzler Shear Force (WBSF). Before SSF, raw-internal steak temperatures were taken in Celsius (C), in the geometric center of the steaks, using a T-type thermocouple (TMQSS-062U-6, OMEGA Engineering, Inc., Stamford, CT) connected to a portable

thermometer (OMEGA 450-ATT, OMEGA Engineering, Inc., Stamford, CT). Raw steak weights were measured with a scale set in grams and recorded. Afterward, all steaks were cooked to an internal temperature of 70°C on a belt grill (TBG60-V3 MagiGril, MagiKitch'n Inc., Quakertown, PA). After cooking, internal steak temperatures and weights were recorded before slice shear force evaluation was conducted using a Slice Shear force blade attached to a Food Texture Analyzer (250 mm/min blade speed, TMS- Pro, Food Technology Corp., Sterling, VA.). The rest of the steak was bagged (PB-90-C, 0.85 mil., 6 x 3 x 15 in., Get Reddi, Inteplast Group, Livingston, NJ) and stored overnight at 2°C. The next day, six cores (1.27 cm diameter) were cored out with a drill press going parallel to the muscle fibers and sheared using the Food Texture Analyzer with a triangular Warner-Bratzler blade (250 mm/min blade speed). An average of the six cores was calculated for each steak for statistical analysis.

Instrumental color and visual discoloration

Steaks (2.54 cm) were placed on Styrofoam trays (21.6 x 15.9 x 2.1 cm, Styro-Tech, Denver, CO) and overwrapped with oxygen-permeable film (Prime Source PSM 18 #75003815, Bunzl Processors Division, North Kansas City, MO). The steaks were subsequently placed under the retail display and repositioned daily to minimize location effects (3°C under white fluorescence lighting at 1000 to 1800 lux, Lamp Type: F32T8/TL741 700 Series 32 watt, Phillips, Inc. New Jersey). Instrumental color measurements were taken daily during the 7-day simulated retail display for each aging period. Commission International de l'éclairage (CIE) L*, a*, and b* values were obtained using a Minolta CR-400 colorimeter (Minolta, Osaka, Japan). Parameters for the colorimeter were set with a D65 illuminant, 2° observer, with an 8 mm diameter measurement area. The colorimeter was calibrated daily with a white ceramic tile (Calibration Plate, Serial No. 14933058, Konica Minolta, Japan) covered with the same

overwrap film as used for the steaks. Lightness (L*) is measured with a range from 0 (black) to 100 (white), a* is a measurement of redness with a range between red (positive values) and green (negative values), and b* is a measure of yellowness with yellow (positive) to blue (negative). Appendix III contains further details and tips for measuring color. Six measurements were recorded per steak and the mean was calculated for each steak for statistical analysis. Color readings were taken at the same time each day.

The visual discoloration was assessed daily during the retail display period, across aging periods, with 5 trained panelists comprised of graduate students from the University of Nebraska-Lincoln. Panelists were trained using a standardized discoloration guide (Appendix IV). Discoloration % was approximated from 0% to 100% with 0% being no discoloration on the surface and 100% being a fully discolored steak.

Lipid oxidation (TBARS)

Lipid oxidation was determined using thiobarbituric acid reactive substances (TBARS), a measure of the amount of malonaldehyde present in the sample. This was done for all aging period samples at 0 and 7 d RD following the protocol described by Ahn et al. (1998; see Appendix V). Five g of powdered meat were placed into a 50 mL conical tube and immersed with 14 mL of distilled deionized water and 1 mL of butylated hydroxyanisole (BHA) solution (10% BHA; 90% ethanol). Samples were homogenized using a Polytron (Kinmatica AG, PT-10-35 GT) for 15 seconds at medium to high speed. The samples were centrifuged (2,000 x g for 5 min at 10°C) and one mL of supernatant was transferred into a 15 mL tube. Two mL of thiobarbituric acid/ trichloroacetic acid (TBA/TCA) solution (15% TCA and 20 mM TBA in deionized distilled water) were added and the samples were placed in a water bath at 70°C for 30 min. Subsequently, samples were cooled in a 20 °C water bath for 10 min then centrifuged

(2,000 x g for 5 min). After centrifugation, 200 µL of the samples were transferred to a 96 well plate in duplicates along with pre-made standards. The plate was read using a microplate spectrophotometer (Model Epoch, Biotek, Winooski, VT) at an absorbance of 540 nm. The measured results were expressed in mg of malonaldehyde per kg of tissue**.**

Proximate composition

Fat, moisture, and ash (%) were determined from powdered-raw meat samples from all aging periods. Moisture and ash (%) were measured in duplicate using a Thermogravimetric Analyzer (Model 604- 100-400, LECO Corporation, St. Joseph, MI). Total fat (%) was measured in triplicate using the Soxhlet method (~48 h) of ether extraction after taking the difference of the already quantified moisture percentage (AOAC, 1990) (Appendix VI). Protein was calculated by the overall difference.

Sarcomere length

Sarcomere length was determined following the method described by Cross et al. (1981) and Dolazza and Lorenzen (2014) using helium-neon laser diffraction. A pinch of powdered meat sample was placed on a clear-glass microscope slide. A single drop of 0.25 M sucrose solution was added to the sample and topped with a glass coverslip. The distance between the base of the laser and the top of the slide was 100 mm. A piece of paper was placed underneath the stand to mark the three diffraction bands. Five single sarcomeres per sample were identified and sarcomere length (µm) was calculated by the equation provided by Cross et al. (1981):

Sarcomere length (µm)=

$$\frac{0.6328 \times D\sqrt{\left(\left(\frac{T}{D}\right)^2 + 1\right)}}{T}$$

D= distance from specimen to diffraction pattern screen (100 mm)

T= spacing between diffraction bands (mm)

Fatty acid analysis

The extraction of the total lipids was conducted using the chloroform-methanol procedure described by Folch et al. (1957). Following the extraction process, lipids were reduced to fatty acid methylated esters according to Morrison and Smith (1964) and Metcalfe et al. (1966). One gram of powdered sample was homogenized with 5 mL of 2:1 chloroform: methanol and kept at room temperature (23°C) for 1 hr. Afterward, the homogenate was filtered through Whatman #2 paper into a screw cap tube and brought to a final volume of 10 mL with the 2:1 chloroform: methanol. Samples were vortexed for 5 s with 2 mL 0.74% KCl solution and centrifuged for 5 minutes at 1,000 x g, and the top layer was aspirated off. After aspirating, the samples were placed in a heating block at 60°C and continually flushed with nitrogen until dried. Once dry, 1 mL of 0.5 NaOH in methanol was added, vortexed for 5 s, and heated for 10 min at 100°C. Subsequently, one mL of 14% boron trifluoride in methanol was added, vortexed for 5 s, and the samples were heated again at 100°C for 5 min. Following the heating period, 2 mL of saturated salt solution and 2 mL of hexane were added to the samples and vortexed (5 s). Samples were then centrifuged at 1,000 x g for 5 min and the hexane layer (top layer) was removed to be analyzed using gas chromatography (TRACE 1310 Gas Chromatography; ThermoFisher Scientific, Waltham, MA). Separation of the fatty acids was done using a capillary column (Chrompack CP-Sil 88- 0.25 mm x 100 m; Inlet temp: 260°C, Oven: 140°C hold for 5 min, increase at 4°C/min to 240°C and hold for 15 min. FID temp: 280°C. Injected at 30:1 ratio) and identified based on their retention times compared to known commercial standards (NU- Check Prep, Inc., Elysiam, MN; # GLC-68D, GLC-79, GLC-87, GLC-455, and GLC-458, see

Appendix VII). Determination of fatty acids percentage was done with the peak areas in the chromatograph and value were converted to mg/100 g tissue:

Fatty acid mg/100 g tissue = (% of fatty acid peak area * fat content of samples) * 1000

Free calcium concentration

Free calcium was quantified using the procedure described by Parrish et al. (1981), with modifications. Three g of powdered sample were placed in a thick-wall, polyallomer ultracentrifuge tube (13 x 55 mm; Beckman Coluter, Brea, CA) and centrifuged at 196,000 x g (Beckman Optima XPN-90 Ultracentrifuge, Type 50.2 Ti rotor, Beckman Coulter, Brea, CA) at 4°C for 30 min. Seven hundred µL of the supernatant were collected and treated with 0.1 mL of 27.5% trichloroacetic acid (TCA), then vortexed until the TCA was thoroughly mixed. Samples were centrifuged at 6,000 x g (accuSpin Micro 17R, ThermoFisher Scientific, Waltham, MA) at 4°C for 10 min. Four hundred µL of supernatant were transferred to a syringe, and the volume was brought to 4 mL with deionized, distilled water (ddH_2O). The diluted calcium sample was then filtered through a 13 mm diameter Millex-LG 0.20 µL syringe filter (Millipore, Bedford, MA). Calcium concentration of samples was quantified at Ward Laboratories (Kearney, NE) using an inductively- coupled plasma emission spectrometer (iCAP 6500 Radial; Thermo Electron, Cambridge, UK) with appropriate calcium concentration standards.

pH analysis

Powdered steak samples from 2 d aging period with 0 RD were weighed into 5 g duplicates with 45 mL of distilled deionized water in 250 mL plastic beakers. A magnetic stir bar was added to each beaker to ensure constant mixing. The pH was measured using a pH meter (Orion 410A plus: ThermoFisher Scientific; Waltham, MA) that was calibrated using 4.0, 7.0,

and 10.0 pH standards. The analysis was conducted from the mean of the measured duplicate samples. The pH probe was rinsed with distilled, deionized water and dried with a Kimwipe between every measurement.

Troponin-T degradation

Powdered steak samples were evaluated for the degree of Troponin-T degradation according to Chao et al. (2018) with modifications described by Ribeiro et al. (2019).

Myofibrillar protein isolation

Myofibrillar proteins were isolated according to Pietrzak et al. (1997) with modifications. Three g of powdered meat were weighed into a 50 mL conical tube and suspended in 15 mL rigor buffer (0.1 M KCl, 2mM $MgCl_2$, 1 mM EDTA, and 10 mM K_2HPO_4; pH 7.4, in 1 L distilled-deionized H_2O (ddH_2O). Samples were homogenized using a polytron (Kinimatica CH-6010, Switzerland) at medium speed (setting 6) for 10-sec bursts until fully mixed. The homogenized sample was filtered through double-layer cheesecloth into a 50 mL conical tube. Filtered sample was pipetted (1.4 mL) into an Eppendorf tube (2 mL safe-lock tubes; 02236352, Eppendorf AG, Hamburg, Germany) and centrifuged at 4,000 x g for 5 min at 4°C. The supernatant was decanted, and the pellet was resuspended in 1 mL rigor buffer. Then the pellet was vortexed until thoroughly mixed into the solution and centrifuged for 5 min at 4,000 x g at 4°C. This process was repeated three times ending with decanting the supernatant thoroughly. One mL of suspension buffer (0.1 M Tris-base, 1.25 mM EDTA, 5% SDS; pH 8, in 1 L ddH_2O) was added to the pellet and vortexed until mixed into the solution. The sample was centrifuged for 5 min at 4,000 x g at 4°C, and the supernatant was pipetted into a new 2 mL Eppendorf tube

for determination of protein concentration. Samples were kept at -80°F for future protein concentration determination.

Protein Concentration

A concentration series (20-2000 µg/mL) of bovine serum albumin (BSA) standards was prepared using suspension buffer as the diluent. Twenty-five µL of BSA standards and diluted myofibrillar protein samples were placed into a designated well on a 96 wells microplate (Microtest III sterile 96-well flat-bottomed microplate; Becton Dickinson & Company, Franklin Lakes, NJ). Two hundred µL of BCA working reagents (50:1 of Reagent A: Reagent B; Pierce Biotechnology, Rockford, IL) were added to each designated well on the microplate and incubated at 37°C for 40 min and then cooled to room temperature. After cooling to room temperature (23°C), absorbance was read at 562 nm, and protein concentrations were expressed as µg/mL. Myofibrillar protein samples were diluted to 2 µg/µL with ddH_2O. Sample dilution was then completed with 2 µg/µL 2x Laemmli sample buffer (65.8 mM Tris-HCl, 2.1% SDS, 26.3% glycerol, 0.01% bromophenol blue) with 5% β-mercaptoethanol to finish with a 50:1 protein ratio. All samples were vortexed and then heated for 5 min at 95°C.

Gel electrophoresis

In duplicate, 10 µL of Bio-Rad Kaleidoscope Pre-stained standard and samples were loaded separately to a well in the 12 well, 4-20% Mini-PROTEAN TGX™ precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) using a Bio-Rad Mini-PROTEIN 2 Cells system (Bio-Rad Laboratories, Hercules, CA) immersed in 1x Tris/Glycine/SDS #161-0732 running buffer (25 mM Tris-base, 192 mM, and 0.1% SDS; pH 8.3). The system was run at the constant 200 voltage (V) for 60 min.

Western Blotting

Standard and samples in the gel were blotted on to polyvinylidene difluoride (PVDF) membranes (0.45 µm; Immobilon-FL transfer membrane; Millipore) using a Bio-Rad Mini-Transfer-Blot Electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA) for 60 min at a constant amperage of 180 mA with transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol, 0.1% SDS at pH 9.2). Membranes were placed in a container with 15 mL of Odyssey Blocking Buffer (LI-COR, Lincoln, NE) for 2 h on a rocking platform and incubated in primary anti-troponin-T (JLT-12; Sigma-Aldrich, St. Louis, MO) antibody at a 1:10,000 dilution in Odyssey blocking buffer containing 0.2% Tween-20 for 1 h. Then the membranes were incubated overnight with the diluted primary antibody at 4°C while rocking. Membranes were washed four times on a platform with 15 mL of Tris Buffered Saline containing 0.2% Tween-20 for 10 minutes each, and incubated in IRDye 680LT Conjugated Goat Anti-Mouse IgG1 (LI-COR, Lincoln, NE) secondary antibody diluted 1:10,000 in Odyssey blocking buffer with 0.2% Tween-20 for 1 h on a rocking platform in darkness. Membranes were washed four times for 10 minutes each with Tris Buffered Saline containing 0.2% Tween-20 while rocking. Then the membranes were washed once with 15 mL of Tris Buffered Saline for 30 minutes to remove Tween20 residue. Membranes were then analyzed using Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) at 700 nm. All intact troponin-T and degraded troponin-T were quantified via band intensities (k. pixels) using Odyssey application software Image Studio Lite Version 5.2. (LI-COR, Lincoln, NE) Bans 1 and 2 (38 and 35 kD, respectively) relate to intact troponin-T while bands 3 and 4 (30 and 28 kD, respectively) relate to degraded troponin-T. Percent troponin-T degraded was measured by band intensities of degraded bands divided by total band intensities of the designated lane.

Isoprostanes

Samples were measured following the guidelines and solutions provided in the OxiSelect 8-iso-Prostaglandin F2α ELISA Kit (Cell Biolabs, San Diego, CA). The kit is a competitive enzyme-linked immunoassay meant to determine levels of 8-iso-PGF2α in biological samples.

Kit Components Provided:

1. Goat Anti-Rabbit Antibody Coated Plate (Part No. 250001): One 96-well strip plate.

2. Anti-8-iso-PGF2α Antibody (Part No. 233701): One 20 µL tube of anti-8-iso-PGF2α rabbit IgG, contains 0.05% sodium azide.

3. Sample Diluent (Part No. 233702): One 50 mL bottle.

4. Neutralization Solution (Part No. 233705): One 20 mL bottle.

5. 10X Wash Buffer (Part No. 310806): One 100 mL bottle, contains 0.2% thimerosal.

6. Substrate Solution (Part No. 310807): One 12 mL amber bottle.

7. Stop Solution (Part. No. 310808): One 12 mL bottle, contains 0.5 N sulfuric acid.

8. 8-iso-PGF2α Standard (Part No. 233703): One 25 µL tube of 200 µg/mL 8-iso-PGF2α in dimethyl sulfoxide (DMSO).

9. 8-iso-PGF2α-HRP Conjugate (Part No. 233704): One 70 µL tube of 8-iso-PGF2α-HRP conjugate.

Sample Preparation

After removing powdered meat from -80 °C storage, 30 mg samples were weighed into 5 mL Eppendorf tubes. Two mL of 2N NaOH was added and the sample was homogenized using a

Micropolytron (Pro Scientific Bio Gen Series PRO200) for 20 secs, cleaning between samples, then vortexed for 20 seconds. Samples were placed in a 45 °C water bath for 2 hours to ensure hydrolysis. Subsequently, the samples were for 20 minutes to reach room temperature, then neutralized with 2 mL of 2N HCL and vortexed for 20 seconds. Two mL were allocated into two 2 mL tubes per sample and centrifuged (10,000 x g for 15 min at 4 °C). After centrifugation, 100 µL of the sample were added to 1.5 mL tube and was mixed with 100 µL of Neutralization Solution to ensure the pH was adjusted to a range of 6-8.

Assay Protocol

Dilution of Anti-8-iso- PGF2α Antibody with sample diluent in a 1:1000 ratio. Once diluted and vortexed, add 100 µL of the diluted Anti-8-iso-PGF2α to the Goat Anti-Rabbit Antibody Coated Plate and incubate at 25 °C on an oscillation shaker for 1 hour. During incubation, prepare 100 µL of 1x Wash Buffer by diluting 10x Wash Buffer Concentrate with deionized water. Remove substrate solution from a cooler, and place in room temperature conditions. Fresh standards were prepared and adjusted to fit the appropriate concentration range of the powdered meat samples (see appendix VIII). After incubation, remove the antibody solution from the wells. Wash the wells 5 times with 300 µL 1x Wash Buffer per well. Ensure excess solution is removed at the last wash by taping the upside-down microwell plate on an absorbent pad. In preparation, dilute 8-iso-PGF2α-HRP conjugate solution with sample diluent in a 1:80 ratio. Combine 55 µL of the 8-iso-PGF2α standard or hydrolyzed sample and 55 µL 8-iso-PGF2α-HRP conjugate in a 0.6 mL microtube and vortex until thoroughly mixed. Transfer 100 µL of the combined solution to each well. Sample Diluent is used as a control. Incubate the coated plate at 25 °C on an oscillation shaker for 1 hour. After incubation, remove solutions from the wells. Wash 5 times with 300 µL of 1x Wash Buffer per well. Ensure excess solution is

removed at the last wash by taping the upside-down microwell plate on an absorbent pad. Add 100 µL of Substrate Solution to each well and incubate at room temperature for 30 minutes on an oscillation shaker. Afterward, add 100 µL of Stop Solution to each well, to stop the enzyme reaction, and read absorbance immediately on a microplate reader at 450 nm wavelength.

Statistical analysis

Color data were analyzed as a 2x2x2 factorial with a split plot design with a repeated measure. The processing method of corn, presence or absence of DDGS, and quality grade served as the main plot factors and the aging period was the split-plot factor. The day of retail display served as the repeated measure. Fatty acid profile, free calcium concentration, isoprostanes, pH, proximate composition, and sarcomere length were analyzed as a 2x2x2 factorial. The TBARS, tenderness determination, and Troponin-T degradation data were analyzed as a 2x2x2 split-split plot design. The first split plot was aging period, and the second split plot was the day of retail display with data sliced with the day of aging. Data were analyzed using PROC GLIMMIX program of SAS with LSMEANS statement. SEM was calculated using the method described in Levenson et al. (2000). Statistical significance was determined at $P < 0.05$.

Results and Discussion

Tenderness determination via Shear Force

The only interactions found were between the aging period and retail display, though the results were sliced by day of aging. The only main effects that were found to be significantly different were between corn processing methods and then quality grade.

Steaks from cattle fed DRC had significantly lower WBSF than steaks from cattle fed SFC *(P*< 0.05*)* indicating that cattle fed DRC were more tender than cattle fed SFC (Table 1). There was no statistical difference in shear force with the presence or absence of distillers grains (*P*> 0.05). This is consistent with multiple reports that found no impact of distillers grains supplementation to a corn diet (Roeber et al., 2005; Gill et al., 2008; Leupp et al., 2009; Koger et al., 2010; Segers et al., 2011; Mello et al., 2012). In contrast, other reports found tenderness improvements when supplemented with distillers grains in exchange for corn (Gordon et al., 2002; Depenbusch et al., 2009; Aldai et al., 2010; Senaratne, 2012; Chao et al., 2018).

There was a detectable difference in tenderness between Upper 2/3 Choice and Select strip loins *(P*< 0.05*)*, with Upper 2/3 Choice having significantly lower WBSF (Table 1). This is consistent with some reports that have determined the impact of quality grade has on tenderness (McBee and Wiles, 1967; Smith et al., 1987; Wheeler et al., 1994; Emerson et al., 2013), though the mechanisms by which marbling impacts tenderness, or the presence of a cause-effect relationship, are unresolved (Simone et al., 1958; Smith and Carpenter, 1976), these data support the claim there is a relationship between marbling and objective tenderness.

As the strip loins were aged, there were significant decreases in the WBSF from 2 to 9 to 16, regardless of treatment *(P*< 0.*05).* There was a further decrease in the WBSF after 23 d of

aging, compared to 16 d aged, but it was not statistically significant ($P > 0.05$; Fig. 1). This was consistent with multiple reports that recognize the improvement of tenderness with postmortem aging (Epley, 1992; Dransfield, 1994; Mello et al., 2012; Domenech-Pérez et al., 2017; Hart et al., 2018; Ribeiro et al., 2019).

Aging followed by 7 d of retail display (RD) reduced the WBSF values compared to the counterparts that were not under RD. However, only 2 d aging followed by 7 d RD and 9 d aging followed by 7 d RD were significantly different ($P < 0.05$) from each other (Fig. 2). The WBSF values decreased from 9 d aging and 7 d RD to 16 d aging and 7 d RD, 16 d and 7 d RD was slightly, but not significantly, more tender than 23 d aging and 7 d RD.

Instrumental color and visual percentage discoloration

Muscle lightness (L*) exhibited aging effects ($P < 0.01$). As aging increased, there was an increase in L* that was significant from 2 d aging and 9 d aging with all other days of aging similar to d 9 (Fig. 3). The decrease of L* with aging was inconsistent with other reports and may be indicative of protein alteration, structurally (MacDougall, 1982). The lack of significance ($P > 0.05$) found between the presence or absence of DDGS versus only SFC or DRC was in contradiction to multiple reports (Gill et al., 2008; Depenbusch et al., 2009; Leupp et al., 2009).

Discoloration and redness (a*) have profound impacts on consumer decisions to purchase beef at retail. Both color traits had a 3-way interaction between corn processing type, length of postmortem aging, and day of retail display, and the presence or absence of DDGS, length of postmortem aging, and day of retail display ($P < 0.05$). Furthermore, there was a quality grade by the length of postmortem aging by day of retail display interaction in the discoloration measurement. The simulated retail display conditions in the laboratory were colder than those

typically observed in retail stores. This provided the opportunity to more carefully study changes in color characteristics during retail display. No differences in any color trait were observed within the first 4 d of retail display. Effects of corn processing method and presence or absence of DDGS were apparent following 5-7 d of retail display. For all treatments, discoloration tended to increase, and redness tended to decrease during days 5–7 of retail display following 2 and 9 d of aging (Tables 2-6). Differences in discoloration and redness between DRC and SFC were significant ($P< 0.05$) following 16 and 23 d of storage, with steaks from cattle fed SFC exhibiting significantly ($P< 0.05$) reduced redness (Table 2) and more discoloration (Table 4) than steaks from cattle fed DRC after 6 or 7 d under simulated retail display conditions. This deterioration in color for SFC is thought to be due to the improved absorption of unsaturated fatty acids during digestion (Zinn et al., 2002), caused by the steaming process, that can negatively impact color stability. This is consistent with the fatty acid results that had increased unsaturated fatty acids (UFA) in SFC, like 18:2, compared to DRC (Table 11). Steaks from cattle fed diets containing DDGS, compared to diets without DDGS, resulted in a significant decrease in redness (Table 3) and increases ($P< 0.05$) in discoloration (Table 5) after 6-7 day of retail display following extended storage (16 and 23 d). The impact of diet on this study was clear for redness and discoloration. This was consistent with a variety of reports that found the addition of DDGS decreased color stability and increased discoloration, likely due to the increase polyunsaturated fatty acids (PUFA) deposited in the cellular membranes (Holman and Elmer, 1947; Morrissey et al., 1998; Roeber et al., 2005; Wood et al., 2008; Depenbusch et al., 2009; Leupp et al., 2009; Segers et al., 2011; Mello et al., 2012; Domenech-Pérez et al., 2017; Chao et al., 2018; Hart et al., 2018; Ribeiro et al., 2018). The continual increase of discoloration and decrease of shelf life and color stability in meat aged or under retail display was an expected

trend when compared to other reports (Epley, 1992; Vitale et al., 2014; English et al., 2016). The increase in discoloration and decrease in color stability and shelf life for our samples was also consistent with other reports (Hood, 1980; Mello et al., 2012; Domenech-Pérez et al., 2017; Ribeiro et al., 2018).

Fresh meat color influences willingness to purchase in that 20% discoloration can lead up to a 50% discounting of the price (Hood and Riordan, 1973). The steaks from cattle fed an addition of DDGS had 37.1% discoloration at 16 d aging and 7 d RD compared to the NO DDGS of 11.9% discoloration. Both DRC and SFC exceeded the 20% mark after 16 d aging and 7 d RD, and all treatments surpassed the 20% discoloration point by 23 d aging and 6 d RD. Reinforcing the premise that postmortem aging and retail display were considered deleterious for color stability and there were negative meat color attributes in steaks from cattle fed DDGS. For a*, there was a tendency towards significance ($P< 0.10$) between grade by aging by the retail display with Upper 2/3 Choice losing redness more quickly than Select steaks. This would be consistent with the premise that higher PUFA content, reported in the Choice quality grade samples, will increase the susceptibility for lipid oxidation and thus decrease of redness as the meat discolors (Greene, 1969; Morrissey et al., 1998; Nute et al., 2007; Grimsrud et al., 2008; Zakrys et al., 2008; Faustman et al., 2010). In terms of discoloration, the difference between Upper 2/3 Choice and Select was significant ($P< 0.05$) particularly due to the 16 d aging and 7 d RD that found a higher level of discoloration noted in Select steaks than Upper 2/3 Choice (Table 6). This is in contradiction to what was expected from the literature. Since lipid oxidation is related to myoglobin oxidation, it would be reasonable to expect lower fat content would improve color stability (Greene, 1969; Morrissey et al., 1998; Nute et al., 2007; Grimsrud et al., 2008).

A 5-way interaction of the processing method of the corn by presence or absence of DDGS by quality grade by aging by retail display was identified for b* values (P= 0.0505; Table 7). After 7 d RD, Select steaks were higher in yellowness than Upper 2/3 Choice at 2 d aging, but Upper 2/3 Choice surpassed Select at 9 d, 16 d, and 23 d of aging with 7 day RD (P< 0.05). Yellowness continued to decrease as aging continued, especially notable between 2 d and 23 d aging (P< 0.05). Treatment DRC had higher b* values than SFC, and No DDGS was had a higher b* than DDGS. Also, DRC with no DDGS had significantly higher b* than SFC with DDGS (P< 0.05). Upper 2/3 Choice steaks at 23 d aging, 7 d RD, with an SFC DDGS diet, were significantly higher in b* than Select steaks at 23 d aging, 7 d RD, with an SFC DDGS diet (P< 0.05). Also, Upper 2/3 Choice steaks at 23 d aging, 7 d RD, with an SFC and no DDGS diet were significantly higher in b* than Select steaks at 23 d aging, 7 d RD, with an SFC DDGS diet (P< 0.05).

Lipid oxidation (TBARS)

Detrimental effects of SFC (versus DRC) and DDGS (versus diets without DDGS) were also observed with oxidation (TBARS) following 7 d of retail display (P< 0.05), regardless of days of aging (Tables 8 and 9). This interaction is consistent with reports that increased amount of fat content and particularly PUFA increases the susceptibility of meat lipids to oxidize and produce secondary products that can be detected via the TBARS protocol (Holman and Elmer, 1947; Ahn and Kim, 1998; Gill et al., 2008; Depenbusch et al., 2009; Faustman et al., 2010; Mello et al., 2012; Domenech-Pérez et al., 2017; Chao et al., 2018; Ribeiro et al., 2019). There was no significant impact on lipid oxidation as it related to quality grade.

Regardless of diet, oxidation values continue to increase with aging and 7 d of RD (Fig. 4). A significant difference for lipid oxidation was only found between 9 d and 16 d of aging

after 7 RD ($P< 0.05$). Steaks did not differ in the oxidation level at the beginning of the retail period. Not surprisingly, exposure to oxygen, as occurs during retail display, accelerates oxidation. Lipid oxidation is associated with rancidity and discoloration, so feeding SFC or DDGS may have detrimental effects on the retail value of beef steaks compared to DRC or No DDGS. This suggests that the DRC diet (without DDGS) results in beef that is better able to maintain visual desired color than diets containing SFC or DDGS and thus would take longer to be discounted. This is consistent with several reports that continued periods of aging and retail display result in a continued increase in lipid oxidation, adversely impacting the meat (Mello et al., 2012; Domenech-Pérez et al., 2017; Hart et al., 2018; Ribeiro et al., 2018). The degree of the lipid oxidation can impact not just protein oxidation/discoloration, but also aroma, flavor, and shelf life (Gray et al., 1996; Morrissey et al., 1998; Faustman et al., 2010; Ladeira et al., 2014). Previous research has indicated that surpassing 2.28 mg/kg TBARS would negatively impact beef acceptability (Campo et al., 2006). After 7d RD, DRC, SFC, and presence of DDGS exceeded this mark with the addition of SFC (versus DRC) and presence of DDGS (versus absences of DDGS) being the highest ($P< 0.05$). The absence of DDGS in the diet fed to the cattle was the only dietary source that did not exceed that 2.28 mg/kg TBARS threshold compared to the presence or absence of DDGS at 3.54 and 1.90 mg/kg, respectively. While both corn processing methods of corn also exceeded the TBARS threshold, with DRC at 2.31 and SFC at 3.13, it is important to note these were averages of the corn processing method diets with or without DDGS. Overall, the addition of DDGS led to the greatest increase in lipid oxidation followed by SFC, which helps explain the color stability and overall shelf life results.

Proximate composition

In this study, dietary treatment had no effect ($P > 0.05$) on proximate composition (Table 10). The lack of difference due to dietary treatments are in support of multiple studies that have determined supplementing distillers grains does not alter the proximate composition of the beef (Mello et al., 2012; Domenech-Pérez et al., 2017; Hart et al., 2018; Ribeiro et al., 2019). However, this is a contraction to other reports (Pethick et al., 2004; Segers et al., 2011; Buttrey et al., 2013). However, in terms of quality grade, Upper 2/3 Choice had significantly higher fat content compared to Select steaks ($P<0.05$). Increased fat percentage in the Upper 2/3 Choice samples, compared to the Select steaks, would be expected since the difference of the quality grades is related to the amount of intramuscular fat present (Table 11).

Sarcomere length

There was no statistical difference ($P > 0.05$) in the sarcomere length among the dietary treatments (Table 12). This reported no difference in sarcomere length is consistent with previous reports (Ribeiro et al., 2018; Ribeiro et al., 2019) and suggests that any tenderness differences were not due to differences in muscle shortening.

Fatty acid analysis

The processing method of corn (DRC versus SFC) had an impact on the amount and type of fatty acids present in the meat. Fatty acids such as linoleic acid (C18:2) and some trans-unsaturated fats, in general, were found in significantly higher ($P < 0.05$) amounts (mg/100 g of tissue) in beef steaks from cattle fed SFC than beef steaks from DRC diet (Table 13). This matches the differences in digestibility between SFC and DRC diets. Cattle fed SFC has been found to have increased starch digestibility compared to DRC, presumably due to the steaming

process increasing the moisture content and improving the ability of cattle to uptake nutrients in the corn (Zinn et al., 2002).

Linoleic acid (C18:2), palmitelaidic (C16:1T), and polyunsaturated fatty acids (PUFAs) were significantly higher ($P< 0.05$) in beef steaks from cattle fed DDGS than the diets without DDGS (Table 14). This is consistent with reports that feeding distillers grains to cattle increases the amount of PUFA that were deposited in the muscle and therefore present in the meat (Gill et al., 2008; Depenbusch et al., 2009; Mello et al., 2012).

An interaction between the processing method of corn and the presence or absence of DDGS was seen in a few of the fatty acids. Steaks from cattle fed DRC without DDGS had significantly higher levels ($P< 0.05$) of pentadecanoic acid (C15:0) compared to DRC with DDGS and SFC with DDGS had significantly higher levels ($P< 0.05$) of the heptadecanoic acid (C17:0) compared to DRC with DDGS (Table 15). A decrease of C15:0 in DRC with DDGS is consistent with a report from Depenbusch et al. (2009). A significant difference of C17:0 in SFC with DDGS, compared to DRC with DDGS, seems to be more related to the increased digestibility, caused by the SFC since previous data noted higher amounts of C17:0 in meat from cattle fed distillers grain than fed corn only diet (Mello et al., 2012).

The quality grade was significant for the fatty acid profile in this study (Table 16), with Upper 2/3 Choice-grade steaks having significantly higher levels ($P< 0.05$) of unsaturated fatty acids like 18:2 and total polyunsaturated fatty acids than Select-grade steaks but no differences in color stability or oxidation. Marbling is primarily, but not exclusively, comprised of SFA that were deposited intramuscularly (Legako et al., 2015).

Free calcium concentration

There was no statistical difference ($P > 0.05$) in free calcium content among the treatments at 2 d of aging (Table 17). Free calcium plays a key role in tenderness through activating proteolytic activity. These data indicate that differences in sarcoplasmic calcium cannot explain tenderness differences.

pH analysis

No significant pH differences were observed between treatments (Table 18; $P > 0.05$). This supports the claim that differences in pH did not impact other factors evaluated in this study.

Troponin T degradation

No differences were found for troponin t degradation between the treatments (Table 19). This is consistent with other results that found no impact of the addition of distillers grains on troponin t degradation (Ribeiro et al., 2019), though in contradiction to a study that found a difference (Chao et al., 2018). Although there were no dietary or quality grade impacts, there was a clear aging impact that indicates proteolytic activity (Table 20). Troponin t is a valuable protein to measure the degradation of because it is a target of proteolysis from calpains and indicators of tenderization postmortem (Lonergan et al., 2001; Huff-Lonergan et al., 2010). Therefore, this study's results are consistent with previous reports that troponin t is a target for postmortem tenderization.

Isoprostanes

No differences were found between the treatments in this study (Table 21). In general, SFC had higher isoprostanes (8-iso-Prostaglandin F2α) concentration than DRC, the presence of DDGS results in higher isoprostanes concentration than the absence of DDGS, and Upper 2/3 Choice steaks had higher isoprostanes concentration than Select steaks. The steaming process involved with SFC allows better digestibility than DRC (Zinn et al., 2002), DDGS being a distiller grain that provides a higher energy diet than corn, and increases the deposition of PUFA (Mello et al., 2012; Ribeiro, 2017), as well as Upper 2/3 Choice quality grade having higher fat content and stronger relationship to tenderness, compared to Select, leads to the proposition that greater levels of ROS-induced oxidative stress would occur in the samples and would be reflected in the isoprostanes concentration (Blumer, 1963; McBee and Wiles, 1967; Tatum et al., 1980; Smith et al., 1987; Wheeler et al., 1994; Kim et al., 2008; Wood et al., 2008; D'Alessandro et al., 2012b; Emerson et al., 2013). Though statistical significance was not found in the results, the utility of the measurement warrants its use in further investigating other potential relationships of oxidative stress and meat quality

. **Conclusion**

Results suggest that beef steaks from cattle fed DRC had significantly lower WBSF than SFC and Upper 2/3 Choice steaks had lower WBSF than Select. This is the case up until 16 d of aging where the difference was still in favor of Upper 2/3 Choice steaks, but they were statistically similar. Differences of troponin-T degradation were caused by aging. There were no statistical differences in isoprostanes across treatments. SFC or DDGS has a reduced color and lipid stability compared to DRC or No DDGS, and accordingly lead to reduced shelf life. Furthermore, the fatty acid profile showed higher levels of key fatty acid(s) like linoleic acid and

PUFAs in SFC compared to DRC, DDGS compared to without DDGS, and Upper 2/3 Choice steaks compared to Select steaks. Thus, diet composition can impact beef shelf life and quality, however, the reason for differences in tenderness between Upper 2/3 Choice and Select grade carcasses remains elusive.

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Table 1. Warner Bratzler Shear Force of strip loin steaks (*L. lumborum*) from steers fed either with dry rolled corn with dried distillers grains with solubles, dry rolled corn without dried distillers grains with solubles, steam flaked corn with dried distillers grains with solubles, or steam flaked corn without dried distillers grains with solubles at Upper 2/3 Choice or Select quality grade (n=36).

	Category				
Grain	**DDGS**	**Quality Grade**	**WBSF (kgf)**	***SEM***	**P-value**
DRC			3.46[b]	0.2	0.02
SFC			4.04[a]		
	DDGS		3.68	0.18	0.43
	No DDGS		3.84		
		Choice	3.39[b]	0.23	<0.01
		Select	4.11[a]		

[a,b] Means in the same column within a category without common superscripts differ ($P < 0.05$).
WBSF: Warner Bratzler shear force.
DRC: Dry rolled corn.
SFC: Steam flaked corn.
DDGS: Dried distillers grains with solubles.
No DDGS: Without dried distillers grains with solubles.

Table 2. Redness (a*) of strip loin steaks (*L. lumborum*) from steers fed either dry rolled corn without dried distillers grains with solubles, steam flaked corn without dried distillers grains with solubles with 2, 9, 16, and 23 d of aging at 5 to 7 d retail display (n=36).

A	**a*, 2 d aged**		B	**a*, 9 d aged**	
RD	**DRC**	**SFC**	**RD**	**DRC**	**SFC**
5	19.62[a]	17.45[b]	**5**	18.95[a]	17.54[a]
6	19.75[a]	16.29[b]	**6**	17.66[a]	16.65[a]
7	17.69[a]	16.90[a]	**7**	17.64[a]	15.48[b]
SEM	0.58		***SEM***	0.47	

C	**a*, 16 d aged**		D	**a*, 23 d aged**	
RD	**DRC**	**SFC**	**RD**	**DRC**	**SFC**
5	19.13[a]	17.70[a]	**5**	17.79[a]	16.81[a]
6	17.81[a]	16.37[b]	**6**	15.26[a]	13.57[b]
7	15.33[a]	13.09[a]	**7**	11.46[a]	9.11[b]
SEM	0.88		***SEM***	1.35	

[a,b] Means in the same row of the same aging period and retail display day without common superscripts differ ($P < 0.05$).
A: a* 2 days aged loins.
B: a* 9 days aged loins.
C: a* 16 days aged loins.
D: a* 23 days aged loins.
RD: Retail display.
DRC: Dry rolled corn.
SFC: Steam flaked corn.

Table 3. Redness (a*) of strip loin steaks (*L. lumborum*) from steers fed either with dried distillers grains with solubles or without dried distillers grains with solubles with 2, 9, 16, and 23 d of aging at 5 to 7 d retail display (n=36).

A	**a*, 2 d aged**		B	**a*, 9 d aged**	
RD	**DDGS**	**NO DDGS**	**RD**	**DDGS**	**NO DDGS**
5	18.39[a]	18.67[a]	**5**	17.65[a]	18.84[a]
6	18.15[a]	17.89[a]	**6**	16.21[b]	18.10[a]
7	18.21[a]	16.39[b]	**7**	15.55[b]	17.57[a]
SEM	0.33		***SEM***	0.5	

C	**a*, 16 d aged**		D	**a*, 23 d aged**	
RD	**DDGS**	**NO DDGS**	**RD**	**DDGS**	**NO DDGS**
5	18.21[a]	18.62[a]	**5**	16.79[a]	17.81[a]
6	16.19[b]	17.99[a]	**6**	13.42[b]	15.41[a]
7	12.40[b]	16.02[a]	**7**	9.13[b]	11.44[a]
SEM	0.94		***SEM***	1.35	

[a,b] Means in the same row of the same aging period and retail display day without common superscripts differ ($P < 0.05$).
A: a* 2 days aged loins.
B: a* 9 days aged loins.
C: a* 16 days aged loins.
D: a* 23 days aged loins.
RD: Retail display.
DDGS: Dried distillers grains with solubles.
No DDGS: Without dried distillers grains with solubles.

Table 4. Percentage discoloration of strip loin steaks (*L. lumborum*) from steers fed either dry rolled corn without dried distillers grains with solubles, steam flaked corn without dried distillers grains with solubles, with 2, 9, 16, and 23 d of aging at 5 to 7 d retail display (n=36).

A	**% discolor, 2 d aged**	
RD	**DRC**	**SFC**
5	0.00[a]	0.32[a]
6	0.48[a]	2.58[a]
7	0.94[a]	5.65[a]
SEM	0.88	

B	**% discolor, 9 d aged**	
RD	**DRC**	**SFC**
5	0.03[a]	0.52[a]
6	0.58[a]	2.75[a]
7	1.63[b]	9.01[b]
SEM	1.38	

C	**% discolor, 16 d aged**	
RD	**DRC**	**SFC**
5	1.28[a]	1.86[a]
6	6.75[a]	7.81[a]
7	21.47[a]	27.50[a]
SEM	4.43	

D	**% discolor, 23 d aged**	
RD	**DRC**	**SFC**
5	3.65[a]	7.59[a]
6	19.73[b]	40.18[a]
7	46.14[b]	74.49[a]
SEM	10.99	

[a,b] Means in the same row of the same aging period and retail display day without common superscripts differ ($P < 0.05$).

A: Percentage discoloration 2 days aged loins.
B: Percentage discoloration 9 days aged loins.
C: Percentage discoloration 16 days aged loins.
D: Percentage discoloration 23 days aged loins.
RD: Retail display.
DRC: Dry rolled corn.
SFC: Steam flaked corn.

Table 5. Percentage discoloration of strip loin steaks (*L. lumborum*) from steers fed either dried distillers grains with solubles or without dried distillers grains with solubles with 2, 9, 16, and 23 d of aging at 5 to 7 d retail display (n=36).

A	**% discolor, 2 d aged**		B	**% discolor, 9 d aged**	
RD	**DDGS**	**NO DDGS**	**RD**	**DDGS**	**NO DDGS**
5	0.22[a]	0.10[a]	**5**	0.35[a]	0.20[a]
6	1.88[a]	1.17[a]	**6**	2.36[a]	0.97[a]
7	4.47[a]	2.12[a]	**7**	7.24[a]	3.40[a]
SEM	0.66		***SEM***	1.09	

C	**% discolor, 16 d aged**		D	**% discolor, 23 d aged**	
RD	**DDGS**	**NO DDGS**	**RD**	**DDGS**	**NO DDGS**
5	2.25[a]	0.90[a]	**5**	5.79[a]	5.45[a]
6	11.09[a]	3.46[b]	**6**	35.81[a]	24.10[b]
7	37.12[a]	11.85[b]	**7**	70.93[a]	49.70[b]
SEM	5.53		***SEM***	10.48	

[a,b] Means in the same row of the same aging period and retail display day without common superscripts differ ($P < 0.05$).

A: Percentage discoloration 2 days aged loins.

B: Percentage discoloration 9 days aged loins.

C: Percentage discoloration 16 days aged loins.

D: Percentage discoloration 23 days aged loins.

RD: Retail display.

DDGS: Dried distillers grains with solubles.

No DDGS: Without dried distillers grains with solubles.

Table 6. Percentage discoloration of strip loin steaks (*L. lumborum*) with either Upper 2/3 Choice or Select quality grade with 2, 9, 16, and 23 d of aging at 5 to 7 d retail display (n=36).

A	% discolor, 2 d aged	
RD	**Choice**	**Select**
5	0.26[a]	0.06[a]
6	2.10[a]	0.96[a]
7	4.97[a]	1.62[a]
SEM	0.73	

B	% discolor, 9 d aged	
RD	**Choice**	**Select**
5	0.56[a]	0.00[a]
6	2.90[a]	0.43[a]
7	8.44[a]	2.21[a]
SEM	1.29	

C	% discolor, 16 d aged	
RD	**Choice**	**Select**
5	1.25[a]	1.90[a]
6	6.16[a]	8.40[a]
7	17.55[b]	31.41[a]
SEM	4.72	

D	% discolor, 23 d aged	
RD	**Choice**	**Select**
5	4.64[a]	6.61[a]
6	29.63[a]	30.28[a]
7	61.94[a]	58.70[a]
SEM	10.02	

[a,b] Means in the same row of the same aging period and retail display day without common superscripts differ ($P < 0.05$).
A: Percentage discoloration 2 days aged loins.
B: Percentage discoloration 9 days aged loins.
C: Percentage discoloration 16 days aged loins.
D: Percentage discoloration 23 days aged loins.
RD: Retail display.

Table 7. Yellowness (b*) of strip loin steaks (*L. lumborum*) from steers fed either dry rolled corn with dried distillers grains with solubles, dry rolled corn without dried distillers grains with solubles, steam flaked corn with dried distillers grains with solubles, or steam flaked corn without dried distillers grains with solubles either Upper 2/3 Choice or Select quality grade with 2, 9, 16, and 23 d of aging at 5 to 7 d retail display (n=36).

Grain	DDGS	Quality Grade	Aging	RD	b*
DRC	DDGS	**Choice**	2	5	9.26[ab]
DRC	NODDGS	**Choice**	2	5	9.95[a]
SFC	DDGS	**Choice**	2	5	8.69[ab]
SFC	NODDGS	**Choice**	2	5	8.37[b]
DRC	DDGS	**Choice**	2	6	9.97[a]
DRC	NODDGS	**Choice**	2	6	10.07[a]
SFC	DDGS	**Choice**	2	6	8.54[b]
SFC	NODDGS	**Choice**	2	6	8.83[ab]
DRC	DDGS	**Choice**	2	7	8.99[a]
DRC	NODDGS	**Choice**	2	7	9.57[a]
SFC	DDGS	**Choice**	2	7	8.92[a]
SFC	NODDGS	**Choice**	2	7	8.46[a]

Grain	DDGS	Quality Grade	Aging	RD	b*
DRC	DDGS	**Select**	2	5	9.35[a]
DRC	NODDGS	**Select**	2	5	9.97[a]
SFC	DDGS	**Select**	2	5	8.69[a]
SFC	NODDGS	**Select**	2	5	8.56[a]
DRC	DDGS	**Select**	2	6	9.28[a]
DRC	NODDGS	**Select**	2	6	10.03[a]
SFC	DDGS	**Select**	2	6	8.88[ab]
SFC	NODDGS	**Select**	2	6	7.25[b]
DRC	DDGS	**Select**	2	7	9.56[a]
DRC	NODDGS	**Select**	2	7	9.20[a]
SFC	DDGS	**Select**	2	7	9.56[a]
SFC	NODDGS	**Select**	2	7	9.06[a]

Grain	DDGS	Quality Grade	Aging	RD	b*
DRC	DDGS	**Choice**	9	5	9.28[ab]
DRC	NODDGS	**Choice**	9	5	10.63[a]
SFC	DDGS	**Choice**	9	5	8.54[b]
SFC	NODDGS	**Choice**	9	5	8.37[b]
DRC	DDGS	**Choice**	9	6	7.88[b]
DRC	NODDGS	**Choice**	9	6	10.17[a]
SFC	DDGS	**Choice**	9	6	8.42[b]
SFC	NODDGS	**Choice**	9	6	8.35[b]
DRC	DDGS	**Choice**	9	7	8.98[ab]
DRC	NODDGS	**Choice**	9	7	10.08[a]
SFC	DDGS	**Choice**	9	7	7.97[b]

Grain	DDGS	Quality Grade	Aging	RD	b*
DRC	DDGS	**Select**	9	5	8.63[a]
DRC	NODDGS	**Select**	9	5	8.75[a]
SFC	DDGS	**Select**	9	5	8.42[a]
SFC	NODDGS	**Select**	9	5	9.13[a]
DRC	DDGS	**Select**	9	6	8.58[a]
DRC	NODDGS	**Select**	9	6	8.83[a]
SFC	DDGS	**Select**	9	6	8.42[a]
SFC	NODDGS	**Select**	9	6	9.04[a]
DRC	DDGS	**Select**	9	7	7.96[a]
DRC	NODDGS	**Select**	9	7	8.71[a]
SFC	DDGS	**Select**	9	7	8.52[a]

SFC	NODDGS	**Choice**	9	7	8.21[b]

SFC	NODDGS	**Select**	9	7	8.61[a]

Grain	DDGS	Quality Grade	Aging	RD	b*
DRC	DDGS	**Choice**	16	5	10.08[a]
DRC	NODDGS	**Choice**	16	5	9.83[a]
SFC	DDGS	**Choice**	16	5	8.95[a]
SFC	NODDGS	**Choice**	16	5	9.14[a]
DRC	DDGS	**Choice**	16	6	9.43[a]
DRC	NODDGS	**Choice**	16	6	9.53[a]
SFC	DDGS	**Choice**	16	6	8.71[a]
SFC	NODDGS	**Choice**	16	6	8.90[a]
DRC	DDGS	**Choice**	16	7	8.99[a]
DRC	NODDGS	**Choice**	16	7	9.45[a]
SFC	DDGS	**Choice**	16	7	8.26[a]
SFC	NODDGS	**Choice**	16	7	8.05[a]

Grain	DDGS	Quality Grade	Aging	RD	b*
DRC	DDGS	**Select**	16	5	9.17[a]
DRC	NODDGS	**Select**	16	5	9.16[a]
SFC	DDGS	**Select**	16	5	8.55[a]
SFC	NODDGS	**Select**	16	5	8.63[a]
DRC	DDGS	**Select**	16	6	8.71[ab]
DRC	NODDGS	**Select**	16	6	9.62[a]
SFC	DDGS	**Select**	16	6	8.24[b]
SFC	NODDGS	**Select**	16	6	7.82[b]
DRC	DDGS	**Select**	16	7	8.14[a]
DRC	NODDGS	**Select**	16	7	8.75[a]
SFC	DDGS	**Select**	16	7	7.53[a]
SFC	NODDGS	**Select**	16	7	7.66[a]

Grain	DDGS	Quality Grade	Aging	RD	b*
DRC	DDGS	**Choice**	23	5	9.32[a]
DRC	NODDGS	**Choice**	23	5	9.22[a]
SFC	DDGS	**Choice**	23	5	8.36[a]
SFC	NODDGS	**Choice**	23	5	9.54[a]
DRC	DDGS	**Choice**	23	6	8.47[a]
DRC	NODDGS	**Choice**	23	6	8.91[a]
SFC	DDGS	**Choice**	23	6	7.86[a]
SFC	NODDGS	**Choice**	23	6	9.06[a]
DRC	DDGS	**Choice**	23	7	8.40[a]
DRC	NODDGS	**Choice**	23	7	8.24[a]
SFC	DDGS	**Choice**	23	7	8.51[a]
SFC	NODDGS	**Choice**	23	7	8.69[a]

Grain	DDGS	Quality Grade	Aging	RD	b*
DRC	DDGS	**Select**	23	5	8.74[a]
DRC	NODDGS	**Select**	23	5	8.63[a]
SFC	DDGS	**Select**	23	5	7.92[a]
SFC	NODDGS	**Select**	23	5	8.52[a]
DRC	DDGS	**Select**	23	6	7.89[a]
DRC	NODDGS	**Select**	23	6	8.11[a]
SFC	DDGS	**Select**	23	6	7.22[a]
SFC	NODDGS	**Select**	23	6	8.19[a]
DRC	DDGS	**Select**	23	7	8.38[a]
DRC	NODDGS	**Select**	23	7	7.68[a]
SFC	DDGS	**Select**	23	7	7.54[a]
SFC	NODDGS	**Select**	23	7	8.14[a]

[a,b] Means in the same row of the same aging period and retail display day without common superscripts differ ($P < 0.05$).
RD: Retail display.

Table 8. Lipid oxidation values of strip steaks (*L. lumborum*) from steers fed either dry rolled corn without dried distillers grains with solubles or steam flaked corn without dried distillers grains with solubles at 0 and 7 d retail display (n=36).

	TBARS	
	0 d RD	**7 d RD**
DRC	0.48[a]	2.31[b]
SFC	0.49[a]	3.13[a]

***SEM*=** 0.67.
[a,b] Means within day without common superscripts differ ($P < 0.05$).
TBARS: Lipid oxidation; mg malonaldehyde/ kg of meat.
RD: Retail display.
DRC: Dry rolled corn.
SFC: Steam flaked corn.

Table 9. Lipid oxidation values of strip steaks (*L. lumborum*) from steers fed either with dried distillers grains with solubles or without dried distillers grains with solubles at 0 and 7 d retail display (n=36).

	TBARS	
	0 d RD	**7 d RD**
DDGS	0.52[a]	3.54[a]
No DDGS	0.44[a]	1.90[b]

***SEM*=** 0.73.

[a,b] Means within day without common superscripts differ ($P < 0.05$).

TBARS: Lipid oxidation; mg malonaldehyde/ kg of meat.

RD: retail display.

DDGS: Dried distillers grains with solubles.

No DDGS: Without dried distillers grains with solubles.

Table 10. Proximate composition[1] of strip loin steaks (*L. lumborum*) aged for 2 d from steers fed either with dry rolled corn without dried distillers grains with solubles, steam flaked corn without dried distillers grains with solubles, dry rolled corn with dried distillers grains with solubles, or steam flaked corn with dried distillers grains with solubles at Upper 2/3 Choice or Select quality grade (n=36).

Grain	DDGS	Quality Grade	Moisture, %	Ash, %	Fat, %	Protein[2], %
DRC	DDGS	Choice	70.90	1.68	9.09	18.33
DRC	DDGS	Select	73.80	1.78	5.01	19.41
DRC	No DDGS	Choice	70.11	1.55	9.62	18.72
DRC	No DDGS	Select	73.49	1.70	5.38	19.43
SFC	DDGS	Choice	72.48	1.47	7.02	19.03
SFC	DDGS	Select	74.38	1.76	4.13	19.72
SFC	No DDGS	Choice	71.78	1.62	7.89	18.72
SFC	No DDGS	Select	74.03	1.71	4.21	20.05
SEM			0.55	0.04	0.77	0.20

[1] There is no difference between dietary treatments ($P > 0.05$).
[2] Protein was calculated by subtracting moisture, ash, and fat from 100.
DRC: Dry rolled corn.
SFC: Steam flaked corn.
DDGS: Dried distillers grains with solubles.
No DDGS: Without dried distillers grains with solubles.

Table 11. Percentage fat of strips loin steaks (*L. lumborum*) at Choice or Select quality grade.

Fat, %		
Choice	**Select**	***SEM***
7.86[a]	4.55[b]	0.77

[a,b] Means without common superscripts differ ($P < 0.05$).

Table 12. Sarcomere length[1] of strip loin steaks (*L. lumborum*) aged for 2 d from steers fed either with dry rolled corn without dried distillers grains with solubles, steam flaked corn without dried distillers grains with solubles, dry rolled corn with dried distillers grains with solubles, or steam flaked corn with dried distillers grains with solubles at Upper 2/3 Choice or Select quality grade (n=36).

Grain	DDGS	Quality Grade	SL (µm)
DRC	NO DDGS	Choice	1.69
DRC	DDGS	Choice	1.69
SFC	NO DDGS	Choice	1.70
SFC	DDGS	Choice	1.71
DRC	NO DDGS	Select	1.65
DRC	DDGS	Select	1.87
SFC	NO DDGS	Select	1.67
SFC	DDGS	Select	1.71

SEM= 0.03

[1] There is no difference between dietary treatments ($P > 0.05$).

SL: Sarcomere length.

DRC: Dry rolled corn.

SFC: Steam flaked corn.

DDGS: Dried distillers grains with solubles.

No DDGS: Without dried distillers grains with solubles.

Table 13. Amount of fatty acids for strip steaks from steers fed different processed corn diets of dry rolled corn without dried distillers grains with solubles or steam flaked corn without dried distillers grains with solubles[1] (n=36).

Fatty Acid, mg/100g	DRC	SFC	*SEM*	P-value
C10:0	3.74	5.59	1.05	0.31
C12:0	6.13	6.23	0.78	0.95
C13:0	2.21	1.37	0.69	0.52
C14:0	213.53	223.00	10.67	0.65
C14:1	74.56	63.95	5.65	0.27
C15:0	31.83	34.00	1.94	0.56
C15:1	65.03	62.10	4.49	0.74
C16:0	1610.54	1616.92	68.01	0.96
C16:1T	22.65	21.65	1.05	0.62
C16:1	254.00	237.12	13.49	0.51
C17:0	74.67	84.94	5.56	0.28
C17:1	84.45	86.07	4.26	0.85
C18:0	710.08	715.18	33.82	0.94
C18:1T	109.39[b]	320.85[a]	64.97	<0.05
C18:1	2129.77	1968.75	158.61	0.60
C18:1V	245.79	196.16	136.91	0.86
C18:2T	34.81	30.08	2.27	0.20
C18:2	328.79[b]	414.82[a]	30.22	0.02
C18:3w6	1.39	0.48	0.47	0.25
C18:3w3	12.72[b]	15.53[a]	1.05	0.05
C20:1	30.30	33.08	2.56	0.57
C20:3	19.43	18.22	0.96	0.51
C20:4w6	62.71	62.16	4.73	0.95
C22:1	2.32	2.30	2.11	0.99

C22:4	2.68	1.43	0.73	0.33
C22:5	15.55	16.66	1.05	0.58
Other	16.20	4.79	6.25	0.45
SFA[2]	2652.74	2687.22	113.74	0.88
UFA[3]	3498.91	3551.42	137.25	0.85
MUFA[4]	3018.28	2992.04	125.85	0.92
PUFA[5]	480.63	559.38	32.17	0.09
Trans[6]	166.85[b]	372.59[a]	63.64	<0.01
w6	64.10	62.64	4.72	0.88
w3	12.72[b]	15.53[a]	1.05	0.05
Total Lipids (mg/ 100g)	6149.09	6238.64	484.93	0.85

[a,b] Means in the same row without common superscripts differ ($P < 0.05$).
[1] Interactions between Table 13 and 14 are reported in Table 15
DRC: Dry rolled corn.
SFC: Steam flaked corn.
[2] SFA- Saturated fatty acids.
[3] UFA- Unsaturated fatty acids.
[4] MUFA- Monounsaturated fatty acids.
[5] PUFA- Polyunsaturated fatty acids.
[6] Trans- Trans-unsaturated fatty acids.

Table 14. Amount of fatty acids for strip steaks from steers fed diets with or without dried distillers grains with solubles (n=36).

Fatty Acid, mg/100g	No DDGS	DDGS	*SEM*	P-value
C10:0	4.91	4.42	0.91	0.79
C12:0	7.14	5.22	0.96	0.23
C13:0	2.21	1.37	0.69	0.52
C14:0	232.84	203.69	13.31	0.17
C14:1	76.17	62.35	6.20	0.16
C15:0	35.78	30.05	2.47	0.13
C15:1	63.40	63.73	4.41	0.97
C16:0	1618.65	1608.81	68.05	0.94
C16:1T	19.91[b]	24.39[a]	1.64	0.04
C16:1	260.30	230.82	15.19	0.25
C17:0	83.34	76.27	5.12	0.46
C17:1	89.74	80.78	4.96	0.30
C18:0	649.43	775.83	49.73	0.07
C18:1T	237.65	192.60	25.76	0.32
C18:1	2088.46	2010.06	153.32	0.80
C18:1V	99.05	342.91	153.28	0.38
C18:2T	31.14	33.75	1.96	0.48
C18:2	316.24[b]	427.37[a]	36.41	<0.05
C18:3w6	0.47	1.40	0.47	0.24
C18:3w3	14.00	14.25	1.05	0.85
C20:1	28.36	35.03	3.10	0.18
C20:3	17.51	20.15	1.17	0.15
C20:4w6	60.41	64.45	4.87	0.67
C22:1	0.00	4.62	2.50	0.28
C22:4	0.95	3.15	0.90	0.01

C22:5	16.10	16.10	1.00	1.00
Other	3.64	17.35	7.39	0.24
SFA[1]	2634.31	2705.65	115.16	0.76
UFA[2]	3422.41	3627.92	148.75	0.46
MUFA[3]	2963.02	3047.29	127.96	0.74
PUFA[4]	459.39[b]	580.63[a]	41.75	0.01
Trans[5]	288.70	250.74	25.34	0.41
w6	60.88	65.85	4.92	0.60
w3	14.00	14.25	0.67	0.85
Total Lipids (mg/ 100g)	6054.16	6333.57	484.93	0.57

[a,b] Means in the same row without common superscripts differ ($P < 0.05$).
DDGS: Dried distillers grains with solubles.
No DDGS: Without dried distillers grains with solubles.
[1] SFA- Saturated fatty acids.
[2] UFA- Unsaturated fatty acids.
[3] MUFA- Monounsaturated fatty acids.
[4] PUFA- Polyunsaturated fatty acids.
[5] Trans- Trans-unsaturated fatty acids.

Table 15. Amount of fatty acids for strip steaks from steers fed different processed corn diets of dry rolled corn or steam flaked corn and with or without dried distillers grains with solubles (n=36).

Fatty Acid, mg/100g	DRC	DRC+DDGS	SFC	SFC+DDGS	*SEM*	P-value
C15:0	39.97[a]	23.70[b]	31.59[ab]	36.41[ab]	15.59	<0.01
C16:1T	23.12[ab]	22.18[ab]	16.70[b]	26.60[a]	8.91	0.01
C17:0	89.70[ab]	59.65[b]	76.99[ab]	92.89[a]	32.80	0.02
C17:1	104.07[a]	64.83[b]	75.40[ab]	96.73[a]	40.36	<0.01
Total Lipids (mg/ 100g)	6427.49	5870.68	5680.83	6796.45	0.012	0.10

[a,b] Means in the same row without common superscripts differ ($P < 0.05$).
DRC: Dry rolled corn.
SFC: Steam flaked corn.
DDGS: Dried distillers grains with solubles.
No DDGS: Without dried distillers grains with solubles.

Table 16. Amount of fatty acids for either Upper 2/3 Choice or Select quality grade strip steaks (n=36).

Fatty Acid, mg/100g	Choice	Select	*SEM*	P-value
C10:0	6.55[a]	2.78[b]	1.41	<0.05
C12:0	8.35[a]	4.01[b]	1.48	0.01
C13:0	0.00[b]	3.58[a]	1.95	0.01
C14:0	285.47[a]	151.05[b]	40.15	<0.01
C14:1	86.39[a]	41.29[b]	24.96	<0.01
C15:0	42.37[a]	23.46[b]	5.76	<0.01
C15:1	74.57[a]	52.56[b]	7.74	0.02
C16:0	2087.33[a]	1140.13[b]	281.76	<0.01
C16:1T	27.05[a]	17.25[b]	3.00	<0.01
C16:1	312.31[a]	178.81[b]	58.18	<0.01
C17:0	102.50[a]	57.11[b]	13.92	<0.01
C17:1	108.80[a]	61.72[b]	14.24	<0.01
C18:0	882.84[a]	542.43[b]	103.92	<0.01
C18:1T	287.53[a]	142.72[b]	47.35	<0.01
C18:1	2524.68[a]	1573.84[b]	313.59	<0.01
C18:1V	370.15	71.81	161.11	0.28
C18:2T	41.25[a]	23.64[b]	5.40	<0.01
C18:2	425.80[a]	317.81[b]	35.61	<0.01
C18:3w6	0.23	1.64	1.76	0.08
C18:3w3	17.76[a]	10.49[b]	2.20	<0.01
C20:1	42.00[a]	21.38[b]	6.43	<0.01
C20:3	20.33	17.33	1.24	0.11
C20:4w6	63.15	61.71	8.61	0.88
C22:1	2.32	2.30	2.11	0.99
C22:4	1.57	2.54	1.72	0.45
C22:5	14.44	17.77	2.53	0.11
Other	16.08	4.91	6.58	0.24
SFA[1]	3415.41[a]	1924.54[b]	445.04	<0.01
UFA[2]	4426.09[a]	2624.24[b]	537.74	<0.01
MUFA[3]	3841.56[a]	2168.75[b]	498.97	<0.01
PUFA[4]	584.53[a]	455.49[b]	43.65	<0.01
Trans[5]	355.83[a]	183.61[b]	54.71	<0.01
w6	63.39	63.35	4.70	0.99
w3	17.76[a]	10.49[b]	2.20	<0.01
Total Lipids (mg/ 100g)	7841.50[a]	4546.23[b]	484.93	<0.01

[a,b] Means in the same row without common superscripts differ ($P < 0.05$).
[1] SFA- Saturated fatty acids.
[2] UFA- Unsaturated fatty acids.
[3] MUFA- Monounsaturated fatty acids.
[4] PUFA- Polyunsaturated fatty acids.
[5] Trans- Trans-unsaturated fatty acids.

Table 17. Free calcium concetration[1] of strip loin steaks (*L. lumborum*) aged for 2 d from steers fed either with dry rolled corn without dried distillers grains with solubles, steam flaked corn without dried distillers grains with solubles, dry rolled corn with dried distillers grains with solubles, or steam flaked corn with dried distillers grains with solubles at Upper 2/3 Choice or Select quality grade (n=36).

Grain	DDGS	Quality Grade	Ca^{2+} (µM)	*SEM*
DRC	NO DDGS	Choice	57.15	2.48
DRC	DDGS	Choice	48.03	
SFC	NO DDGS	Choice	63.12	
SFC	DDGS	Choice	54.47	
DRC	NO DDGS	Select	59.98	
DRC	DDGS	Select	62.93	
SFC	NO DDGS	Select	46.39	
SFC	DDGS	Select	58.14	

[1] There is no difference between dietary treatments, quality grade, or interactions ($P > 0.05$).
Ca^{2+}: Free calcium.
DRC: Dry rolled corn.
SFC: Steam flaked corn.
DDGS: Dried distillers grains with solubles.
No DDGS: Without dried distillers grains with solubles.

Table 18. pH measurement[1] of strip loin steaks (*L. lumborum*) aged for 2 d from steers fed either with dry rolled corn without dried distillers grains with solubles, steam flaked corn without dried distillers grains with solubles, dry rolled corn with dried distillers grains with solubles, or steam flaked corn with dried distillers grains with solubles at Upper 2/3 Choice or Select quality grade (n=36).

Grain	DDGS	Quality Grade	pH	*SEM*
DRC	DDGS	Choice	5.48	0.01
DRC	NO DDGS	Choice	5.51	
SFC	DDGS	Choice	5.54	
SFC	NO DDGS	Choice	5.50	
DRC	DDGS	Select	5.44	
DRC	NO DDGS	Select	5.53	
SFC	DDGS	Select	5.51	
SFC	NO DDGS	Select	5.48	

[1] There is no difference among dietary treatments, quality grade, or interactions ($P > 0.05$).
DRC: Dry rolled corn.
SFC: Steam flaked corn.
DDGS: Dried distillers grains with solubles.
No DDGS: Without dried distillers grains with solubles.

Table 19. Troponin T degradation of strip steaks (*L. lumborum*) from steers fed either dry rolled corn without dried distillers grains with solubles, steam flaked corn without dried distillers grains with solubles, dry rolled corn with dried distillers grains with solubles, or steam flaked corn with dried distillers grains with solubles at Upper 2/3 Choice or Select quality grade and with 2 and 23 d of aging at 0 d retail display (n=36).

Grain	DDGS	Quality Grade	Aging	% TnT Degraded
DRC	DDGS	Choice	2	14.95[bc]
DRC	DDGS	Select	2	9.71[c]
DRC	NODDGS	Choice	2	8.01[c]
DRC	NODDGS	Select	2	5.33[c]
SFC	DDGS	Choice	2	9.06[c]
SFC	DDGS	Select	2	8.09[c]
SFC	NODDGS	Choice	2	1.29[c]
SFC	NODDGS	Select	2	6.50[c]
DRC	DDGS	Choice	23	51.58[a]
DRC	DDGS	Select	23	47.31[a]
DRC	NODDGS	Choice	23	44.02[a]
DRC	NODDGS	Select	23	61.00[a]
SFC	DDGS	Choice	23	37.47[ab]
SFC	DDGS	Select	23	42.80[ab]
SFC	NODDGS	Choice	23	45.67[a]
SFC	NODDGS	Select	23	43.00[ab]
P-value				0.37
SEM				3.43

[a,b,c] Means within day without common superscripts differ ($P < 0.05$).
TnT: Troponin t.
DRC: Dry rolled corn.
SFC: Steam flaked corn.
DDGS: Dried distillers grains with solubles.
No DDGS: Without dried distillers grains with solubles.

Table 20. Troponin T (TnT) degradation of strip steaks (*L. lumborum*) with 2 and 23 d of aging at 0 d retail display (n=36).

Aging	% TnT Degraded
2	7.87[b]
23	46.61[a]
SEM	3.43

[a,b] Means within day without common superscripts differ ($P < 0.05$).
TnT: Troponin t.

Table 21. Concentration of 8-iso prostaglandin F2α[1] of strip loin steaks (*L. lumborum*) from steers fed either with dry rolled corn without dried distillers grains with solubles, steam flaked corn without dried distillers grains with solubles, dry rolled corn with dried distillers grains with solubles, or steam flaked corn with dried distillers grains with solubles at Upper 2/3 Choice or Select quality grade (n=36).

Category				
Grain	**DDGS**	**Quality Grade**	**8-iso**	***SEM***
DRC			122.90	
SFC			186.10	
	DDGS		171.29	
	No DDGS		137.71	
		Choice	165.78	
		Select	143.22	
				19.35

[1] There is no difference among treatment categories ($P > 0.05$).
8-iso: Isoprostanes concentration.
DRC: Dry rolled corn.
SFC: Steam flaked corn.
DDGS: Dried distillers grains with solubles.
No DDGS: Without dried distillers grains with solubles.

Figure Legends

Figure 1. Warner Bratzler Shear Force (WBSF) of strip loin steaks (*L. lumborum*) across treatments with 2, 9, 16, and 23 d of aging at 0 d retail display (n=36).

SEM: 0.18.

[a,b,c] Means without common superscripts differ ($P < 0.01$).

Figure 2. Warner Bratzler Shear Force (WBSF) of strip loin steaks (*L. lumborum*) with 2, 9, 16, and 23 d of aging and 7 d retail display (n=36).

SEM: 0.18.

[a,b] Means without common superscripts differ ($P < 0.01$).

Figure 3. Lightness (L*) of strip loin steaks (*L. lumborum*) with 2, 9, 16, and 23 d of aging (n=36).

SEM: 0.11

[a,b] Means in the same aging period without common superscripts differ ($P < 0.05$)

Figure 4. Lipid oxidation of strip steaks (*L. lumborum*) with 2, 9, 16, and 23 d of aging at 0 d and 7 d retail display (n=36).

SEM: 0.19.

[a,b] Means within day without common superscripts differ ($P < 0.05$).

TBARS: Lipid oxidation; mg malonaldehyde/ kg of meat.

RD: Retail display.

Figure 1.

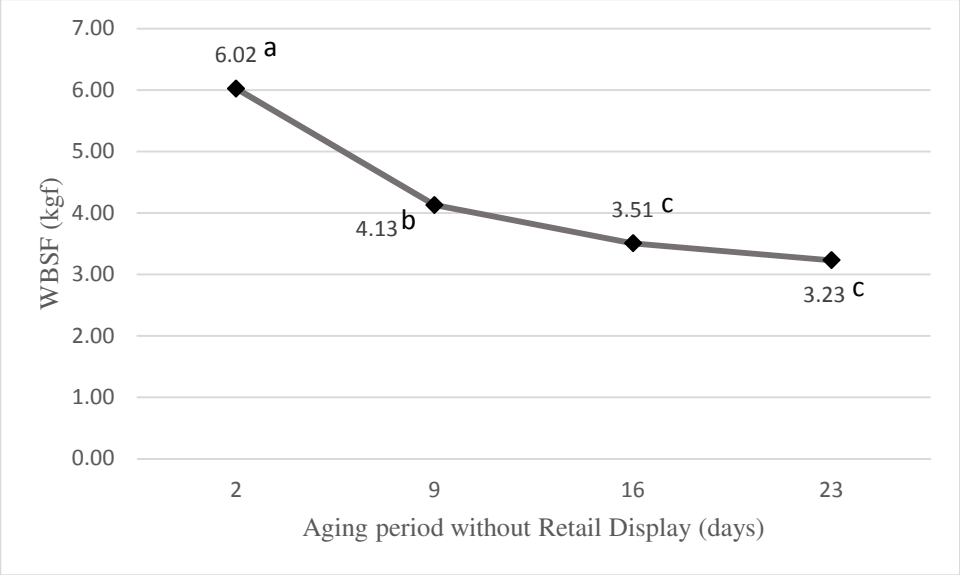

7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
WBSF (kgf)
6.02 a
4.13 b
3.51 c
3.23 c
2
9
16
23
Aging period without Retail Display (days)

Figure 2.

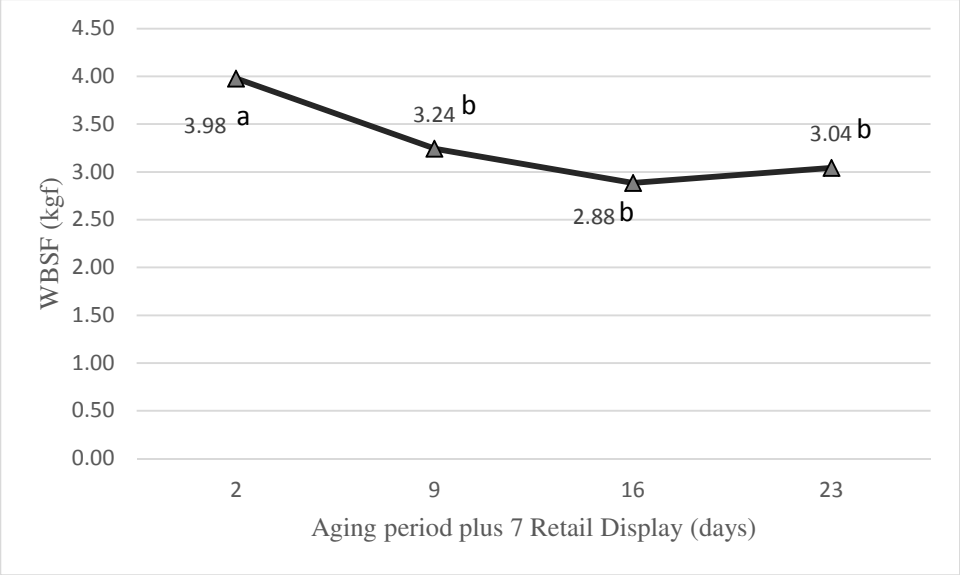

4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
WBSF (kgf)
3.98 a
3.24 b
2.88 b
3.04 b
2
9
16
23
Aging period plus 7 Retail Display (days)

Figure 3.

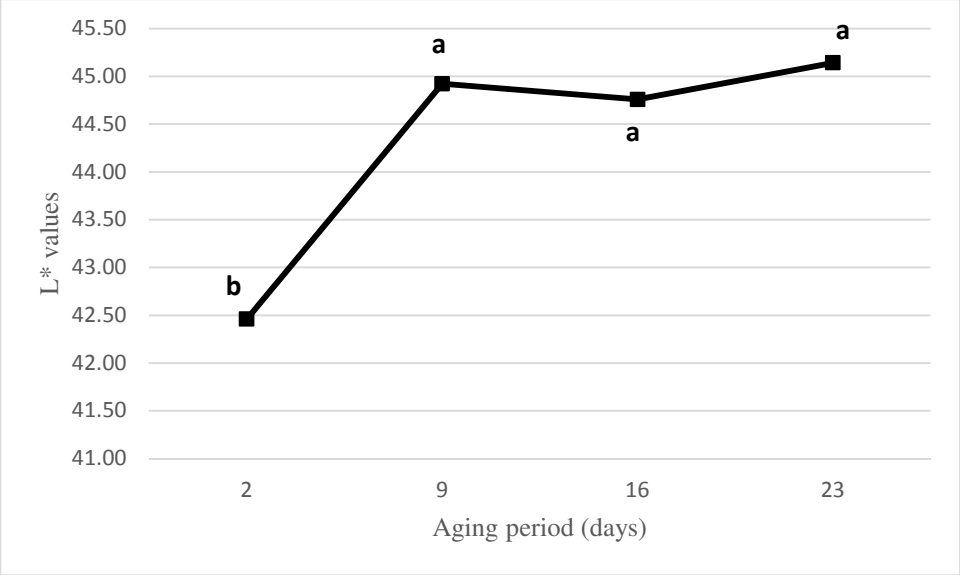
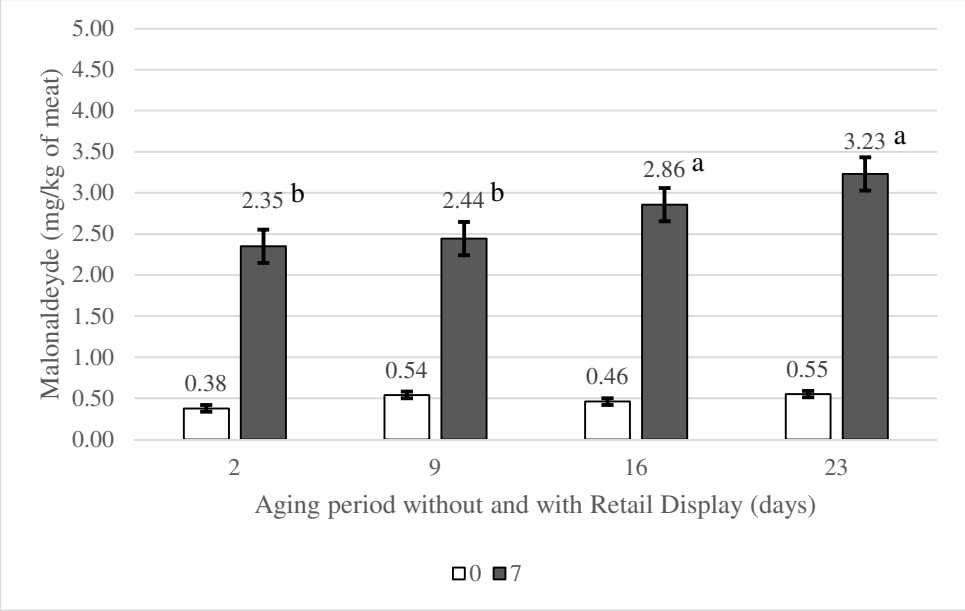

45.50
45.00
44.50
44.00
43.50
43.00
42.50
42.00
41.50
41.00
L* values
a
a
a
b
2
9
16
23
Aging period (days)

Figure 4.


5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Malonaldeyde (mg/kg of meat)
0.38
2.35 b
0.54
2.44 b
0.46
2.86 a
0.55
3.23 a
2
9
16
23
Aging period without and with Retail Display (days)
0
7

Impact of Diet and Quality Grade on Proteomic Analysis of Oxidized Proteins in Beef

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Abstract

The explanation of the relationship between marbling and beef tenderness is still unclear, highlighting the need to evaluate the relationship between these valuable aspects of a positive consumer eating experience. This study was conducted to evaluate the effects of feeding diets with different types of processed corn and with or without distiller grains on byproducts of oxidative stress and how that relates to tenderness and oxidative damage of proteins in beef steaks of differing quality grade. Steers (n= 240) were finished on diets containing dry rolled corn (DRC), DRC + 30% dried distillers grains (DDGS), steam flaked corn (SFC), or SFC + 30% DDGS. Cattle were fed 10 per pen. Only pens with both upper 2/3 Choice and Select-grade carcasses were sampled, with a goal of two (and a minimum of one) of each grade per pen. Three pens per treatment met the selection criterion and 36 carcasses were chosen (21 upper 2/3 Choice and 15 Select). Strip loins aged for 2 d postmortem were fabricated in steaks and evaluated for Warner-Bratzler shear force (WBSF) and proteomic analysis evaluating oxidative damage in proteins. Upper 2/3 Choice grade steaks were found to be more tender than Select grade steaks ($P< 0.05$). Across comparisons, proteomic analysis revealed increased oxidative damage of proteins like heat shock, glycolytic, apoptotic, and structural proteins. Upper 2/3 Choice steaks were found to generally have increased oxidative damage in glycolytic, structural, and heat shock proteins, compared to Select quality grade, similar to those identified in previous research evaluating tender meat ($P< 0.05$). Samples from cattle fed DRC had increased oxidative damage from feeding distillers grains while the SFC-related treatments had the inverse. Overall results support the relationship between marbling and tenderness and suggest oxidative stress may be a factor involved in the phenomenon.

Introduction

Proteomic analysis is a recent technology that has been utilized to measure factors like the degree of regulation or oxidative damage that has occurred between comparisons (D'Alessandro et al., 2012b; Picard et al., 2015; Malheiros et al., 2019). This has become invaluable in measuring the differences in protein class indiscriminately to better grasp how treatments can influence proteins like those involved in structure, apoptosis, energy metabolism, or heat shock, and how they can impact factors like meat quality or related to animal management (Picard and Gagaoua, 2017). Recently, research utilizing proteomics has been conducted to evaluate differences in tender and tough samples of meat (Malheiros et al., 2019). Malheiros et al. (2019) results indicated the contribution of different protein classes on tenderness along with implicating oxidative stress as a factor increasing the oxidative damage of antioxidant enzymes, structural, and heat shock proteins. When an imbalance between ROS and antioxidant protection favors the former, oxidative stress occurs (Dalle-Donne et al., 2001; Sies, 2015; Savini et al., 2016). This oxidative stress can lead to greater cellular damage impacting DNA, protein, and lipids (Halliwell et al., 1993; Morrissey et al., 1998; Halliwell, 2002; Halliwell and Gutteridge, 2015).

The ROS-induced oxidative stress is a recognized consequence of feeding high-fat diets to humans (Savini et al., 2016). In cattle, degrees of oxidative stress appear to impact tenderness of beef (Smuder et al., 2010; D'Alessandro et al., 2011; Guillemin et al., 2011; D'Alessandro et al., 2012b; Chen et al., 2019; Malheiros et al., 2019). Feeding high-fat diets like distillers grains would, therefore, be implicated in potentially inducing oxidative stress in the cattle. It is been hypothesized the feeding of distillers grains can influence the degree of oxidative stress in by increasing the deposition of PUFA in the membranes (Chao et al., 2018). There is evidence to

support that feeding distillers grains impact tenderness of beef (Gordon et al., 2002; Depenbusch et al., 2009; Aldai et al., 2010; Senaratne, 2012; Chao et al., 2018). Furthermore, its commonly recognized that feeding high-fat diets, like distillers grains, in the finishing stage of growth promotes increased rate of marbling development (Blanchard et al., 1999; Houben et al., 2000; Pethick et al., 2004; Smith and Johnson, 2016). Like tenderness, marbling is one of the most important and commonly recognized factors that influence consumers' willingness to pay premiums for the higher marbled product (Boleman et al., 1997; Feuz et al., 2004; Smith and Johnson, 2016; USDA, 2020). Marbling and tenderness have long been recognized as associated characteristics (Morrison, 1937; Jeremiah et al., 1970; Smith et al., 1987; D'Alessandro et al., 2012a; Emerson et al., 2013). There are currently several hypotheses that attempt to explain why the relationship exists, but none adequately resolve the relationship (Smith and Carpenter, 1976). This implies that multiple factors are likely involved.

Recognizing high-fat diets' ability to provide increased marbling (Pethick et al., 2004; Smith and Johnson, 2016), perhaps the relationship between marbling and tenderness is related to the occurrence of oxidative stress. By analyzing oxidative damage in the proteins, perhaps results can highlight proteins that have already been recognized as biomarkers to tenderness (D'Alessandro et al., 2012b; Picard and Gagaoua, 2017; Malheiros et al., 2019) to also have increased oxidative damage in the higher marbled product. Inconsistency in tenderness hinders the profitability of beef (Warner et al., 2005). The causal relationship between marbling and tenderness is still unclear. Therefore, the objectives of this study were to evaluate the effects of diet and marbling on tenderness and the impact those factors have on oxidative damage in proteins, as it relates to tenderness.

Material and Methods

Cattle and dietary treatments

A total of 240 crossbred steers (288 ± 0.54 kg) were blocked into three body weights and randomly assigned to one of 24 pens, (n=10/pen), then fed (University of Nebraska feedlot at Ithica, NE) for 202 days. Four dietary treatments were divided among the pens (6 pens/treatment). Cattle were fed either dry rolled corn (DRC), steam flaked corn (SFC), dry rolled corn plus 30% dried distillers grains with solubles (DRC+ DDGS), or steam flake corn plus 30% dried distillers grain with solubles (SFC+ DDGS). Inclusion of distillers grains was calculated on a dry matter basis. All diets contained 8% sorghum silage, 5% supplement (1.393% fine ground corn, 1.69% limestone, 0.125% tallow, 1.4% tallow, 0.3% salt, 0.05% beef trace mineral, 0.015% Vitamin A-D-E, 0.0165% Rumensin-90, and 0.011% Tylan-40).

Sample collection

Thirty-six USDA Upper 2/3 Choice and Select strip loin (21 Upper 2/3 Choice and 15 Select) were vacuum packaged and obtained from Greater Omaha Packing, Omaha, NE before subsequently being transported to the Loeffel Meat Laboratory at the University of Nebraska-Lincoln. Prior to fabrication, loins kept at (2 ± 5 °C) in dark storage.

Sample Fabrication

Beef loins aged for 2 d were fabricated into steaks for objective tenderness and proteomic analysis. One 2.54 cm steak was used for Warner-Bratzler shear force (WBSF) measurement at 2 d postmortem aging, and one 2.54 cm steak was trimmed of all subcutaneous fat for proteomic analysis. Steak samples for proteomic analysis were frozen for further analysis (-80°C). All steaks subsequently used for proteomic analyses were diced into small portions before freezing

in liquid nitrogen and powdering in a metal blender cup (Model 51BL32, Waring Commercial, Torrington, CT). Samples for proteomic analysis were kept frozen (-80°C).

Tenderness determination via Shear Force

Steaks 2.54 cm thick, never frozen, were measured for tenderness via Warner-Bratzler Shear Force (WBSF). On day 1, raw-internal steak temperatures were taken in Celsius (C), in the geometric center of the steaks, using a T-type thermocouple (TMQSS-062U-6, OMEGA Engineering, Inc., Stamford, CT) connected to a portable thermometer (OMEGA 450-ATT, OMEGA Engineering, Inc., Stamford, CT). Raw steak weights were measured with a scale set in grams and recorded. Afterward, all steaks were cooked to an internal temperature of 70°C on a belt grill (TBG60-V3 MagiGril, MagiKitch'n Inc., Quakertown, PA). After cooking, internal steak temperatures and weights were recorded. The steak was bagged (PB-90-C, 0.85 mil., 6 x 3 x 15 in., Get Reddi, Inteplast Group, Livingston, NJ) and stored overnight at 2°C. The next day, six cores (1.27 cm diameter) were cored out with a drill press going parallel to the muscle fibers and sheared using the Food Texture Analyzer (TMS- Pro, Food Technology Corp., Sterling, VA.) with a triangular Warner-Bratzler blade (blade speed: 250 mm/ min). An average of the six cores was calculated for each steak for statistical analysis.

Proteomics

Samples were evaluated by Dr. Camila Braga and Dr. Jiri Adamec's lab at UNL's Department of Biochemistry. The protocol follows Malheiros et al., (2019) with modifications.

Protein extraction and derivatization procedure

About 50 mg of powdered meat samples were utilized to measuring protein concentrations via the bicinchoninic acid (BCA) method with bovine serum albumin as the

standard (Thermo Scientific, BCA Protein Assay Kit, Waltham, MA). Following, electrophoretic runs with derivatized muscle samples were performed for the treatment groups.

Following procedures described in Tamarit et al. (2012) and Boone et al. (2016) with minor modifications, two cyanine dyes coupled with hydrazides, Cy3-Hz and Cy5-Hz, were used in the derivatization of carbonylated proteins. Cyanine dyes were obtained from Lumiprobe (Hunt Valley, Maryland) and reconstituted to 50 mM in dimethyl sulfoxide (DMSO) and then diluted with a derivatization buffer (1:10, 0.1 M sodium acetate pH 5, 1 mM EDTA and 1% SDS). To reach a final concentration of 1 mg mL^{-1} , derivatization solution (cyanine dyes and derivatization buffer) was added to the protein extracts (62.5 µg of protein) and incubated for 2 hours on a gyratory shaker, in the absence of light at 4°C. Following incubation, 34.5 µL of 2 M Tris and 9.5 µL of 0.2 M $NaCNBH_4$ were added to each tube containing protein extracts with samples being left, in the dark, at room temperature for 15 min to quench the reaction.

2D-DIGE – Electrophoresis

Samples were labeled with Cy5-Hz and subsequently precipitated with acetone (100%, 1:4 v/v, cold acetone, -20°C, for 2 hours) before centrifugation at 16,000 x *g* for 15 min at 4°C. To maintain the solubilized proteins, pellets were resuspended in 125 µL of rehydration buffer with minimal agitation. A buffer mixture of 7 M urea, 2 M thiourea, 2% (w/v) CHAPS (sulfate 3-[(3-cloroaminopropil)-dimethylammonio]-1-propane), 0.5% (v/v) ampholytes at pH ranging from 3 to 10 with 1% 1,4 dithiothreitol (DTT) was used for the electrophoretic separations. The combined samples, labeled with fluorophores, were added to a 7-cm IEF strip (BioRad, Hercules, California) for 12 h passive rehydration. Strips were placed gel-side down onto the sample and overlaid with mineral oil. After the 12 h, the rehydrated strip was isoelectrically

focused to resolve the first dimension (IEF, Step 1: 250 V for 15 min, Step 2: 10,000 V for 3 hours, Step 3: 10,000 V to 50,000 V for 4 h).

The strip was equilibrated, following separation in the first dimension, in two steps. Initially, strips were incubated in a solution containing 6 M urea, 2% (w/v) SDS, 30% glycerol (v/v), 50 mM Tris-HCl (pH 8.8), and 10 mg mL^{-1} (w/v) DTT, and then the strips were incubated using the same solution with 25 mg mL^{-1} (w/v) iodoacetamide (IAA) in place of the DTT. Each step lasted for 15 min and was performed at room temperature, under low agitation. To resolve the second dimension, the strips were applied to 12% polyacrylamide gels. The strips were sealed to the gels with an agarose solution (0.5% w/v) and run for approximately 1.5 h at 125 V. After the 1.5 h, the proteins were fixed using a destain solution containing 10% acetic acid (v/v) and 40% (v/v) ethanol. The labeled proteins were visualized by fluorescence using a Typhoon FLA-9500 imager (GE Healthcare, Uppsala, Sweden) with the 532 nm laser voltage set at 530 and a 570 BP 20 filter for Cy3, and the 635 nm laser voltage set at 700 and a 650 LPR filter for Cy5.

Images were aligned, normalized, and analyzed using Progenesis SameSpots version 4.5. Intergel matching of the Cy3-Hz pool was performed across all gels, to discern statistical significance between the groups, for comparative cross gel statistical analysis of all spots based on normalized spot volumes, enabling the detection of differentially oxidized spots between groups ($P < 0.05$). The gels were stained with Coomassie Blue G-250, and spots of interest were manually excised for protein identification.

Protein identification and pathway enrichment analysis

Treating in 15 min cycles, excised gel fragments were chemical washed and incubated with acetonitrile (ACN) and 50 mM ammonium bicarbonate (ABC) solution (40:60% v/v) at

room temperature. The process was repeated until all of the stains were completely removed, and the gel fragments were opaque. Afterward, the gel slice was shrunk in 100% (ACN). Trypsin working solution (10 µg mL^{-1} in 50 mM ABC buffer), was then added to the gel fragment and incubated overnight at 37°C, in the dark, for digestion of the proteins. The following day, the supernatant was transferred to new 1.5 mL Eppendorf tubes (Hamburg, Germany), and subjected to sonication after the addition of 60% ACN and 5% trifluoroacetic acid (TFA), using a volume sufficient to cover the gel fragment. This was done so the tryptic peptides would be further extracted from the gel. The supernatant was combined with the previous supernatant and the process was repeated two additional times. The pooled supernatants were placed in a SpeedVac (Labconco, Kansas City, MO) to be dried, and the tryptic peptides were resuspended in 25 µL of 0.1% formic acid (FA).

Peptide analysis was performed on a LTQ XL™ Linear Ion Trap mass spectrometer (ThermoFisher Scientific, Waltham, MA) coupled to a dual pump Dionex 3000 RSLCnano LC (ThermoFisher Scientific, Waltham, MA) with mobile phases composed of Solvent A (0.1% formic acid) and Solvent B (0.1% formic acid in ACN). From the resuspended peptides, 5 µL were loaded onto a C18 PepMap100 µ-precolumn (300 µm i.d. x 5 mm; ThermoFisher Scientific, Waltham, MA) at a flow rate of 40 µL/min with 3% Solvent B for 4 minutes. Elution of the peptides was at 400 nL/min from a 15 cm PicoFrit Reprosil-PUR analytical column (New Objective, Woburn, MA) with the following gradient: held in 3% B for 3 min, increased to 15% B over 7 min, increased to 28% B over 30 min, then increased to 99% B in 6 minutes and held for 1 min, followed by returning to 3% B over 1 min, and held for 22 min at 3% B to allow for column re-equilibration.

In a data-dependent mode, Spectra (m/z 400 to 2000) were acquired with the top 3 ions selected for Collision-induced dissociation (CID) fragmentation at 35% normalized collision energy and a dynamic exclusion of 30 s. Nano spray voltage was set at 1.0 kV with a heated capillary temperature of 250°C. Conversion of the raw data files (.raw) were done by MSConvert (Palo Alto, CA) to .mgf format. This process allowed for protein identification with an 'in house' Mascot database (SwissProt 51.6; Uniprot, Geneva, Switzerland). Mascot parameters were set as follows: 1 missed cleavage, variable methionine oxidation, and both peptide and fragment ion mass tolerance at 0.8 Da.

Statistical analysis

The processing method of corn, presence or absence of DDGS, and quality grade served as the main plot factors. For proteomic analysis, if the protein oxidative damage score was greater than 31, then the comparison was significant. Tenderness determination was presented as a 2x2x2 factorial design. Data were analyzed using PROC GLIMMIX program of SAS with LSMEANS statement. SEM was calculated using the method described in Levenson et al. (2000). Statistical significance was determined at $P < 0.05$.

Results and Discussion

Assessment of protein carbonylation was completed using specific derivatization of the proteins' carbonyl groups, 2D-DIGE protein separation, and MS/MS analysis, to identify proteins. Protein spots, on 2D-DIGE, were distinguished by Progenesis SameSpots and assigned a spot number differing in fluorescent intensity ($P<0.05$). The spot identification (ID), protein ID, abbreviation, name, score value, the experimental and theoretical pI, for each protein are shown in Table 1-4. Based on the results tables were constructed to highlight the differences found in the paired treatment comparisons.

The proteins found across the comparison were grouped into one of six specific classes: Structural and cytoplasmic proteins Troponin T, slow skeletal muscle (TNNT1), Troponin T, fast skeletal muscle (Tnnt3), Tropomyosin α-1 chain (TPM1), Tropomyosin β chain (TPM2), Cofilin-2 (CFL2), Desmin (DES), Myosin regulatory light chain 2, skeletal muscle isoform (MYLPF), Profilin-1 (PFN1), α-actinin-2 (ACTN2), α-actinin-3 (ACTN3). Oxidative stress proteins Carbonic anhydrase 3 (CA3), Thioredoxin (TXN), and Transitional endoplasmic reticulum ATPase (TERA). Heat shock proteins α-crystallin B chain (CRYAB), Heat shock protein 60 kDa, mitochondrial (HSPD1), Heat shock 70 kDa protein 1A (HSPA1A), Heat shock cognate 71 kDa protein (HSPA8). Apoptotic proteins Galectin-1 (LGALS1), Cytochrome b-c1 complex subunit 1, mitochondrial (UQCRC1), and Cytochrome c oxidase subunit 5A (COX5A). Glycolytic proteins β-enolase (ENO3), α-enolase (ENO1), Malate dehydrogenase, cytoplasmic (MDH1), Phosphoglycerate mutase (PGAM2), Glycogen phosphorylase, muscle form (PYGM), Phosphoglucomutase-1 (PGM1), Glucose-6-phosphate isomerase (GPI), Fructose-1,6-bisphosphatase isoenzyme 2 (FBP2), Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic (GPD1), β-enolase OS=Bos Taurus (Q3ZC09), Phosphoglycerate kinase 1 (PGK1),

L-lactate dehydrogenase A chain (LDHA), and Glyceraldehyde-3-phosphate dehydrogenase (G3P_BOVIN). Oxidative metabolism ATP synthase subunit alpha, mitochondrial (ATP5F1A), Aconitate hydratase, mitochondrial (ACO2), Creatine kinase M-type (CKM), Cytochrome b-c1 complex subunit 1, mitochondrial (UQCRC1), and Cytochrome c oxidase subunit 5A (COX5A). Nucleotide metabolism protein Adenylate kinase isoenzyme 1 (AK1).

When comparing Upper 2/3 Choice versus Select within the DRC without DDGS diet (Table 1), there was an increase of oxidative damage in the protein adenylate kinase (AK1) for Upper 2/3 Choice. This nucleotide protein is involved in maintaining homeostasis in the cell (Baldassini et al., 2015). Malheiros et al. (2019) found increased oxidative damage to AK1 in tender meat, compared to tough meat. These results agree as Upper 2/3 Choice was shown to be more objectively tender than Select in this study (Table 5). Upper 2/3 Choice steaks from cattle fed DRC with DDGS were found to have more oxidative damage than Select in some heat shock proteins (HSPA8 and HSPA1A). Heat shock proteins are crucial in their ability to stabilize the cell and prevent further damage from pathways like proteolysis and apoptosis (Escobedo et al., 2004; Beere, 2005; Picard et al., 2014). This is consistent with other reports that evaluated the relationship between heat shock proteins and tenderness. Malheiros et al. (2019) also found increased oxidative damage of HSPA8 and HSPA1A in tender meat. The protein HSPA1A has been negatively associated with tenderness (Bjarnadóttir et al., 2012; Carvalho et al., 2014). Conversely, Select steaks from cattle fed SFC without DDGS were found to have increased oxidative damage in HSPA8, compared to Upper 2/3 Choice (Table 3). Upper 2/3 Choice steaks from cattle fed SFC with DDGS had increase oxidative damage in multiple glycolytic enzymes like β-enolase, α-enolase, and phosphoglycerate kinase. The glycolytic enzymes are not only valuable to producing energy in low oxygen conditions but during slaughter when the lack of

oxygen shunts energy production to mostly glycolysis and the lactic acid pathway (Hamm, 1982; Guillemin et al., 2011; Bjarnadóttir et al., 2012; Malheiros et al., 2019). This is consistent with reports from Guillemin et al., (2011a) and Laville et al. (2009) who found a negative relationship between β-enolase and tenderness. In contrast, Malheiros et al. (2019) found increased oxidative damage of β-enolase in tough meat.

The structural protein α-actinin was another protein with increased oxidative damage in Upper 2/3 Choice steaks from cattle fed SFC with DDGS. As a part of the Z disc, α-actinin binds actin to anchor the thin filament to the Z- disc (Otey and Carpen, 2004), increased oxidative damage of α-actinin could compromise the structural integrity of the myofibril and perhaps enhance accessibility to proteolytic enzymes which could enhance tenderness. In total, these proteomic results support the notion that increased oxidative damage in high marbled beef is consistent with increased oxidative damage in tender meat (Malheiros et al., 2019).

Previous research has shown that diets containing DDGS can improve tenderness early postmortem, possibly by accelerating the release of calcium postmortem, thereby enhancing proteolysis (Chao et al., 2018). In the present study, tenderness differences were not consistent enough to be significantly different. The presence of DDGS in the diet of cattle fed DRC increased oxidative damage of many proteins (Table 2) in Upper 2/3 Choice-grade beef. Both Troponin T, slow skeletal muscle and Troponin T, fast skeletal muscle were found to have increased oxidative damage in Upper 2/3 Choice steaks from cattle DRC with DDGS. The degradation of troponin T, postmortem, has been recognized as an indicator of tenderness, as the damage likely impacts the structure of the myofibril (Huff-Lonergan et al., 2010; Zakrys-Waliwander et al., 2012). This is consistent with another proteomic report that associated intact troponin T with decreased objective tenderness (Polati et al., 2012).

The protein β-enolase had increased oxidative damage in Upper 2/3 Choice steaks from cattle DRC with DDGS (Table 2), in contrast to Malheiros et al. (2019). However, its detection in meat from cattle fed with DDGS is consistent with reports that indicate a negative relationship between β-enolase and tenderness (Laville et al., 2009; Guillemin et al., 2011). Malate dehydrogenase was another glycolytic enzyme with increased oxidative damage in Upper 2/3 Choice steaks from cattle DRC with DDGS. Jia et al. (2009) found the intact form of the enzyme was positively related to tenderness.

A comparison was made with Select steaks from cattle fed DRC without DDGS versus from cattle fed DRC with DDGS. Select steaks from cattle DRC without DDGS had greater oxidative damage of apoptotic proteins galectin and cytochrome-c oxidase compared to the DRC with DDGS treatment. Ouali et al. (2006) hypothesized that apoptotic cell death in muscle cells can partially explain variation in meat tenderness. The inference is that the production of apoptotic pathways and cell death could improve tenderness. Therefore, increase oxidative damage of proteins associated with apoptosis may impact meat tenderness. The further degradation of galectin and cytochrome-c oxidase, which can indicate apoptosis, could prevent further cellular damage, and potentially increase tenderness. Previous research suggested that a lower expression of galectin-1 would be associated with greater tenderness (Zapata et al., 2009; Bjarnadóttir et al., 2012). Select steaks from DRC with DDGS were found to have increased oxidative damage in the structural protein desmin. Degradation of desmin could be an indicator of calpain activity and overall beef tenderness caused by the proteolytic process (Huff-Lonergan et al., 2010). Increased oxidative damage of the protein suggests increased tenderness, compared to the Select steaks from cattle fed DRC without DDGS. Heat shock protein 60 kDa was also found to have increased oxidative damage in the Select steaks from cattle fed DRC without

DDGS, which implies a decreased ability to protect against cellular damage as increased activity of heat shock proteins are generally related to increased toughness in meat (Kim et al., 2008; Zapata et al., 2009; Guillemin et al., 2011; Baldassini et al., 2015).

In contrast to DRC, Upper 2/3 Choice steaks from cattle fed SFC without DDGS had increased oxidative damage in multiple structural proteins, compared to Upper 2/3 Choice steaks from cattle fed SFC with DDGS (Table 4). Myosin regulatory light chain and two isoforms of tropomyosin had increased oxidative damage in Upper 2/3 Choice steaks from cattle fed SFC without DDGS. With myosin being one of the main proteins involved in the actomyosin crossbridge, and thus the degree of contraction, oxidative damage could impact the degree of tenderness in the meat. Tropomyosin helps regulate the attachment of myosin to actin (Herrmann, 1989). The increased damage to tropomyosin observed in Upper 2/3 Choice steaks from cattle fed SFC without DDGS might indicate potential tenderness in the product. There was also increased oxidative damage in carbonic anhydrase in Upper 2/3 Choice steaks from cattle fed SFC without DDGS. Carbonic anhydrase is an enzyme that catalyzes CO_2 into bicarbonate, and therefore, is related to detoxification of the cell (Ouali et al., 2013). Carbonic anhydrase has been suggested as a potential marker of beef tenderness (Zapata et al., 2009; D'Alessandro and Zolla, 2013). Increased oxidative damage of carbonic anhydrase was also found in tough meat (Malheiros et al., 2019). Cytochrome b-c1 complex subunit 1 was found to have increased oxidative damage in Upper 2/3 Choice steaks from cattle fed SFC without DDGS. While cytochrome c is a key protein involved in the electron transport chain of oxidative metabolism, it also can signal for apoptotic pathways (Li et al., 2006; Hüttemann et al., 2011). By having increased oxidative damage of the protein, then one would expect a decrease in energy production and the signaling for apoptosis. Therefore, in this study, the differences that are

present in DRC with or without DDGS seem to contradict the impacts of SFC with or without DDGS, indicating the need for further investigation.

Conclusion

Upper 2/3 Choice steaks were found to generally have increased oxidative damage in proteins, compared to Select quality grade, similar to those found in tender meat. This gives credence to the hypothesis that there is a relationship between marbling and tenderness. Samples related to the dietary treatment of DRC followed the hypothesis that feeding distillers grains can improve tenderness by creating conditions where oxidative stress can occur, while the SFC-related treatments do not support such a relationship.

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Table 1. Characteristics of carbonylated protein spots derived from Upper 2/3 Choice- or Select-grade cattle fed dry rolled corn with or without distillers grains.

Spot ID	Protein ID	ABV	Protein	Score	PI/Mw experimental	PI/Mw theoretical	Spot ID	Protein ID	ABV	Protein	Score	PI/Mw experimental	PI/Mw theoretical
Increased oxidative damage in DRC without DDGS Choice							Increased oxidative damage in DRC without DDGS Select						
289	P00570	AK1	Adenylate kinase	1873	9.52/ 24,579	8.40/ 21,664							
Increased oxidative damage in DRC with DDGS Choice							Increased oxidative damage in DRC with DDGS Select						
110	P19120	HSPA8	Heat shock cognate 71 kDa protein	139	4.82/ 64,857	5.37/ 71,241	293	P02510	CRYAB	Alpha-crystallin B chain	2675	8.05/ 24,026	6.76/ 20,037
	Q27975	HSPA1A	Heat shock 70 kDa protein 1A	100		5.67/ 70,259	133	P19483	ATP5F1A	ATP synthase subunit alpha, mitochondrial	668	9.29/ 59,143	9.21/ 59,720

Comparisons are made across the table.

Table 2. Characteristic of carbonylated protein spots derived from cattle fed dry rolled corn with or without distillers grains at the same quality grade.

Spot ID	Protein ID	ABV	Protein	Score	PI/Mw experimental	PI/Mw theoretical	Spot ID	Protein ID	ABV	Protein	Score	PI/Mw experimental	PI/Mw theoretical
Increased oxidative damage in DRC without DDGS Choice							Increased oxidative damage in DRC with DDGS Choice						
293	P02510	CRYAB	Alpha-crystallin B chain	2675	8.05/ 24,026	6.76/ 20,037		Q8MKH6	TNNT1	Troponin T, slow skeletal muscle	903		5.71/ 31,284
							200	Q8MKI3	Tnnt3	Troponin T, fast skeletal muscle	280	6.67/ 38,640	5.99/ 32,126
								Q3T145	MDH1	Malate dehydrogenase, cytoplasmic	468		6.16/ 36,438
							156	Q3ZC09	ENO3	Beta-enolase	506	6.93/ 52,000	7.60 /47,096
								Q9XSJ4	ENO1	Alpha-enolase	473		6.37 /47,326
							71	P79334	PYGM	Glycogen phosphorylase, muscle form	248	9.05/ 102,222	6.65 / 97,293
Increased oxidative damage in DRC without DDGS Select							Increased oxidative damage in DRC with DDGS Select						
360	O97680	TXN	Thioredoxin	358	5.02/ 16,030	4.97/ 11,813	306	Q148F1	CFL2	Cofilin-2	571	6.69/ 22,842	7.66/ 18,737
358	P11116	LGALS1	Galectin-1	802	5.41/ 16,446	5.32/ 14,744	126	P31081	HSPD1	60 kDa heat shock protein, mitochondrial	686	5.28/ 61,048	5.60/ 61,108
	P00426	COX5A	Cytochrome c oxidase subunit 5A	564		6.42/ 16,735	125	O62654	DES	Desmin	3187	5.14/ 61,048	5.21/ 53,532
104	Q08DP0	PGM1	Phosphoglucomutase-1	1362	8.82/ 68,190	6.36/ 61,589							
	Q3ZBD7	GPI	Glucose-6-phosphate isomerase	730		7.33/ 62,855							

Comparisons are made across the table.

Table 3. Characteristics of carbonylated protein spots derived from Upper 2/3 Choice- or Select-grade cattle fed steam flaked corn with or without distillers grains.

Spot ID	Protein ID	ABV	Protein	Score	PI/Mw experimental	PI/Mw theoretical	Spot ID	Protein ID	ABV	Protein	Score	PI/Mw experimental	PI/Mw theoretical
Increased oxidative damage in SFC without DDGS Choice							Increased oxidative damage in SFC without DDGS Select						
							150	P19120	HSPA8	Heat shock cognate 71 kDa protein	4116	3.45/ 65,684	5.37/ 71,241
Increased oxidative damage in SFC with DDGS Choice							Increased oxidative damage in SFC with DDGS Select						
282	P19858	LDHA	L-lactate dehydrogenase A chain	2382	9.33/ 36,495	8.12/ 36,598	390	Q0P571	MYLPF	Myosin regulatory light chain 2, skeletal muscle isoform	3298	4.87/ 14,602	4.88/ 19,010
	Q9XSC6	CKM	Creatine kinase M-type	2068		6.63 /42,989		Q5EA88	GPD1	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	1463		6.42/ 37,648
249					8.01/ 41,484		245					7.88/ 42,000	
	Q5EA88	GPD1	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	1475		6.42/ 37,648		Q2KJJ9	FBP2	Fructose-1,6-bisphosphatase isozyme 2	449		7.52/ 36,767
	ENOB_BOVIN	Q3ZC09	Beta-enolase OS=Bos taurus	9527		7.60 /47,096							
	Q9XSJ4	ENO1	Alpha-enolase	4021		6.37 /47,326							
218					9.17/ 48,296								
	Q3T0P6	PGK1	Phosphoglycerate kinase 1	1453		8.48/ 44,538							
181	P10096	G3P_BOVIN	Glyceraldehyde-3-phosphate dehydrogenase	379	9.60/ 59,368	8.51/ 35,868							
88	Q3ZBT1	TERA	Transitional endoplasmic reticulum ATPase	1603	4.98/ 85,600	5.13 / 89,330							

Q0III9	ACTN3	Alpha-actinin-3	1514	5.31/ 103,151
Q3ZC55	ACTN2	Alpha-actinin-2	1300	5.31/ 103,779

Comparisons are made across the table.

Table 4. Characteristic of carbonylated protein spots derived from cattle fed steam flaked corn with or without distillers grains at the same quality grade.

Spot ID	Protein ID	ABV	Protein	Score	PI/Mw experimental	PI/Mw theoretical	Spot ID	Protein ID	ABV	Protein	Score	PI/Mw experimental	PI/Mw theoretical
Increased oxidative damage in SFC without DDGS Choice							Increased oxidative damage in SFC with DDGS Choice						
455	Q0P571	MYLPF	Myosin regulatory light chain 2, skeletal muscle isoform	94	4.87/ 14,602	4.88/ 19,010	456	P02584	PFN1	Profilin-1	238	9.25/ 14,472	8.46/ 15,057
390	Q0P571	MYLPF	Myosin regulatory light chain 2, skeletal muscle isoform	3298	4.87/ 14,602	4.88/ 19,010							
335	Q3SZX4	CA3	Carbonic anhydrase 3	3126	9.46/ 28,237	7.71/ 29,370							
239	Q5KR49	TPM1	Tropomyosin alpha-1 chain	466	3.57/ 43,111	4.69/ 32,695							
	Q5KR48	TPM2	Tropomyosin beta chain	294		4.66/ 32,837							
179	P31800	UQCRC1	Cytochrome b-c1 complex subunit 1, mitochondrial	951	5.98/ 59,368	5.94/ 52.736							
Increased oxidative damage in DRC without DDGS Select							Increased oxidative damage in DRC with DDGS Select						
100	P79334	PYGM	Glycogen phosphorylase, muscle form	1870	8.73/ 76,945	6.65/ 97,293							
109	P20004	ACO2	Aconitate hydratase, mitochondrial	518		7.87/ 85,359							

Comparisons are made across the table.

Table 5. Warner Bratzler Shear Force of strip loin steaks (*L. lumborum*) from steers fed either with dry rolled corn with dried distillers grains with solubles, dry rolled corn without dried distillers grains with solubles, steam flaked corn with dried distillers grains with solubles, or steam flaked corn without dried distillers grains with solubles at Upper 2/3 Choice or Select quality grade (n=36).

Category					
Grain	**DDGS**	**Quality Grade**	**WBSF (kgf)**	***SEM***	**P-value**
DRC			3.46[b]	0.2	0.02
SFC			4.04[a]		
	DDGS		3.68	0.18	0.43
	No DDGS		3.84		
		Choice	3.39[b]	0.23	<0.01
		Select	4.11[a]		

[a,b] Means in the same column within a category without common superscripts differ ($P < 0.05$).
WBSF: Warner Bratzler Shear Force.
DRC: Dry Rolled Corn.
SFC: Steam Flaked Corn.
DDGS: Dried Distillers Grains with Soluble.
No DDGS: without Dried Distillers Grains with Soluble.

RECOMMENDATIONS FOR FUTURE RESEARCH

The results indicated that Upper 2/3 Choice grade steaks were found to be more tender than Select grade steaks. I would recommend further separating the treatments to better elucidate potential differences related to tenderness, marbling, and oxidative stress. This can include comparing Prime versus Select samples or having greater extremes in the energy of the diet by feeding cattle corn with distillers grains (high energy) versus a forage-based diet (low energy). This study utilized two types of high-fat diets (corn vs corn with DDGS). Although there were numeric distinctions to suggest differences in oxidative stress, via isoprostanes, caused by the changes in diet, lack of difference in factors like objective tenderness may suggest the differences in diet were not significant enough to test this study's hypothesis. The initial design of this project was to obtain 96 carcasses. In actuality, only 36 carcasses met the parameters for this study, and thus the study may not have enough power to identify significant differences. Proteomic results supported the relationship between marbling and tenderness. Further analysis between greater extremes in marbling and factors impacting oxidative stress, and selecting based on quality grade, might better reveal the protein biomarkers that relate marbling and tenderness, and if it relates to oxidative stress. Making a diving deeper to better elucidate the potential impacts oxidative stress might have in the relationship between marbling and tenderness could be valuable in trying to produce consistently tender meat.

Additionally, genetics can be another factor that can be manipulated. Cattle could be genetically selected to create and evaluate extreme differences in response to oxidative stress and how that may impact marbling and postmortem tenderness.

To summarize, based on the findings, some suggestions for future research should include:

1. Conduct a feeding trial with cattle being fed corn with distillers grains versus cattle fed only a forage-based diet.
2. From the same contemporary group, obtain samples from cattle that graded Prime versus Select. This could also include utilizing differing energy diets and selecting for these differences in quality grade.
3. Ensure sufficient samples are collected, based on the power of the experiment, to give the study better potential to elucidate differences that exist.
4. Continuing to evaluate oxidative stress via isoprostanes and oxidative damage via proteomic analysis in tandem with tenderness-related measurements could be a valuable addition to the other additions described above.

Appendix I

Finishing diet composition (Study 1)

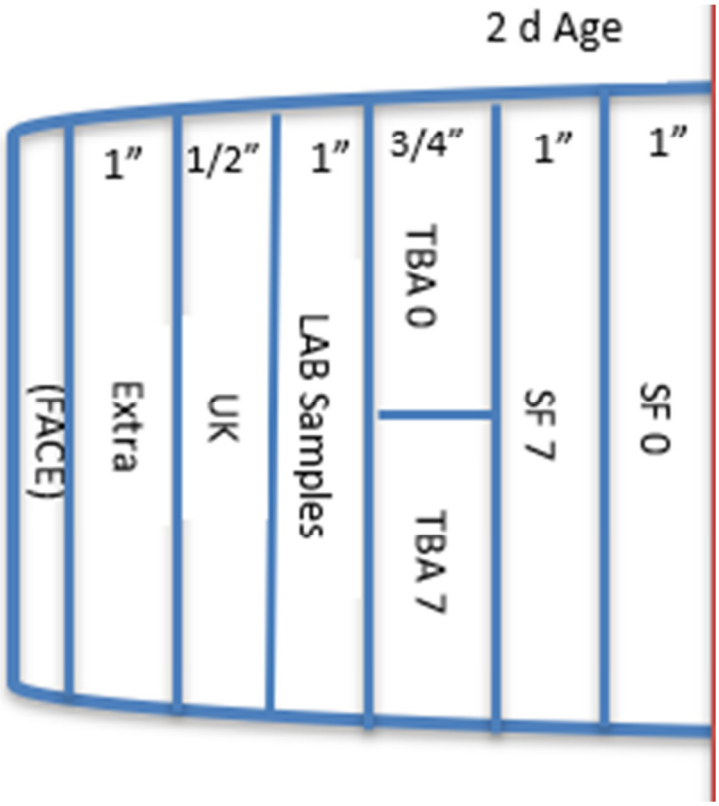
Diet composition (DM basis)

Table 1. Diet Composition (DM Basis) for beef cattle fed either steam flaked corn or dry rolled corn based diets with high protein dried distillers grains

	CON		DDGS	
	DRC	SFC	DRC	SFC
Dry Rolled Corn	87.0	-	57.0	-
Steam Flaked Corn	-	87.0	-	57.0
DDGS	-	-	30.0	30.0
High Protein DDGS	-	-	-	-
Sorghum Silage	8.0	8.0	8.0	8.0
Supplement	5.0	5.0	5.0	5.0
Fine Ground Corn	1.393	1.393	2.793	2.793
Limestone	1.690	1.690	1.690	1.690
Tallow	0.125	0.125	0.125	0.125
Urea	1.400	1.400	-	-
Salt	0.300	0.300	0.300	0.300
Beef Trace Mineral	0.050	0.050	0.050	0.050
Vitamin A-D-E	0.015	0.015	0.015	0.015
Rumensin-90	0.0165	0.0165	0.0165	0.0165
Tylan-40	0.0110	0.0110	0.0110	0.0110

APPENDIX II

Fabrication map (followed for all aging periods)



Appendix III

Objective color (L*, a*, b*) calibration instructions and helpful tips

Minolta Calibration Procedures

1. Before Calibration:

Calibrate Minolta in the same temperature conditions as the measurements being taken.

- Place the Minolta in the environment where samples will be measured about 5 or 10 minutes before calibrating so it can become equilibrated with the temperature.

Calibrate with the same materials as you will be taking measurements.

- If the measurements will not be taken directly on the meat surface, you must calibrate the Minolta with the same material it will be measuring through. For example, if you want to take readings from samples that are wrapped in overwrap, you must put some overwrap around the measuring head "eye" while calibrating using the white tile.

2. Turn the power to the measuring head ON.

3. Turn the power to the data processor ON while holding down the [DELETE/UNDO] key at

the same time.

- Release the [DELETE/UNDO] key when you hear a BEEP. (This deletes any previous data that might still be stored in the data processor)

4. When the screen turns on, the question "Initial set ok?" appears, press the [Measure Enter] key.

5. Once you get to the measurement screen, press the [Index Set] key.

- Use the arrows and the [Measure Enter] key to adjust all the following settings:
  - Printer: On
  -Color space: Off
  -Protect: On
  -Auto Average: However, many readings wanted per sample (1-30)
  -Illuminant: D65
  -Backlight: Off
  -Buzzer: On
  -Disp. Limit

- Press the [Esc] key to return to the measurement screen.

6. Press the [Calibrate] key while in the measurement screen.

7. Enter in the numbers listed on the calibrating white tile for the D65 setting using the following:

[< >] keys and the numeric pad
- (The [< >] keys move the cursor)
-D65 settings:
Y: 93.13 x: 0.3164 y: 0.3330

8. Set up the measuring head so that it is resting on the LCD screen and the “eye” is facing up.

- Place the white calibration tile on the measuring head, near the middle of the tile.

9. Press either the measurement button on the measuring head OR the [Measure Enter] key on the data processor after making sure the ready lamp is ON.

- Make sure the white tile is completely on the measuring head “eye”.
- The Calibration is complete after the lamp flashes 3 times and the screen returns to the measurement screen.
- Do not move the measuring head during calibration.

10. Press the [Color Space] key until the L*, a*, b* screen shows up.

11. Calibration is finished, and the Minolta is ready

- To save battery life, turn both the measurer and data collector off after calibration is finished until you need it for measuring. The calibration and settings will not be erased.

- When turning back on for measurements, ONLY turn on the power buttons. DO NOT hold down the [DELETE/UNDO] key at the same time. This will delete the calibration and settings and all of the steps will have to be repeated.

Cleaning

- Wipe machine down with a soft, clean dry cloth. Never use solvents such as thinner or benzene.

- If the white calibration tile becomes dirty, wipe it gently with a soft, clean dry cloth. If dirt is difficult to remove, wipe it with lens cleaner and cloth, then dry.

Appendix IV

Visual guide for percent surface discoloration

0%

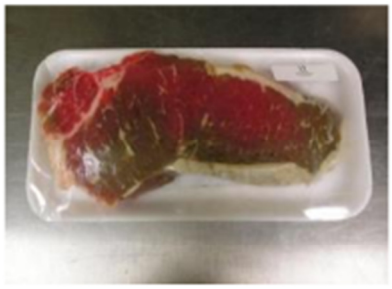
5%

10%

20%

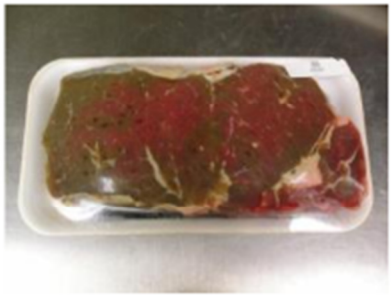
30%

40%



50%

60%



70%

80%

90%

100%

Appendix V

Thiobarbituric Acid Reactive Substances Assay

(Buege and Aust, 1978), Modified by Ahn et al., (1998)

TEP solution (1, 1, 3, 3-Tetraethoxypropane) (Make new weekly)

Stock Solution: Dilute 99 µl TEP (97%) bring volume to 100 mL ddH2O

Working Solution: Dilute stock solution to 1:3 (TEP Solution: ddH2O) (1x10-3M)

TBA/TCA (2-Thiobarbituric Acid/Trichloroacetic Acid) Stock Solution: 1L

15% TCA (w/v) and 20 mM TBA (MW 144.5) reagent in ddH2O

Dissolve 2.88 b TBA in warm ddH2O first, then add TCA (150g) and ddH2O to 1L

BHA (Butylated HydroxyAnisole) Stock Solution:

Make 10% stock solution by dissolving in 90% ethanol

10g BHA dissolved in 90mL ethanol (90%) + 5mL ddH2O

Standards: In Duplicate

Blank	1 mL ddH_2O	Moles of TEP
Standard 5	100 µL working TEP + 1.90 mL ddH_2O	(5×10^{-5} M)
Standard 4	1 mL Std. 5 + 1 mL ddH_2O	(2.5×10^{-5} M)
Standard 3	1 mL Std. 4 + 1 mL ddH_2O	(1.25×10^{-5} M)
Standard 2	1 mL Std. 3 + 1 mL ddH_2O	(0.625×10^{-5} M)
Standard 1	1 mL Std. 2 + 1 mL ddH_2O	(0.3125×10^{-5} M)

Remove 1 mL of Standard 1 and discard it, leaving 1 mL behind.

TBA Procedure:

1. Mix all reagents and standards before beginning.

2. Transfer 5g powdered sample into a 50mL conical tube; add 14 mL of ddH_2O and 1.0 mL of BHA.

3. Homogenize for 15 sec with a polytron.

4. Centrifuge for 2000xg for 5 min.

5. Transfer 1mL of homogenate or standard to 15mL conical tube.

6. Add 2 mL of TBA/TCA solution, vortex.
7. Incubate in a 70°C water bath for 30 min. to develop color.
8. Cool samples in a cold-water bath for 10 min.
9. Centrifuge tubes at 2000 x g for 15 min.
10. Transfer duplicate aliquots of 200 μL from each tube into wells on a 96-well plate.
11. Read absorbance at 540 nm.

Calculations: mg of malonaldehyde/kg of tissue

K (extraction) = (S/A) x MW x (106/E) x 100

Where:

S= Standard concentration (1 x 10-8 moles 1, 1, 3,3-tetraethoxypropane)/5ml

A= Absorbance of standard

MW= MW of malonaldehyde

E= sample equivalent

P= percent recovery

Final Calculation: .012 x concentration x 72.063 x 10-6 = mg of Malonaldehyde/kg tissue

Reagents (Sigma): TBA –T5500; TCA –T9159; TEP –T9889; BHA –B1253

Appendix VI

Fat extraction with Soxhlet method

WARNING: ETHER IS EXTREMELY FLAMMABLE AND PRODUCES EXPLOSIVE PEROXIDES. NEVER BRING A RADIO OR ANY OTHER POTENTIALLY SPARK-PRODUCING ITEM INTO THE FAT EXTRACTION ROOM.

1. Check ground glass connections. They should be wiped clean with a dry paper towel and given a thin coating of stopcock grease.

2. Each boiling flask must contain boiling stones. This helps prevent the violent boiling of the solvent which could be dangerous.

3. Load samples into soxhlet tubes, arrange them so that no samples are above the level of the top bend in the narrower tubing on the outside of the soxhlet. (The soxhlet will only fill with the solvent up to this point before cycling back down into the boiling flask.) In general, the large soxhlets will hold about 20 two-gram samples and the small soxhlets from 4-6.

4. Fill the large (500ml) boiling flasks with 400ml of solvent and the small (125ml) flasks with 100ml of solvent. Do this under the fume hood.

5. Fit the soxhlet onto the boiling flask. Very carefully, bring the assembly into the extraction room and fit it onto the condenser. Make sure all ground glass connections are snug and each boiling flask is resting on the heating element. The ceramic fiber sheet should be covering the bare metal surfaces on the burners completely.

6. Turn on the water supply to the condensers (usually a quarter turn). Check later to make sure condensers are cool enough –if not, increase water flow.

7. Turn heating element control dials between three and four. Each burner has a dial. Never turn the burner beyond five. Ether has a very low boiling point and violent boiling is dangerous. Double-check fittings, boiling stones, etc.

8. Fat extraction will take from 24 to 72 hours depending on the sample. (Beef– 48 hours, Bacon– 72 hours). Check extractions twice daily to see that everything is alright while they are running.

9. When done, turn off the burners and let the solvent cool completely before removing samples.

10. After it has cooled down, slowly uncouple the flask and soxhlet tube from the condenser. Cover the top of the soxhlet with one palm to reduce ether vapors while transporting it to the fume hood. Air dry samples in the fume hood for two hours to get rid of the remaining ether in the samples. Pour ether back slowly into an approved container for reuse or discarding. Do not leave ether out of the hood or the flammable cabinet.

11. Place samples in drying oven (105°C) for about four hours or overnight before weighing back.

Calculation: ([(Original weight including filter paper and paper clip –Fat extracted sample weight) / Sample weight] * 100) -% Moisture = % Fat

Appendix VII

Fatty acid determination

1. Weigh out 1 g of pulverized muscle tissue. If extracting subcutaneous fat, weigh out 0.1 g of pulverized subcutaneous fat into a centrifuge tube.

2. Add 5 mL of 2:1 chloroform: methanol (v/v) for muscle tissue or 3 mL for subcutaneous fat.

3. Vortex for 5 s and let stand for 1 h at room temperature.
4. Filter homogenate through Whatman #2 filter paper into 13 x 150 mm screw cap tube bringing the final volume with chloroform: methanol to 10 mL for muscle lipid and 5 mL for subcutaneous fat extract. If stopping at this point, purge the test tube with nitrogen, cap tube, and store at -80°C. Add 2 mL of a 0.74% KCl solution for muscle lipid extract or 1 mL for subcutaneous fat tissue extract and vortex for 5 s. If stopping at this point, purge test tube with nitrogen, cap tube, and store at 0°C for no more than 24 h.

5. Centrifuge samples at 1000 x g for 5 min. Following centrifugation, aspirate off the aqueous phase (top layer). If stopping at this point, purge the test tube with nitrogen, cap tube, and store at -80°C.

6. Evaporate to dryness under nitrogen at 60°C.

7. Add 1 mL of a 0.5 M NaOH in methanol. Vortex for 5 sec. Heat for 10min at 100°C.

8. Add 1 mL of boron trifluoride in 14% methanol. Vortex for 5 sec. Heat for 5 min at 100°C.

9. Add 2 mL of a saturated salt solution and 2mL of hexane. Vortex for 5 sec.

10. Centrifuge samples at 1,000 x g for 5 min. Following centrifugation, remove the hexane layer (top layer) **making sure not to disrupt the aqueous phase** (lower layer) and place in GC vial. Purge GC vial with nitrogen, cap and crimp cap, and store at -80°C until the sample is ready to be read on the GC.

GC Settings

Column- Chrompack CP-Sil 88 (0.25 mm x 100 m)

Injector Temp- 270°C Detector Temp- 300°C

Head Pressure- 40 psi

Flow Rate- 1.0 mL/min

Temperature Program- Start at 140°C and hold for 10 min. Following 10 min, raise temperature 2°C/min until temperature reaches 220°C. At 220°C, hold for 20 min.

Appendix VIII

Isoprostanes: Preparation of Standards

Standard Tubes	**8-iso-PGF2α Standard (µL)**	**Sample Diluent (µL)**	**8-iso-PGF2α Standard (pg/mL)**
7	1	4999	40,000
6	250	750	10,000
5	250	750	2,500
4	250	750	625
3	250	750	156.25
2	250	750	39.0625
1	250	750	9.7656
0	0	200	0

Appendix IX

Impact of Green Grass Diet on Fat Composition of Beef Steaks

Cattle and dietary treatments

A total of 240 crossbred steers (340 ± 23.6 kg) were blocked into three body weights (BW) and randomly assigned to one of 24 pens, (n=10/pen), then fed (University of Nebraska feedlot at Mead, NE) for 203 days. Since there was an uneven distribution of initial BW, replication 1 (40 hd) was assigned to block 1, replications 2, 3, and 4 (120 hd) were assigned to block 2, and replications 5 and 6 (80 hd) were assigned to block 3. Four dietary treatments were divided among the pens (6 pens/treatment). Cattle were fed Green Grass at 0, 10, 20, or 30 % of diet DM, displacing dry-rolled corn (DRC) in the diet (Appendix VIV). The Green Grass diet is a product that's composed of several plants and plant seeds that are high in omega 3 fatty acids, specifically α-linolenic acid (ALA). Inclusion of distillers grains was calculated on a dry matter basis. All diets also consisted of 15 % WDGS, 20 % corn silage, and 6 % liquid supplement on DM basis along with 33 mg/kg Rumensin (Elanco Animal Health, Greenfield, IN) and 9.7 mg/ kg Tylan® (Elanco Animal Health, Greenfield, IN) on a DM basis. Logistical issues resulted in a shortage of Green Grass products to feed at the end of the feeding period. On d 150 to 176, Green Grass 10, 20, and 30 diets were dropped to 7.5%, 15%, and 22.5% Green Grass inclusion, respectively. On d 177 to 187, Green Grass 10, 20, and 30 diets were dropped to 5%, 7.5%, and 15% Green Grass inclusion, respectively. On d 188 through the remainder of the trial, Green Grass 10 and 20 were switched to the control diet, while Green Grass 30 was dropped to 7.5 % Green Grass inclusion. On day 189 through the remainder of the trial, Green Grass 30 was switched to the control diet.

Sample collection

All steers were transported to J. F. O'Neil Packing Co. (Omaha, NE) and held 12 h prior to slaughter. Collection days were January 4th, 14th, and 17th following a 48 h chill, post-slaughter. Carcasses were tagged with a university number corresponding to the pen within which the cattle were fed. In total, hot carcass weight, liver abscess rare, ribeye area, marbling score, and 12th rib back fat was recorded, and yield grade was calculated (Appendix X). Steak samples were collected by cutting 3.81 cm steak at the 5th rib. Steak samples were transported on ice to the University of Nebraska meat lab for fatty acid analysis. Selected loins were subsequently vacuum packaged with laminated and numbered ID tags and transported to the Loeffel Meat Laboratory at the University of Nebraska-Lincoln.

Fabrication

Upon arrival at Loeffel Meat Laboratory, the 2 d aged beef steaks, cut at the 5th rib, were removed from the vacuum packaging and were trimmed of all muscles except the *longissimus thoracis* muscle. Steaks were vacuum packaged (MULTIVAC 500, Multivac, Inc., Kansas City, MO) in Prime Source Vacuum pouches (3 mil STD barrier, Prime Sources, St. Louis, MO) and kept frozen (-80 °C) until ready to powder for future analyses. All steaks subsequently used for laboratory analyses were diced into small portions before freezing in liquid nitrogen and powdering in a metal blender cup (Model 51BL32, Waring Commercial, Torrington, CT) from February 4-8, 2019 and kept frozen (-80°C) for following analyses.

Fatty acid analysis

Extraction of the total lipids were completed with the chloroform-methanol procedure described by Folch et al. (1957). See the previous section of fatty acid analysis for more details (pg. 62).

Proximate composition

See the previous section of proximate composition for more details (pg. 61).

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) as randomized block design. The pen was used as the experimental unit while kill block nested within BW block was included in the model as fixed effects.

Results and Discussion

As the inclusion of Green Grass diet increased, there were a linear decrease in some saturated fatty acids (C12:0, C15:0, C17:0), monounsaturated fatty acids (C14:1, C16:1, C17:1, C18:1), and in C20:3 ω6, and total ω6 (omega-6) in mg/100 g of lean tissue ($P \leq 0.02$; Appendix XI). Inversely, as the inclusion of the Green Grass diet increased, there was a linear increase for concentrations of C18:1T, C18:2T, C18:2, C18ω3, C20:5ω3, and C22:5 in mg/100 g of lean tissue ($P \leq 0.01$). There was an increase of 304 % for C18:3ω3 and 302 % for total ω3 (omega-3) fatty acids, respectively, comparing 30 Green Grass to control. Polyunsaturated fatty acids (PUFA), and trans-unsaturated fatty acid (Trans) concentrations also linearly increased ($P \leq 0.01$) in mg/100 g of lean tissue, as Green Grass inclusion, increased in the diet. This is consistent with reports that found increased fatty acids like C18:1T and C18:3ω3 from diets that

had higher omega fatty acids present like the supplementation of fish oil and feeding grass diets compared to concentrate (Scollan et al., 2001; Nuernberg et al., 2005; Warren et al., 2008; Najafi et al., 2012). Concentrations of the total ω6, and the ratio of ω6:ω3 linearly decreased ($P \leq 0.01$) as Green Grass inclusion increased in the diet which agrees with Scollan et al. (2001). For total fat % from the proximate analysis, 10 and 20 Green grass had greater percent fat within lean steak samples (11.41 % and 11.51 %) compared to 0 and 30 Green Grass (10.96 % and 10.43 %; $P= 0.04$; Appendix XII). The percent of moisture, from the proximate analysis, in the steak samples, was greater ($P= 0.02$) for 0 and 30 Green Grass (68.13% and 68.75 %, respectively) compared to 10 and 20 Green Grass (67.76 % and 67.71 %, respectively). The increase in the concentration of PUFA, total ω3, and C18:3ω3 support the hypothesis that increasing the amount of dietary omega-3 fatty acids from feeding Green Grass positively influences fatty acids deposited in the meat, with dramatic increases in ω3 (omega-3) fatty acids. Overall, increasing the Green Grass inclusion in the diet resulted in higher PUFA, total ω3, and C18:3ω3.

Appendix X

Table 1. Diet Composition (DM basis) for finishing steers fed 4 inclusions of Green Grass product

	Treatment[1] % Inclusion			
Ingredient	0	10	20	30
Dry-rolled corn	59	49	39	29
Wet Distillers Grains plus Solubles	15	15	15	15
Green Grass[1]	0	10	20	30
Corn Silage	20	20	20	20
Supplement[2]	6	6	6	6
CP, %	46.0	7.0	7.0	7.0
Ca, %	5.7	5.2	5.2	5.2
P, %	0.05	0.09	0.09	0.09
Salt, %	3.1	3.1	3.1	3.1
K, %	2.6	3.2	3.2	3.2
Vitamin A, IU	10,820	10,820	10,820	10,820
Dietary Nutrient Composition[3], %				
DM	54.3	54.3	54.3	54.3
CP, % DM	14.0	14.0	16.3	18.7
ADF, % DM	10.3	12.5	14.7	16.9
Ca, % DM	0.40	0.47	0.56	0.65
P, % DM	0.45	0.51	0.58	0.64
Mg, % DM	0.14	0.17	0.20	0.23
K, % DM	0.81	0.91	0.96	1.02
Na, % DM	0.03	0.04	0.06	0.07
S, % DM	0.17	0.21	0.26	0.30
Fe, PPM	65.8	157.2	248.5	339.9
Zinc, PPM	26.8	32.0	37.8	43.6
Cu, PPM	2.9	6.1	9.2	12.4
Manganese, PPM	15.2	22.6	30.1	37.5
Dietary Fatty Acid Profile[3], % DM				
C12:0	0.00	0.00	0.00	0.00
C14:0	0.00	0.00	0.00	0.00
C16:0	0.62	0.66	0.70	0.73
C16:1	0.00	0.01	0.01	0.02
C18:0	0.08	0.10	0.13	0.16
C18:1	1.05	1.18	1.31	1.44
C18:2	2.33	2.28	2.22	2.17
C18:3	0.11	0.31	0.52	0.73
C20:0	0.01	0.02	0.02	0.02
C20:1	0.01	0.02	0.02	0.02
C20:5	0.00	0.00	0.01	0.01
C22:0	0.00	0.00	0.01	0.01
C22:6	0.00	0.00	0.01	0.01
C24:0	0.01	0.01	0.01	0.01
Other	0.18	0.22	0.27	0.32
Total Fatty Acids	4.40	4.82	5.23	5.65

[1]Differences in dietary treatment were due to Green Grass (Sunseo Omega 3, Chungcheongbuk-do, Korea) inclusion (0,10, 20, 30 % of diet DM)
[2]Supplements were formulated to provide 33 mg/kg Monensin (Rumensin-90®; Elanco Animal Health, DM Basis), 9.7 mg/kg Tylosin (Tylan®; Elanco Animal Health, DM Basis), ≥10,820 IU Vitamin A, supplement in diet 0 provided protein in the form of urea
[3]Nutrient Compositions and fatty acid profiles were formulated from ingredient samples

Appendix XI

Table 2. Effect of increasing inclusion of Green Grass on cattle performance and carcass characteristics

	Treatment[1]					Contrast		
Item,	0	10	20	30	*SEM*	L[2]	Q[3]	C[4]
Carcass adjusted Performance								
Initial BW, kg	390	390	390	390	0.50	0.91	0.20	0.09
Final BW, kg	683	674	684	673	4.63	0.16	0.98	0.11
DMI, kg/d	11.9[a]	12.2[ab]	12.3[b]	12.2[b]	0.13	0.04	0.16	0.78
ADG, kg	1.75	1.70	1.70	1.70	0.022	0.14	0.89	0.13
G:F	0.147[a]	0.139[b]	0.142[b]	0.137[b]	0.0011	< 0.01	0.07	0.02
Carcass characteristics								
HCW, kg	430	425	431	424	2.90	0.16	0.96	0.11
LM area, cm^2	80.7	78.1	80.0	80.0	0.903	0.85	0.16	0.21
Fat depth, cm	1.85[ab]	1.78[a]	1.98[b]	1.78[a]	0.064	0.88	0.33	0.02
Calculated YG[5]	4.45	4.44	4.62	4.30	0.091	0.43	0.12	0.12
Liver abscess, %	8.97	8.97	12.74	10.89	4.075	0.58	0.83	0.60
Marbling[6]	470[a]	470[a]	480[a]	430[b]	9.75	0.05	0.03	0.35

[1] Differences in dietary treatments were due to Green Grass inclusion (0, 10, 20, or 30 % of diet DM).
[2] L= P-value for the linear response to Green Grass inclusion
[3] Q= P-value for the quadratic response to Green Grass inclusion
[4] C= P-value for the cubic response to Green Grass inclusion
[5] Calc. YG (calculated yield grade), Calculated as 2.5 + (2.5 x 12^{th} rib fat, cm) + (0.2 x 2.5 (KPH, %)) + (.0038 x HCW, lbs.) – (0.32 x REA, cm^2)
[6] 400 = $Small^0$, 500 = $Modest^0$
[ab] Means in a row with different superscripts differ ($P < 0.05$).

Appendix XII

Table 3. Fatty acid profile of steak samples collected at the 5th rib from steers fed increasing inclusion of Green Grass product in mg/100g of lean tissue (DM basis)

	Treatment[1]					Contrast		
Fatty acid	0	10	20	30	*SEM*	L	Q	C
C10:0	9.30	7.77	5.93	5.66	1.222	0.03	0.62	0.74
C12:0	5.22[a]	3.89[ab]	2.87[b]	1.80[b]	0.786	< 0.01	0.87	0.93
C14:0	342	361	343	328	11.8	0.28	0.16	0.46
C14:1	103[a]	106[a]	89.8[b]	89.1[b]	4.15	< 0.01	0.64	0.08
C15:0	43.91[ab]	47.51[a]	40.57[b]	37.24[b]	2.345	0.02	0.16	0.20
C15:1	139	162	156	140	8.4	0.95	0.03	0.06
C16:0	2796	2892	2915	2680	83.2	0.39	0.63	0.63
C16:1T	25.90	30.95	23.36	35.78	6.165	0.43	0.56	0.25
C16:1	374[a]	348[a]	345[a]	299[b]	11.7	< 0.01	0.39	0.22
C17:0	117[a]	127[a]	113[ab]	98.7[b]	5.667	< 0.01	0.05	0.40
C17:1	141[ab]	155[b]	127[ab]	116[a]	9.7	< 0.02	0.20	0.18
C18:0	1525	1631	1647	1494	61.2	0.79	0.05	0.77
C18:1T	302[a]	392[b]	425[b]	414[b]	20.4	< 0.01	0.02	0.88
C18:1	4099[a]	4059[a]	4130[a]	3555[b]	139.2	0.02	0.07	0.24
C18:1V	185	181	203	182	9.8	0.74	0.40	0.14
C18:2T	47.00[a]	48.25[a]	52.04[a]	62.80[b]	3.349	< 0.01	0.17	0.77
C19:0	13.57[a]	23.71[a]	31.90[b]	24.30[ab]	3.638	0.02	0.03	0.41
C18:2	355[a]	449[b]	484[bc]	508[c]	14.5	< 0.01	0.03	0.48
C18:3ω6	10.53[a]	4.14[b]	4.57[b]	3.63[b]	2.042	0.04	0.20	0.38
C18:3ω3[2]	21.71[a]	53.04[b]	68.29[c]	87.77[d]	3.819	<0.01	0.14	0.25
C20:0	11.78	17.47	12.08	3.75	5.943	0.28	0.25	0.76
C20:1	47.46	50.80	49.02	51.53	3.980	0.57	0.92	0.60
C20:2	35.35[a]	41.74[a]	23.27[b]	9.29[c]	4.371	< 0.01	0.03	0.15
C20:3 ω6	26.27[a]	24.05[ab]	21.63[bc]	19.71[c]	1.209	< 0.01	0.90	0.90

C20:3 ω3	1.73	1.47	1.65	2.19	1.325	0.79	0.77	0.99
C20:4ω3	0.0	0.0	0.0	0.0	-	-	-	-
C20:4ω6	72.88[a]	79.21[a]	68.84[ab]	61.07[b]	3.125	< 0.01	0.04	0.19
C20:5ω3	0.0[a]	1.87[b]	1.99[b]	7.12[c]	0.511	< 0.01	< 0.01	< 0.01
C22:0	1.47	1.95	1.13	0.00	0.659	0.09	0.24	0.74
C22:1	10.79	3.96	0.00	3.31	2.970	0.06	0.11	0.74
C22:2	0.00	0.00	0.26	0.00	0.124	0.64	0.30	0.17
C22:4	5.59[a]	5.36[a]	3.43[ab]	0.0[b]	1.200	< 0.01	0.20	0.97
C22:5	9.33[a]	18.46[b]	20.48[bc]	24.15[c]	1.511	< 0.01	0.09	0.21
C22:6	0.30	1.14	4.22	5.09	1.410	0.01	0.99	0.49
C23:0	0.99	0.55	0.00	1.68	0.691	0.63	0.14	0.46
C24:1	17.49[a]	6.56[b]	2.06[c]	2.39[c]	1.244	< 0.01	< 0.01	0.78
TOTAL	10,894	11,335	11,417	10,352	336.7	0.32	0.04	0.61
Other	64.00	75.02	90.91	79.21	8.993	0.14	0.22	0.43
SFA[3]	4854	5105	5102	4659	155	0.41	0.04	0.79
UFA[4]	6040	6230	6315	5693	186	0.27	0.04	0.48
SFA:UFA	87.88	93.49	93.19	85.23	2.987	0.54	0.04	0.90
MUFA[5]	5440	5483	5544	4891	175.5	0.06	0.06	0.36
PUFA[6]	600[a]	747[b]	772[b]	803[c]	22.1	< 0.01	0.02	0.21
Trans[7]	376[a]	470[b]	496[b]	510[b]	25.0	< 0.01	0.13	0.62
ω6[8]	112[a]	110[a]	97.2[ab]	86.4[b]	5.09	< 0.01	0.36	0.54
ω3[9]	24.19[a]	56.99[b]	73.01[c]	97.30[d]	4.320	< 0.01	0.34	0.22
ω6:ω3	5.64[a]	2.28[b]	1.55[b]	0.93[b]	0.552	< 0.01	0.02	0.32

[1]Differences in dietary treatment were due to Green Grass (Sunseo Omega 3, Chungcheongbuk-do, Korea) inclusion (0,10, 20, 30 % of diet DM)

[2]C18:3ω3= Alpha linolenic acid [3]SFA = saturated fatty acids, [4]UFA=unsaturated fatty acids, [5]MUFA = monounsaturated fatty acids, [6]PUFA = polyunsaturated fatty acids, [7] Trans= Trans-unsaturated fatty acids, [8]ω6= total omega 6 fatty acids, [9]ω3=total omeg-3 fatty acids

[abcd] Means in a row with different superscripts differ ($P< 0.05$).

Appendix XIII

Table 4. Proximate analysis of lean steak samples from steers fed increasing inclusion of Green Grass product

	Treatment[1]					Contrast		
Item	0	10	20	30	*SEM*	L[2]	Q[3]	C[4]
Fat, %	10.96[ab]	11.41[ab]	11.51[a]	10.43[b]	0.340	0.34	0.04	0.60
Moisture, %	68.13[ab]	67.76[a]	67.71[a]	68.75[b]	0.260	0.20	0.02	0.57

[1]Differences in dietary treatment were due to Green Grass inclusion (0,10, 20, 30 % of diet DM)
[2] L= *P*-value for the linear response to Green Grass inclusion
[3] Q= *P*-value for the quadratic response to Green Grass inclusion
[4] C= *P*-value for the cubic response to Green Grass inclusion
[ab] Means in a row with different superscripts differ ($P < 0.05$).