Ice age: Investigating the impact of freezing and aging order on the palatability, physiochemical properties, and protein degradation of historically tough muscles

by

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Abstract

Beef is commonly aged and frozen to improve and preserve quality. While industry practices favor aging before freezing, some studies suggest freezing then aging may also be viable. This study evaluated how freezing and aging sequences affected consumer eating experience, physicochemical traits, and proteolysis in three muscles. Beef carcasses (N = 12; USDA Choice, A maturity) were collected from a Midwestern plant. Strip loins (*Longissimus lumborum,* LL), *Semitendinosus* (ST), and *Biceps femoris* (BF) were fabricated into 2.54-cm steaks and assigned to age-then-freeze (AF) or freeze-then-age (FA) for 21 or 28 days. Steaks were then assigned to one of the following designations: consumer sensory panels, Warner–Bratzler shear force, and lab assays. All steaks were aged before or after freezing between 1-4˚ C in the absence of light. Steaks were blast frozen before or after aging for 91 days at -20˚C. For all assays, samples were cooked to a peak internal temperature of 71˚C. The consumers (N = 192) evaluated samples for flavor, juiciness, tenderness, and overall liking, as well as acceptability for each sensory trait. On the following day, one steak from each muscle and treatment combination was evaluated for Warner–Bratzler shear force, cook loss, purge loss, and instrumental color. Freezing sequence and aging period did not affect (P > 0.05) consumer ratings of tenderness, flavor liking, or overall liking. However, unsurprisingly muscle type significantly influenced tenderness, flavor liking and overall liking, with the LL rated higher (P < 0.05) than the ST and BF. A freezing order × muscle × aging interaction was detected for juiciness (P < 0.05) in which the 28 d FA LL steaked resulted in the highest juiciness rating compared to all other muscles regardless of aging period or freezing treatment. Warner–Bratzler shear force confirmed LL as the most tender (2.76 kg; P < 0.05), with no difference (P > 0.05) between ST (4.06 kg) and BF (5.26 kg). There was an increase (P < 0.05) in purge loss in the FA compared to the AF samples (12.49% vs 8.57%).

However, the FA samples had a lower ($P < 0.05$) percentage of cook loss compared to the AF samples (15.14% vs 16.31%). Desmin degradation revealed an interaction ($P < 0.05$) between freezing treatment and aging period across all muscles combined. Specifically, the 28-day, FA samples had a lower percentage ($P < 0.05$) of intact desmin compared to the 21 and 28-day, AF samples indicating more proteolytic activity occurred. This study found that reversing the freezing order did not impact the overall palatability of beef steaks from the loin or round. On the contrary, it led to a higher purge loss, increasing the potential for economic loss. Therefore, reversing the sequence of freezing and aging is not a viable strategy for the industry.

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Dedication

This thesis is dedicated to my parents, Cory and Sally. I would not be the person I am today without your unwavering support, encouragement, and constant guidance. Thank you for believing in me and always pushing me throughout my life.

Chapter 1 - Literature Review

Introduction

Freezing is one of the most established and widely utilized methods for preserving beef (Setyabrata et al., 2019). Utilizing this method allows for the preservation of product quality, while extending shelf life, a factor that is critical for the cold chain stability especially for international trade. Beef destined for export is transported over extended distances and remains in transit for extended periods. Freezing is required to ensure product safety and quality until it reaches foreign markets. In 2024, the United States exported $10.45 billion dollars' worth of beef products (USDA, 2024). The effects of freezing on meat quality have been extensively researched, with emphasis placed on the formation of ice crystal and the associated structural damage, alterations in tenderness, and the consumer perceptions of quality. Although it is widely recognized that some degree of tissue damage is unavoidable during the freezing process, this damage does not typically result in diminished palatability.

Advantages of freezing Meat

One of the primary advantages of freezing meat is the increased shelf life and cold chain flexibility achieved by maintaining the quality of the product, by preventing bacteria growth (Dang et al., 2021). Shelf life is defined as the period during which a product maintains safety and quality under recommended storage conditions. It is one of the most critical factors for both consumers and retailers because it directly influences consumer purchasing decisions (Ren et al., 2022)

The added shelf-life that is capitalized on by the utilization of freezing enables the export of meat products to overseas markets and enhances overall supply chain efficiency. The extension of shelf life allows United States beef producers to continue to grow the amount of

beef that is exported throughout the world, without sacrificing quality (Leygonie et al., 2012). The export of $10.45 billion dollars' worth of beef product in 2024 reflects an average growth of 5.2% from 2015 to 2024 (USDA, 2024). Moreover, freezing enables beef to be stored for up to 12 months, compared to significantly shorter storage durations at refrigerated temperatures (USDA, 2024). Additionally freezing serves as an important barrier to microbial spoilage by extending the product's shelf life. Previous research has demonstrated that beef stored for extended periods at refrigerated temperatures exhibits greater growth of spoilage bacteria compared to beef that is frozen (Dang et al., 2021, Savell et al., 2005). The protective effect of freezing is achieved by reducing the availability of water to microorganisms, thereby slowing the rate of chemical reactions. (Rahman and Labuza, 2007). Although freezing does not reduce the total microbial load present, it effectively halts microbial activity because microorganisms become dormant under frozen conditions (Leygonie et al., 2012). Thus, freezing remains a crucial component of the meat industry, as it extends shelf life, enhances food safety, and contributes to greater flexibility for both suppliers and consumers.

Disadvantages of Freezing Meat

Freezing beef, while effective for long-term preservation, presents several disadvantages that can result in economic and quality loss. One of the most significant concerns is purge and cook loss, which directly contribute to reduced yields and profitability for processing plants. The primary driver of these losses is the formation of ice crystals during freezing, which causes structural damage to muscle fibers. This disruption increases drip loss upon thawing and increases cook loss during preparation, ultimately potentially decreasing juiciness (Kim et al., 2015). Beyond yield loss, freezing also influences other quality attributes such as protein denaturation and discoloration, which affect the sensory and visual appeal of beef products

(Leygonie et al., 2012). Then affecting the sensory and visual appeal of beef products (Leygonie et al., 2012). These factors show how freezing, despite its advantages, can decrease product quality and create economic drawbacks for the industry.

Formation of Ice Crystals

One of the most critical factors that can influence meat quality during freezing is the formation of ice crystals. As water transitions to ice within muscle cells, the expanding ice crystals rupture cell membranes and muscle fibers, resulting in weakened structural integrity and reduced water-holding capacity (Quian et al., 2021). Qian et al. (2021) described this decrease in water-holding capacity as being caused by the ice crystals increasing the overall space between each muscle fibers during freezing. The reduction in water-holding capacity is directly linked to the redistribution of intracellular water during phase transition, as the formation of ice enlarges intercellular spaces and develops drip channels that facilitate purge upon thawing (Leygonie et al., 2012, Liu et al., 2010). The mechanical damage caused by these crystals further disrupts the myofibrillar proteins, which compromises the structural integrity of the myofibril lattice and decreases its ability to retain moisture (Xia et al., 2009; Zhang et al., 2018).

It has been widely researched that the freezing rate of meat is highly influential on the overall damage during the freezing process. The rate of freezing strongly determines the size, distribution, and location of ice crystals, and therefore the extent of freeze-induced damage. A slow freezing rate, typically occurs at temperatures around -10˚C to -20˚C, which promotes extracellular nucleation and the growth of large ice crystals (Leygonie et al., 2012; Kiani & Sun, 2011). These large extracellular crystals exert osmotic pressure that drives water migration out of cells, causing dehydration, membrane rupture, and increased drip loss upon thawing (Mulot et al., 2019; Kaale & Eikeyik, 2014). Furthermore, slow freezing extends the time of muscle in the

critical zone of crystallization, typically, between -1 to -7 ˚C, which has been identified as the temperature range where approximately 80% of ice formation occurs.

By contrast, a rapid freezing rate, often achieved through blast freezing, typically between -30 to -40 °C, generates a high nucleation rate, leading to the formation of numerous small ice crystals (Pham, 2006; Kaale & Eikeyik, 2014). These smaller crystals form both intra- and extracellularly, which reduces structural disruption, minimized protein denaturation, and improves overall water-holding capacity compared to slow freezing (Kiani & Sun, 2011; Lu et al., 2022). Rapid freezing is also associated with better preservation of tenderness and juiciness, as smaller ice crystals minimize fiber swelling and Z-line damage (Zhang et al., 2018; Zhu et al., 2004).

In addition to freezing rate, storage temperature fluctuations strongly affect ice crystal formation. Recrystallization is a phenomenon where smaller ice crystals fuse into larger ones during frozen storage, particularly when temperature is near the glass transition point of muscle water (Roos, 2021; Kumar et al., 2019). Larger recrystallized crystals are more damaging than primary crystals, as they continue to rupture cellular membranes and increase drip channels during thawing (Zhang et al., 2018). Recrystallization is especially detrimental in retail and distribution systems where temperature control may be inconsistent, as temperature fluctuations promote ice recrystallization and associated tissue damage, which degrade water-holding capacity and color stability (s). Thus, maintaining constant storage temperatures is recommended to limit recrystallization (Leygonie et al., 2012).

A novel freezing method that has been investigated in recent years is high-pressure freezing, which has been developed to minimize crystal-induced damage. This technology allows instantaneous and uniform distribution of ice crystals throughout the muscle tissue (Bing & Sun,

2002). Under conventional freezing conditions, ice crystals generally form as type I crystals, which are large, extracellular structures, that ultimately led to significant moisture loss and reduce water-holding capacity during thawing (Leygonie et al., 2012, Warner et al., 2017). In contrast, high-pressure freezing promotes the formation of type IV crystals, which are much smaller, denser, and more uniformly distributed within both intra- and extracellular spaces. Because these crystals more closely resemble the size of water molecules, they reduce mechanical rupture of cell membranes, thereby mitigating swelling and drip loss upon thawing. The transition from type I to type IV crystals allow for the minimization of freeze-induced damage to muscle proteins and cellular integrity. This improved structural preservation has been shown to positively impact both physiochemical traits, such as reduced purge, as well as sensory outcomes, including juiciness retention after thawing (Bing & Sun, 2002, Sun & Li, 2003).

The rate and extent of ice crystal formation can also vary by muscle type due to differences in fiber structure, water content, and biochemical composition. For example, Setyabrata et al. (2019) reported that the *Longissimus lumborum* (LL) required approximately 26 hours to pass through the critical zone of water crystallization (–1 to –7 °C), whereas the *Semitendinosus* (ST) froze more rapidly. Differences in freezing kinetics between muscles are attributed to variation in muscle size, fiber orientation, and metabolic type, which influence thermal conductivity and water distribution (King et al., 2009; Hughes et al., 2014).

While ice crystal formation and freezing dynamics primarily describe structural and biochemical changes, their ultimate importance lies in how they influence consumer perception of beef quality. Larger extracellular crystals and recrystallization events manifest as increased purge loss, reduced juiciness, and darker surface color, all of which consumers associate with lower freshness and quality (Mancini & Hunt, 2005; Neethling et al., 2017). Conversely, rapid

freezing and advanced techniques that produce smaller, uniformly distributed crystals help preserve tenderness, juiciness, and visual appeal, resulting in more favorable consumer eating experiences (Dang et al., 2021). These outcomes demonstrate that although consumers cannot directly observe microscopic crystal morphology, the effects of freezing and storage are indirectly perceived through palatability traits and purchasing decisions. Therefore, understanding ice crystal formation is not only essential for improving processing efficiency but also for ensuring consumer satisfaction and maintaining beef's competitive position in global markets.

Freezing Impact on Color

Color is one of the most critical attributes influencing consumer perception and acceptance of meat. It is often considered an indicator of freshness, quality, and safety. However, freezing and subsequent frozen storage can significantly influence meat color, leading to discoloration and quality degradation (Leygonie et al., 2012; Faustman et al., 2010). The primary pigment responsible for meat color is myoglobin, a sarcoplasmic heme protein that stores oxygen in muscle fibers (Faustman et al., 2010). Myoglobin exists in different redox forms, deoxymyoglobin, oxymyoglobin, and metmyoglobin. Each of these bring a distinct color to meat. Oxymyoglobin, which is bright red, is the most desirable to consumers. While metmyoglobin is brown and signals spoilage or quality loss (Mancini and Hunt, 2005).

During freezing, oxidation and physical changes may change the influence between these forms, causing discoloration. When meat is frozen, the formation of large extracellular ice crystals that disrupt muscle fibers and cell membranes (Leygonie et al., 2012). This damage then leads to the release of pro-oxidants, such as free iron that leads to an increase in myoglobin oxidation (Xia et al., 2009). With lipid oxidation, products such as malondialdehyde, promote the

heme degradation and metmyoglobin formation (Liu et al., 2010). This is mainly focused during long-term frozen storage. During the freezing and thawing of meat, the metmyoglobin reducing ability (MRA), the capacity of muscle enzymes to convert oxidized metmyoglobin back to the desirable red oxymyoglobin pigment, is often reduced (Mancini & Hunt, 2005). A decline in MRA limits the muscle's ability to maintain a bright red surface color, accelerating discoloration and browning during storage (Mancini & Hunt, 2005). Mitochondrial damage during freezing reduces the reducing equivalents, then limiting the ability of meat to convert brown metmyoglobin back to its oxymyoglobin. When meat is frozen, the rate of freezing plays a critical role in determining the amount of ice crystal damage that happens. The rapid freezing leads to smaller ice crystals, allowing the color to be preserved more effectively (Kim et al., 2015). Additionally, storage at lower temperatures improves oxidative stability by slowing oxidative reactions compared to higher freezing temperatures (Jeong et al., 2011). However, as frozen storage time increases, cumulative oxidative damage and residual enzymatic activity gradually reduce color stability, leading to greater discoloration over time (Leygonie et al., 2012). Kim et al. (2011) reported that beef loins stored at –20°C for more than six months exhibited significantly lower redness values and greater browning compared to loins stored for shorter durations.

Freezing has a Positive Impact on Tenderness

Tenderness remains one of the most critical attributes determining consumer acceptance of beef (Miller et al., 2001). The underlying mechanisms within freezing and its impact on tenderness include structural alterations, enzymatic activity, and moisture loss. The primary physical mechanism by which freezing affects tenderness is through the formation of ice crystals. As water transitions to ice, ice crystals are formed and disrupt the cell membranes. These

structural changes weaken the muscle matrix, thereby reducing the force required during chewing or instrumental shear force values (Zhang et al., 2018). Structural damage caused by freezing also results in the rupture of lysosomal membranes, which can release proteolytic enzymes such as cathepsins into the sarcoplasm (Bekhit and Faustman, 2005). These enzymes degrade cytoskeletal proteins during the thawing process, contributing to an increase in tenderness (Koohmaraie and Geesink, 2006). Additionally, freezing alters the distribution of water within muscle, leading to structural disruption that influences both purge loss and tenderness. Upon thawing, drip loss increases as water is expelled from damaged fibers, resulting in a looser fiber matrix and reduced water-holding capacity (Xia et al., 2012). The extent of these effects is largely dependent on freezing parameters, particularly freezing rate and storage conditions. Although this damage may sometimes increase perceived tenderness due to fiber weakening, it typically occurs at the expense of juiciness (Kiani & Sun, 2011; Leygonie et al., 2012).

There is consensus across research that with the utilization of freezing and thawing within beef can increase the overall tenderness of a product when utilizing shear force methodology (Leygonie et al., 2012). This decrease in shear force values has been widely correlated to the period of time in which the sample was frozen as well as the confounding factor of how long the steak was aged prior to freezing (Leygonie et al., 2012). Moreover, this increase in tenderness has been thought to be a combination of enzymatic action in proteolysis breaking down the muscle fibers, aging, and ice crystal formation causing the loss of structural integrity. Moreover, the freezing sequence utilized within beef has been shown to see differences when the sequence is switched. When utilizing aging and then freezing allows for proteolytic activity from endogenous enzymes such as calpains to occur prior to freezing, this results in improved

tenderness (Kim et al., 2018). This is the industry standard as it ensures proteolysis before the potential inactivation of enzymes during frozen storage. The freeze and then age approach can allow for increased enzymatic activity upon thawing due to the membrane rupture and the release of enzymes (Rehman et al., 2024). This proteolysis is crucial to postmortem tenderization. The calpain system and its inhibitor calpastatin, play a critical role in degrading myofibrillar proteins and overall meat tenderness (Koohmaraie, 1996).

Calpastatin

Calpastatin is the endogenous inhibitor of the calpain system, which plays a key role in postmortem proteolysis and meat tenderization. Calpastatin is a specific inhibitor of μ- and m-calpain and plays a critical role in regulating proteolysis during meat aging (Rehman et al., 2024). Calpastatin is a large (approximately 120 kDa), heat stable protein that is a multiheaded inhibitor that has four reaping inhibitory domains. The domains are I, II, III, and IV (Wendt et al., 2004). Within each of these inhibitory domains, they can inhibit one calpain molecule (Mellgren et al., 1982). The calpain system that included both calpastatin and calpains are largely responsible for the degradation of myofibrillar proteins such as titin, nebulin, troponin-T, and desmin during postmortem aging (Koohmaraie, 1992). Calpastatin modulates this system by binding to the calpain heterodimer, inhibiting its catalytic activity through interaction with both catalytic and regulatory domains (Maddock et al., 2005). The interaction between calpain activation and calpastatin inhibition then influences postmortem proteolysis and overall meat tenderness. In terms of tenderness, high levels of calpastatin inhibit the calpain-mediated proteolysis, resulting in lower postmortem degradation of cytoskeletal proteins and led to tougher meat (Koohmaraie et al., 2002). An example of this would be that Bos indicus breeds and older animals tend to have a higher calpastatin activity compared to younger Bos taurus

cattle, correlating to an increased toughness (Shackelford et al., 1991; Wheeler et al., 2002). Another major player in the activity of calpastatin is the utilization of freezing. There have been studies that have shown that calpastatin is more susceptible to freezing and that calpastatin decreases as time progresses during freezing periods (Koohmaraie et al., 1991). Freezing causes structural damage, affecting both the sarcoplasmic and myofibrillar proteins (Zhou et al., 2010). Freezing halts most enzymatic activities, however the utilization of thawing can then reactivate calpains. The extent to which calpastatin inhibits this reactivation depends on its structural integrity after frozen storage (Zhang et al., 2019). Calpastatin tends to be more stable than calpain during freezing, however oxidative stress can modify its methionine and cysteine residues, affecting its inhibitory potential (Liu et al., 2008). This damage can shift the balance of calpain activation upon thawing, resulting in higher levels of post-thaw proteolysis (Koohmaraie, 1996; Koohmaraie et al., 1995). These changes then directly influence the ability of the calpain system to function properly. Denatured proteins can lose activity due to the changes in their tertiary and quaternary structures (Devine et al., 2001). A big impact on calpastatin activity is the duration that the product is frozen. Studies have demonstrated that prolonged frozen storage (over 6 months) at temperatures at or less than -10˚C leads to substantial decline in calpastatin inhibitory capacity (Leygonie et al., 2012). Moreover, it has been shown that a slower freezing rate can cause extensive damage to the muscle structure, resulting in a greater amount of protein denaturation and potential loss of calpastatin function (Devine et al., 2001). Lower storage temperatures (-80˚C vs -20˚C) have been associated with greater retention of calpastatin activity due to reduced molecular motion and oxidative degradation (Farouk et al., 2003). Additionally, the sequence of freezing and aging can significantly impact calpastatin and calpain dynamics. Utilizing freezing before aging preserves the calpastatin activity, resulting in reduced proteolysis

post-thaw. In contrast, aging before freezing reduces calpastatin levels through natural degradation, allowing for continued proteolysis during frozen storage (Koohmaraie, 1996). Kim et al. (2018) demonstrated that freeze-then-age treatments can result in higher shear force values compared to age-then-freeze treatments, due to greater calpastatin presence. Finally, different muscle can exhibit varying levels of calpastatin. Muscle with inherently high calpastatin content, such as the ST and *Biceps femoris* (BF), tend to show greater resistance to proteolysis after freezing (Shackelford et al., 1994). Additionally, muscles that have a higher percentage of oxidative fibers can experience more oxidative stress during freezing, altering the calpastatin stability. Calpastatin plays a pivotal role in regulating meat tenderness by inhibiting the activity of calpains.

Calpains

Tenderness is one of the most critical attributes in determining consumer eating satisfaction within beef. Postmortem proteolysis plays a pivotal role in meat tenderization. The calpain system has been widely recognized as a key player within this process. Within meat science, there are two different calpains that are present, u- and m- calpains. Calpains are non-lysosomal, calcium activated proteases involved in proteolysis of structural proteins. The stoppage of blood flow and depletion of ATP lead to increased intracellular calcium levels in the muscle cells. This rise in calcium activates calpains. This then allows the Calpain to degrade key myofibrillar and cytoskeletal proteins such as desmin, titin, nebulin and troponin-T (Koohmaraie et al., 1996). The u-Calpain required micromolar concentrations of calcium for activation, while the m-calpain requires millimolar concentrations of calcium to become activated. However, it has been found that u-calpain is the primary factor in postmortem meat tenderization as it has a lower calcium activation level and is the most susceptible to calpastatin (Koohmaraie et al.,

1994). However, Calpains do not degrade actin and myosin, indicating that tenderization is primarily the result of weakening the myofibrillar structure rather than complete protein breakdown (Huff-Lonergran & Lonergran, 2005). Currently, there is only one known inhibitor for calpains and that is Calpastatin (Ono and Sorimachi, 2011). It binds to calpain in a calcium-dependent manner and prevents its proteolytic activity. High levels of calpastatin activity have been associated with tougher meat, such as Bos indicus breeds (Koohmaraie et al., 1991). U-calpain is activated within the first few hours' postmortem and contributes to early proteolysis. It is the most active around 24 hours postmortem and then subsides due to autolysis. However, m-calpain is rarely involved due to its higher calcium threshold not being met in physiological conditions (Morgan et al., 1993). Koohmaraie (1990) discovered that the activity of μ- and m-calpain in beef is stable for 6 weeks when stores at -70˚C. Boehm (1998) discovered that between day 1 postmortem and day 7 postmortem, there is a change from 83% to 63% of activity of m-calpains. It has also been discovered that the activity both u-calpains and m-calpains were both consistent across both -20˚C and -80˚C when frozen (Kristensen et al., 2006). In previous studies, it was shown that during storage the m-calpain activity changes. (Boehm et al., 1998, Koohmaraie et al., 1990).

Desmin

The structural integrity and postmortem proteolysis of muscle fibers significantly influence beef tenderness (Koohmaraie et al., 2002). Desmin is a type III intermediate filament protein that is located at the Z-line region of the sarcomere and forms a filamentous network around the myofibrils (Taylor et al., 1995). In which it links together myofibrils and connects them to the plasma membrane and mitochondria (Paulin & Li, 2004). This allows it to maintain myofibrillar alignment and transmits contractile forces across muscle fibers. Desmin helps with

structure during contraction and relaxation. It's part in the organization of muscle fibers influences meat texture and palatability (Ertbjerp, 2022). This is because desmin is one of the earliest cytoskeletal proteins to undergo proteolytic degradation during postmortem aging (Taylor et al., 1995; Huff Lonergran et al., 1996). This then correlates with the weakening of muscle structure and the improvement of tenderness over times (Koohmaraie, 1996; Melody et al., 2004). Western blotting has consistently shown a decrease in intact desmin bands within the first 24 to 72 hours postmortem (Huff Lonergan et al., 2010; Zhang et al., 2006). The degradation is initiated by proteolytic enzymes, such as μ-calpain. This then targets demin's head domain causing the subsequent breakdown of muscle fiber architecture (Geesink et al., 2000). It has a α-helical rod domain with a non-helical head and tail domain, which allows it to polymerize into filaments (Herrmann et al., 2007). Genetics studies in desmin-knockout mice showed that there were disorganized myofibrils, mitochondrial dysfunction, and progressive muscle degeneration. This highlighted it as essential in the role of muscle maintenance (Milner et al., 2000; Thornell, 2019). Desmin degradation has been extensively correlated with improved beef tenderness across various breeds and aging times (Rhee et al., 2004). Western blot analysis of desmin integrity is often used as a molecular market for tenderness potential (Qian et al., 2020). A faster rate of desmin degradation is associated with greater myofibrillar index and lower Warner-Bratzler shear force values (Calkins & Sullivan, 2007; Zhang et al., 2006). Breed differences in tenderness are partly attributes to differences in degradation kinetics. Bos indicus breeds, which generally have tougher meat, show delayed desmin degradation and higher calpastatin activity compared to Bos taurus breeds (Shackelford et al., 2001; Koohmaraie et al., 2002). Postmortem aging time is a critical factor influencing desmin degradation. Aging beef for up to 14 days increases desmin proteolysis, coinciding with improved tenderness (Wheeler et al.,

1994; Koohmaraie, 1996). The use of electrical stimulation can accelerate glycolysis and calcium release, enhancing calpain activation and desmin breakdown (Devine et al., 1999). Early electrical stimulation promotes faster tenderization and more rapid desmin degreadation compared to non-stimulated controls (Savell et al., 1978). One of the products that is often seen during major degradation is a polypeptide of 38 kDa. When compared, this degradation product is often present with μ-calpain digested myofibrils. Because of this, it is thought that μ calpain may be partially responsible for desmin degradation (Lonegran et al., 2010). It has been previously shown that the freezing and then aging sequence of beef leads to the highest protein degradation (Grayson et al., 2014).

Aging

Aging of beef is one of the most widely utilized techniques in the meat industry to improve palatability, with tenderness being the primary trait influenced (Smith et al., 2008). The aging process relies heavily on endogenous enzymatic activity to degrade structural proteins within muscle fibers, thereby weakening the muscle matrix and enhancing tenderness (Koohmaraie, 1994). The principal enzymatic system responsible for postmortem tenderization is the calpain proteolytic system, which is composed of μ-calpain, m-calpain, and their endogenous inhibitor, calpastatin (Koohmaraie, 1992; Goll et al., 2003). Calpains are activated by calcium ions and act on key myofibrillar proteins such as titin, nebulin, desmin, and troponin-T (Taylor et al., 1995; Huff-Lonergan et al., 2010). Elevated calpastatin activity inhibits calpain function, thereby limiting proteolysis and slowing postmortem protein degradation (Whipple et al., 1990).

One of the most studied protein substrates during aging is troponin-T, as its degradation products serve as biochemical markers of proteolysis and tenderization (MacBride & Parrish, 1977). Western blot analyses have consistently demonstrated a decline in intact troponin-T bands

and the concurrent appearance of lower molecular weight fragments as postmortem aging progresses (Taylor et al., 1995). These changes reflect the proteolytic activity of calpains and are commonly used to assess the extent of muscle protein degradation during storage.

There are two principal aging methods employed commercially: dry aging and wet aging. Among these, wet aging is the predominant practice in the beef industry due to its efficiency, yield advantages, and widespread adoption in large-scale production systems.

Freezing/Aging Sequence

Within the meat industry, practice of aging followed by freezing has become the standard due to its efficiency and logistical practicality for processors and distributors. Aging allows for the development of tenderness through endogenous enzymatic activity, and subsequent freezing halts further biochemical deterioration, enabling products to be stored and distributed for extended periods without compromising quality (Bekhit et al., 2014; Leygonie et al., 2012). From a supply-chain perspective, aging then freezing offers clear advantages for cold chain management and consistency in product handling.

The idea that reversing this sequence might improve tenderness originates from work in the early 1990s. Whipple et al. (1990) suggest that freezing could inactivate calpastatin, the endogenous inhibitor of the calpain proteolytic system, potentially increasing proteolysis after thawing. This hypothesis created a narrative that “freeze then age” might enhance tenderness by indirectly allowing greater calpain activity (Koohmaraie, 1996).

Several studies have investigated the interaction between freezing sequence and postmortem aging, though results have been inconsistent. Some studies have shown that aging prior to freezing results in an increase in tenderness. Geesink et al. (2011) found that beef loins aged for 21 days prior to freezing had significantly lower Warner–Bratzler shear force values

compared to loins that were frozen first and then aged. Similarly, Savell (2012) reported approximately a 15% reduction in Warner–Braztler shear force values for samples aged 21 days before freezing compared to those frozen immediately postmortem. However, other studies have demonstrated that freezing before aging can enhance tenderness. Grayson et al. (2014) found that samples frozen and then aged displayed the lowest shear force values when compared to fresh or freeze-only counterparts.

Beyond tenderness, freezing order also influences water-holding capacity and yield. Freeze–then–age samples often display greater drip loss and cook loss due to ice crystal damage and post-thaw proteolysis (Farouk et al., 2018; Zhou et al., 2010). This reduction in water-holding capacity can potentially negatively affect juiciness and diminish yield, presenting both sensory and economic challenges.

Lipid oxidation provides another quality attribute where freezing sequence matters. Freeze then age samples have been associated with increased lipid oxidation due to extended exposure of compromised membranes to oxidative environments following thawing (Kim et al., 2015). Bekhit et al. (2014) reported higher thiobarbituric acid reactive substances (TBARS) values in lamb frozen immediately after slaughter and aged post-thaw, indicating greater oxidative rancidity compared to age then frozen samples. Flavor compounds such as free amino acids and peptides are better preserved when aging occurs before freezing, resulting in age then freeze samples with a more intensive beef flavor and fewer oxidative off-flavors (Kim et al., 2015; Bekhit et al., 2014).

Ultimately, while aging before freezing remains the dominant industry practice due to convenience and consistency in processing. Evidence suggests that freezing before aging may yield biochemical advantages under certain conditions, particularly related to proteolytic activity.

Importantly, while biochemical and instrumental measures have been well characterized, few studies have evaluated how freezing order influences consumer perception of palatability traits such as tenderness, juiciness, and flavor.

Conclusion

Freezing has been a long-term staple in cold chain management, and there has been extensive research that have investigated the role of freezing and its effects on beef quality and physiochemical characteristics. However, limited research has examined how the order of freezing and aging, rather than the length of aging alone, influences consumer eating experience. Most previous work has focused on biochemical characteristics such as proteolytic activity, yet little is known about how these freezing then aging sequences affect consumer perception of palatability traits. Therefore, the objective of the current research was to determine how altering the freezing sequences affect not only the proteolytic degradation of muscle proteins but the consumer sensory rating of historically tough muscles.

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Chapter 2 - Ice age: Investigating the impact of freezing and aging order on the palatability, physiochemical properties, and protein degradation of historically tough muscles

Introduction

Freezing is a longstanding practice within the meat industry that extends shelf life by slowing the growth of spoilage microorganisms and pathogens (Leygonie et al., 2012). In recent years, the importance of freezing and specialized cold-chain management has increased due to growing demand for exported frozen beef (USDA, 2024). As a result, freezing has become a critical preservation strategy that allows processors to maintain quality and ensure product safety across long distribution channels.

Extensive research has looked at the effects of freezing on the quality and physicochemical properties of meat (Zhang et al., 2018; Qian et al., 2021). Freezing damages muscle tissue at the cellular level through the formation of ice crystals, which rupture cells and disrupt myofibrillar structure (Leygonie et al., 2012). The rate and temperature of freezing strongly influence ice crystal morphology; slower freezing promotes the development of larger extracellular crystals, which exacerbate cellular rupture, reduce water-holding capacity, and increase purge loss (Aroeira et al., 2016; Qian et al., 2021). Despite these negative outcomes, freezing has also been associated with beneficial effects, such as improved tenderness, which results from physical disruption of muscle fibers by ice crystals (Beyer et al., 2024).

While much of the literature has focused on the general impact of freezing on meat quality, relatively few studies have investigated how the sequence of freezing and aging influences palatability traits. Traditionally, beef is aged and then frozen for convenience and

supply chain efficiency. However, alternative sequences have been proposed as a means of altering proteolysis and tenderness development., (Grayson et al., 2014; Aroeira et al., 2016). This impact has been attributed to the deactivation of calpastatin, the inhibitor of the main proteolytic system, the calpains (Grayson et al., 2014; Aroeira et al., 2016). Grayson et al. (2014) found a clear improvement in tenderness while aging after a freezing period in the *Semitendinosus* and *Longissimus lumborum.* However, Setyabrata et al. (2019) demonstrated that freezing prior to aging increased purge loss compared to the industry standard. However, no studies have evaluated the impact of reversing the freezing order on palatability.

Therefore, the objective of this study was to evaluate palatability, physicochemical properties, and protein degradation in three beef muscles subjected to two freezing sequences (age then freeze; freeze then age) and two aging periods (21 and 28 days).

Materials and Methods

The North Dakota State University (NDSU) Institutional review board (IRB) approved all procedures for use of human participants in sensory panel evaluations (IRB # 0005179, March 2024)

Sample Collection and fabrication

Beef carcasses ($N = 12$) were selected from a Midwestern beef processing plant on the same kill date. The carcasses graded USDA Low Choice and were A maturity. Beef carcasses were selected to be uniform for yield and quality grade, using the same rail designation for consistency. The research team from North Dakota State University collected carcass data (marbling scores, lean maturity, skeletal maturity, ribeye area, preliminary yield grade, and hot carcass weight.) Trimmed strip loins (IMPS #180) and goosenecks (IMPS #170) were collected

from the right side of the carcasses and then transported to North Dakota State University (Fargo, North Dakota) for processing the day after collection. During fabrication, the *Semitendinosus* and *Biceps femoris* were separated from the rest of the gooseneck. The *Longissimus lumborum* (LL), *Semitendinosus* (ST), and *Biceps femoris* (BF) were denuded and sliced into twelve 2.54-cm steaks. Each steak was randomly assigned a 4-digit code with a tag and assigned to one of the following destinations: age then freeze (AF) (21 days), age then freeze (28 days), freeze then age (FA) (21 days), or freeze then age (28 days). Then steaks were assigned to one of the following assays: consumer sensory panels, shear force, or lab assays. Steaks designated for lab shear force were also utilized to collect raw and cooked color readings. Additionally, steaks designated for lab assays were used for proximate analysis and proteolysis. All steaks were aged between 1-4° C in the absence of light. All steaks were frozen in a commercial freezer located at North Dakota State University and held at -20°C for 91 days before being placed in a refrigerator to thaw for 24 hours before the time of use. The treatments including freezing then aging reversed the freezing and aging order using the same time and temperatures as described above.

Consumer sensory panels

Consumer sensory panelists (n = 96 per aging period, N = 192 total) from the Fargo, North Dakota area were recruited and compensated for their participation. Eight panels were hosted at North Dakota State University (NDSU), each with 24 panelists. Consumer sensory panels procedures followed protocols done at Kansas State University and were modified to fit the parameters for NDSU facilities (Farmer et al., 2022; Beyer et al., 2021).

Panelists used Samsung and Lenovo tablets (Lenovo TB-8505F) to complete Qualtrics surveys (v. 2417833; Qualtrics Software). Surveys collected demographic data, purchasing motivators, and evaluations for six beef samples. Within the demographic survey, it contained

questions pertaining to gender, age, household size, income level, ethnicity, education level, palatability preferences, and weekly beef consumption. Purchasing motivators included animal welfare, antibiotic or hormone use, brand, diet (corn vs. forage), natural/organic claims, nutrient content, familiarity with the cut, packaging, price, size, color, marbling, eating satisfaction, and fresh vs. frozen claims. Responses were recorded on a 100-point sliding scale with 0 representing extremely unimportant, 50 being neither unimportant nor important and 100 representing extremely important.

Consumers were fed 6 samples, containing all treatment and muscle combinations within a single aging period. Samples were fed one aging period at a time due to the freezing logistics and consumer panel capability. Consumers were provided with a napkin, fork, water cup, expectorant cup, apple juice, and unsalted crackers. The unsalted crackers, water, and apple juice were used as a palate cleanser between samples. Before the panels began, consumers were given instructions on how to operate the tablet, ballot, and the use of palate cleansers.

Steaks were thawed 24 hours prior to consumer panels. Samples were cooked to a peak temperature of 71°C as outlined by the AMSA sensory guidelines (AMSA, 2015) monitored using a temperature probe (Thermopen mk4) inserted into the geometric center of the steak on a Cuisinart GR-150 Griddler Deluxe (Cuisinart, Stamford, CT, USA). Peak temperatures were monitored and recorded. Each muscle was sliced using a guide into cubes (2.54 cm thick x 1 cm x 1 cm cubes) and two cubes were served immediately to consumers. The consumers evaluated each sample for juiciness, tenderness, flavor liking, and overall liking on a 0–100-point sliding scale. On the scale, 0 represented extremely dry, extremely tough, dislike extremely; 50 represented neither juicy nor dry / tough nor tender / like nor dislike; and 100 represented

extremely juicy, extremely tender, and like extremely. Additionally, consumers determined if the samples were acceptable for juiciness, tenderness, flavor liking, and overall liking (yes/no).

Warner–Bratzler shear force, color, and cook loss

Warner Bratzler shear force, raw and cooked color, and cook loss were all measured on the same steak the day after consumer panels. Before the steaks were cooked, they were weighed for cook loss and each sample was allowed 20 minutes to bloom. A raw color reading (CIE *L*, a*, b*)* was taken using a HunterLab Miniscan spectrophotometer (Illuminant A/10, 2.54 cm aperture). Three readings were taken in different locations on the steak and an average *L*, a*, b** readings were recorded. Next, samples were cooked to a peak temperature of 71˚C as outlined by the AMSA sensory guidelines (AMSA, 2015) and as described above. Once peak temperatures were recorded, steaks were reweighed for cook loss. Cook loss was calculated as the difference between raw and cooked weight and represented as a percentage. After weighing, each steak was sliced using a double blade knife parallel to the muscle fibers to expose a 1-inch internal surface. After a 3-minute bloom time, steaks had 3 color readings taken along the internal cut surface and an average CIE *L*, a*,* and *b** were recorded. Spectral data was collected from the spectrophotometer to calculate oxymyoglobin, metmyoglobin, deoxymyoglobin percentages using formulations published in the AMSA color guidelines (King et al., 2023). The remaining portion of the steak was used for Warner Bratzler shear force following the guidelines in the AMSA sensory guidelines (AMSA, 2015). After 24 hours of chilling, 6 cores were taken parallel to the muscle fiber. The six cores were sheared using a V-shaped blade perpendicular to the muscle fibers using a Warner-Bratzler shear force machine (225 mm/min head speed; Tallgrass Products, Manhattan, KS). The six readings were recorded and averaged in kg of force to represent shear force values.

Purge Loss

Samples designated for laboratory assays were used to calculate purge loss. The day after consumer sensory panels, steaks thawed for 24 hours. While still in the package, each sample was weighed and recorded as the total weight. Next, the package was opened, and the steak was reweighed as a raw weight. The packaging was rinsed with water, dried with a paper towel, and reweighed. Final purge loss was calculated by subtracting the combined weights of the steak and package from the initial packaged weight and represented as a percentage.

Sarcoplasmic Protein Extraction

After determining purge loss, samples were pulverized in a food processor, stored in whirlpack bags, and frozen at -80 °C. Pulverized samples were used for proteolysis and proximate analysis.

Protein extraction began by adding 1.5 g of frozen sample to 1X Post Rigor Extraction Buffer (PREB; containing: ethylenediaminetetracetin (EDTA, Sigma EDS-500G) and Tris (VWR JT4109-06)). 200 mM of PMSF was added to the sample. Samples were homogenized for 4.5 seconds in a FastPrep-24 Classic (MP Biomedicals Cat #116004500) beat mill homogenizer. Samples were centrifuged at 21,000 x g at 4˚C for 30 minutes using a Allegra 25R centrifuge with TA-14-50 fixed angle rotor (Beckman Coulter, Fullerton, CA) Samples were stored at -80°C. Samples were thawed and 6 mL of Whole Muscle Extraction Buffer containing Sodium Phosphate buffer and 2% (wt/vol) SDS, was added. Samples were homogenized using a polytron Kinematical (10/35 with controller and PTA 10S Generator, Brinkmann, Westbury, NY) until the sample was completely dispersed. Samples were centrifuged at 4500 x g for 15 minutes at 10˚C utilizing an Allegra 25R Centrifuge, Beckman Coulter with TA-14-50 fixed-angle rotor).

Western Blotting

The western blot analysis began by casting polyacrylamide gels. The stacking gel was created by mixing the separating gels. We utilized 12.5% separating gels, that contained 30% Acrylamide: bis BioRad 161-0158 at a 37.5:1 ratio, 4x Separating Gel Buffer, 18MΩ H_2O, 10% wt/vol AMPER, and TEMED. A BioRad Mini-PROTEAN Tetra Cell gel casting system was used to cast the gels. Once the system was set up, the separating gel was poured into the casting system utilizing a syringe and needle. Then 1 mL of 18MΩ H_2O was added onto the top to prevent oxygen from inhibiting polymerization. The separating gel was allowed to sit for 90 minutes to allow the gel to polymerize. Next the stacking gel was prepared. A 5% gel was utilized containing 30% Acrylamide: bis (37.5:1) Biorad 161-0158, 4x stacking gel, 18MΩ H_2O, 10% (wt/vol) AMPER, and TEMED. Combs were inserted and gels were allowed to polymerize for 90 minutes. After samples had been polymerized, they were wrapped and set in a fridge overnight. The next day, gels were allowed to come to room temperature. Gels were then clamped into the BioRad Mini-PROTEAN Tetra Cell gel electrophoresis unit. 1X running buffer containing Tris, Glycine, EDTA, and 0.1% SDS, was added between the gels to determine if there was any leaking. Combs were removed, and the wells were washed using a pipet. 2.5 µL of Precision Plus Kaleidoscope Protein Standard (BioRad 161-0375) was loaded into the first well of the gel. 5 µL of pooled controlled protein was added into the second and tenth well of the gel. Samples were randomly assigned a gel and well number within the same muscle and 5 µL of sample protein were loaded into wells. 1X Running Buffer was then added into the reservoir of the electrophoresis chamber and a stir bar was added. The unit was placed on a stir plate. The electrophoresis chamber was covered with electrode leads and connected to a BrioRad PowerPac HC Power Supply #164-5025. The power supply was then set to 150 V and set to run for 90 minutes. Filter papers (BidRad Cat: 1703966) were soaked for 3 minutes in 1X transfer buffer.

The Polyvinylidene Fluoride transfer membrane (PVDF) (Millipore Immobilon-P, IPVH00010; 0.45 µm) was soaked in methanol and then transferred to the 1X transfer buffer. The gels were then transferred to a Hoefer TE 22 tank to complete the transfer. This was done at a voltage of 120 V for 12 minutes. After transferring, membranes were soaked in methanol for 10 seconds and then allowed to dry for 15 minutes. Membranes were then soaked in methanol and then transferred to a container that had 5% methanol and 1X PBS, 0.1% Tween. Samples were then incubated in the blocking solution for one hour on a rocker. After an hour, the blocking solution was poured off and the primary antibody in 1X PBS, 0.1% Tween was added. Samples were then incubated overnight at 4˚C. The next day, samples were washed 3 times for 20 minutes each with 1X PBS, 0.1% Tween. Then the second antibody, that was diluted with 1X PBS, 0.1% Tween, and Precision Protein Streptactin-HRP conjugate (BioRad 161-0380) was added and incubated on a rocker for 1 hour. Samples were then washed again with 1X PBS, 0.1% Tween 3 times for 10 minutes each. Then chemiluminescent Detection began by adding ECL Prime Western Blotting Detection Reagents (GE Healthcare Life Sciences, Piscataway, NJ). Membranes were laid on a flat surface and both reagents were added to the membrane and allowed to incubate for 5 minutes. Samples were then read on a FluorChem FC2CCD camera on autoexposure.

Proximate Analysis

Twenty-four hours prior to laboratory assays, samples were thawed and prepared for scanning. Each sample was pressed evenly into a petri dish, ensuring the absence of air pockets or surface irregularities. Proximate composition was determined using a FOSS FoodScan™ 2 (FOSS Analytical, Hillerød, Denmark), utilizing near-infrared (NIR) transmittance and reflectance spectroscopy. This estimates moisture, fat, protein, and collagen content by measuring the absorption of NIR light at specific wavelengths. The FoodScan™ 2 system was

calibrated against standard reference methods. Samples were inserted into the instrument, and spectral data were collected and processed using FOSS software.

Statistical Analysis

The statistical analysis was conducted using SAS (Version 9.4; SAS Inst., Inc., Cary, NC) PROC GLIMMIX. The experimental unit was individual carcasses. The fixed effects were muscle, freezing treatment, and aging period. Data was analyzed as a split-split plot design. The whole plot factor was muscle, the subplot factor was aging period, and the sub-subplot was freezing treatment. An alpha of 0.05 was set for a level of significance.

Results

Consumer Demographics

Demographic characteristics of consumers ($N = 192$) are summarized in Table 2.1. Panelists were predominantly female (53.13%) compared to male (46.88%). Participants were primarily between the ages of 20-39 years old (48.43%). Additionally, a majority of the consumers were Caucasian (77.08%), while African American, Asian, Latino, Mixed Race, and others were represented in smaller proportions. Most consumers were married (61.98%). Additionally, household income over $75,000 (56.77%) and have at least one college degree (81.77%). Flavor was selected as the most important palatability trait (47.92%) and the most common beef consumption frequency was 3 times per week (29.17%).

Consumers rated the importance of 16 purchasing motivators by the importance in their household (Table 2.2). Price was rated the most ($P < 0.05$) important compared to all motivators besides color. While color and marbling were, was rated more important ($P < 0.05$) than familiarity with cut, nutrient content, animal-welfare claims, eating satisfaction, fresh vs. frozen

claims, and antibiotic use. Natural/organic claims, brand, and grain-fed claims were rated less important ($P < 0.05$) than hormone use and packaging type.

Consumer Sensory Evaluation

Consumer sensory evaluation data is presented in Table 2.3 and 2.4. There was an interaction ($P < 0.05$) for juiciness, but not for tenderness, flavor liking, or overall liking, therefore, main effects were evaluated. Overall, the freezing treatment and aging period did not impact ($P > 0.05$) tenderness, flavor liking, or overall liking scores for the consumer. However, as expected, the tenderness scores were significantly greater for the *Longissimus lumborum* (LL) being the most tender ($P < 0.05$) followed by the *Semitendinosus* (ST) and then the *Biceps femoris* (BF) ($P < 0.05$). Within flavor liking, the consumers rated the LL the highest ($P < 0.05$) compared to all other muscles while, the ST and BF were similar ($P > 0.05$). For overall liking, the consumers rated the LL the highest ($P < 0.05$) followed by the ST and then the BF. There was an interaction ($P < 0.05$) for juiciness within freezing order, muscle, and aging period. Overall, there were no differences ($P > 0.05$) for the LL between aging periods and treatment combinations for juiciness ratings. Moreover, there were no differences ($P > 0.05$) in the BF across all aging periods and freezing treatments for juiciness ratings, showing that there is no perceived increase in juiciness. However, , the 21-day AF ST samples had a lower ($P < 0.05$) juiciness rating compared to the 28-day AF ST samples while there were no differences ($P > 0.05$) between the 28-day AF ST samples and the 28-day FA ST samples. Therefore, the juiciness interaction appeared to be driven by the impact of aging period and treatment on the ST. Within juiciness, flavor liking, tenderness, and overall acceptability, there were no differences ($P > 0.05$) in the percentage of consumers that rated the freezing treatment and aging period steaks as acceptable. Similar to the sensory scores, muscle impacted ($P < 0.05$) each

acceptability trait. For juiciness and flavor acceptability, the LL had the highest ($P < 0.05$) percentage of consumers rate it as acceptable while the ST and BF resulted in similar ($P > 0.05$) percentages in acceptability. Finally, for tenderness and overall acceptability, yet again, the LL yielded the highest ($P < 0.05$) percentage of consumers rating it as acceptable while the lowest ($P < 0.05$) percentage of consumers rated the BF steaks as acceptable.

Warner–Bratzler Shear Force

The Warner–Bratzler Shear Force data is presented in Table 2.5 which supports the consumer sensory results. There were no significant differences ($P > 0.05$) in shear force values between the AF (3.93 kg) and FA (4.12 kg) steaks. Additionally, there were no differences ($P > 0.05$ kg) between the 21-day aged samples (3.81kg) and the 28-day aged samples (4.24 kg). As expected, these data indicated the LL resulted in the lowest shear force ($P < 0.05$) value (2.76 kg), being the most instrumentally tender, while there were no significant differences ($P > 0.05$) between the ST (4.06 kg) and the BF (5.26 kg).

Raw Color

Raw color is presented in Tables 2.6, 2.7, and 2.8. Within raw color there was an interaction ($P < 0.05$) for L^* between aging period, freezing treatment, and muscle. The LL steaks displayed similar ($P > 0.05$ L^* values regardless of aging period and freezing treatment. However, the 21-day AF ST had the highest ($P < 0.05$) L^* reading compared to all treatment combinations, besides the 21-day AF BF, 21-day FA BF, and the 28-day AF ST ($P > 0.05$). Moreover, the 28-day AF BF had a lower ($P < 0.05$) L^* reading compared both the 21-day AF and FA BF. Finally, there were no differences ($P > 0.05$) in L^* values between muscles for the 28-day FA samples.

There was also an interaction in a^* values between the freezing treatment and muscle ($P < 0.05$), with the AF ST having the highest ($P < 0.05$) a^* value being the brightest, most cherry red in appearance. Surprisingly, the AF LL had a lower ($P < 0.05$) a^* value compared to the FA LL. There were no differences ($P > 0.05$) in a^* values between the AF BF and the FA BF.

Moreover, there was an interaction ($P < 0.05$) in b^* readings between the freezing treatment and muscle ($P < 0.05$), with the AF ST, having the highest b^* value. The FA LL had a greater ($P < 0.05$) b^* reading compared to the AF LL. There were no differences ($P > 0.05$) for BF in b^* values between the AF and FA freezing treatments. Moreover, the AF ST samples had a larger ($P < 0.05$) b^* value compared to the BF and LL. Finally, the FA ST had a greater ($P < 0.05$) b^* value compared to the FA BF.

Additionally, there was an interaction for metmyoglobin, deoxymyoglobin, and oxymyoglobin between aging period and muscle ($P < 0.05$). For metmyoglobin, the 21-day aged BF resulted in the highest percentage of metmyoglobin. However, there were no differences ($P > 0.05$) between aging periods for the LL and ST in the percentage of metmyoglobin. While each muscle resulted in different ($P < 0.05$) metmyoglobin percentages within the 21-day aged samples (BF > LL > ST), there were no differences ($P > 0.05$) between muscle for the 28-day aged samples. For deoxymyoglobin, the 21-day aged LL had a higher percentage ($P < 0.05$) of deoxymyoglobin compared to the 28-day aged LL. However, the 28-day aged ST had a higher percentage ($P < 0.05$) of deoxymyoglobin compared to the 21-day aged ST. There were no differences ($P > 0.05$) in percentages of deoxymyoglobin between aging periods for the BF samples. For oxymyoglobin, the 28-day aged steaks, the LL had a higher percentage ($P < 0.05$) of oxymyoglobin compared to the 21-day aged LL. In contrast to the LL, the 21-day aged ST had a greater ($P < 0.05$) percentage of oxymyoglobin compared to the 28-day aged ST. Similar to the

LL, the 28-day aged BF had a higher percentage ($P < 0.05$) of oxymyoglobin compared to the 21-day aged BF.

Cooked Color

For cooked color (Tables 2.9 and 2.10), all three main effects were displayed. Freezing treatment, aging period, and muscle all impacted ($P < 0.05$) the L^* values. For freezing treatment, the AF samples had a higher ($P < 0.05$) L^* value compared to the FA samples. Additionally, the aging period was significant ($P < 0.05$) as the 21-day aging period samples had a higher L^* value compared to the 28-day aging period samples. Lastly, the ST samples had the highest ($P < 0.05$) L^* values, followed by the LL, and then the BF. However, there were no notable differences for a^* values ($P > 0.05$) between any factor evaluated. Finally, only the aging period was significant ($P < 0.05$) for b^* values as the 21-day aged steaks had higher ($P < 0.05$) b^* values compared to the 28-day aged samples.

There were no significant differences *(*$P > 0.05$) in metmyoglobin percentages for the freezing treatment, aging period, or muscle. However, there was a significant difference ($P < 0.05$) between the aging periods for oxymyoglobin with the 21-day aged samples resulting in a higher percentage of oxymyoglobin compared to the 28-day aged samples. Additionally, the ST resulted in a higher ($P < 0.05$) percentage of oxymyoglobin compared to the BF, being similar ($P > 0.05$) to the LL. ($P < 0.05$). Finally, there was an interaction ($P < 0.05$) in the percentage of deoxymyoglobin between the aging periods and muscle. The 21-day aged LL had a lower ($P < 0.05$) percentage of deoxymyoglobin compared to the 28-day aged LL. Likewise, the 21-day aged ST had a lower ($P < 0.05$) percentage of deoxymyoglobin compared to the 28-day aged ST. There were no differences ($P > 0.05$) between the 21-day aged and 28-day aged BF. Finally, the 21-day aged BF had a higher ($P < 0.05$) percentage of deoxymyoglobin compared to the LL and

ST of the same aging period while all three muscles resulted in similar ($P > 0.05$) percentages after being aged for 28 days.

Purge Loss

Purge loss results are presented in Table 2.5. There were no interactions ($P < 0.05$) for purge loss, therefore, main effects were evaluated. There was an increase ($P < 0.05$) in purge loss in the FA samples compared to the AF samples (12.49% vs 8.57%). Additionally, the LL had the lowest ($P < 0.05$) percentage of purge loss, while there were no differences ($P > 0.05$) between the ST (12.79%) and BF (12.59%). There were no significant differences ($P > 0.05$) between the 21-day aged (10.39%) and the 28-day aged (10.67%) samples.

Cook Loss

Cook loss results are presented in Table 2.5. There were no interactions ($P > 0.05$) for cook loss, therefore, main effects were evaluated. The LL had the lowest ($P < 0.05$) percentage (11.51%) of cook loss, followed by the ST (19.56%) and then the BF (16.10%) ($P < 0.05$). Moreover, the FA samples had the lowest ($P < 0.05$) percentage of cook loss compared to the AF samples (15.14% vs 16.31%, respectively). There was no significant difference ($P > 0.05$) between the two different aging periods.

Proteolysis

Desmin degradation (Table 2.11 and Table 2.12) revealed an interaction between freezing treatment and aging period across all muscles combined. Specifically, the 28-day FA samples had a lower percentage ($P < 0.05$) of intact desmin compared to the 21 and 28-day AF samples indicating more proteolytic activity occurred. Similarly, the 28-day FA steaks had a greater ($P < 0.05$) amount of degraded desmin compared to the 21-day AF steaks. There was no significant

difference ($P > 0.05$) for intact desmin among the three different muscles. However, the BF had a greater amount ($P < 0.05$) of degraded desmin compared to the LL and ST.

Discussion

Freezing and aging are two of the most common postmortem technologies in beef production. Many studies have compared how freezing order affects physicochemical traits (Lagerstedt et al., 2008; Leygonie et al., 2012). However, relatively few have examined consumer perception across multiple muscles. Addressing this gap, the present study found that freezing sequence did not significantly affect consumer ratings of tenderness, flavor, or overall acceptability. Consumers could not distinguish between samples aged then frozen and frozen then aged. This indicates that although freezing order can alter biochemical processes, these changes do not always reach a perceptible threshold for consumers (Warner et al., 2017; Setyabrata et al., 2019; Kim et al., 2018).

Previous studies have provided a mixed picture regarding the potential sensory impact of altering the freezing sequence. Setyabrata et al. (2019) utilized samples that were aged only, frozen first, then-thawed/aged, and aged first, then-frozen/thawed, where each sample was aged for seven days. This study found no difference in shear force values or desmin degradation between the freezing first then-thawed/aged and aging first then-frozen/thawed samples, suggesting minimal improvement in tenderness between the two treatments (Setyabrata et al., 2019). In contrast, Lagerstedt et al. (2008) found that freezing prior to aging reduced shear force values in LL steaks. However, consumer responses did not always align with the instrumental tenderness results. These studies indicated that while freezing order can influence instrumental tenderness and proteolytical pathways. This potentially occurs through partial inactivation of calpastatin activation (Koohmaraie et al., 1995; Kim et al., 2015). These changes are often subtle

and inconsistently perceived by consumers. These current findings provide further evidence that biochemical shifts induced by the freezing sequence do not always support differences in eating quality.

The lack of sensory differences observed in the present study can be attributed to several factors. First, the magnitude of biochemical changes caused by the freezing sequence may not have been large enough to exceed the consumer detection thresholds, particularly in untrained panels that require larger contrasts to register perceivable differences (Miller et al., 2001). Second, variability among steaks within a muscle can mask treatment effects (Leygonie et al., 2012; Warner et al., 2017). Finally, aging itself remains a driver of tenderization. Once sufficient postmortem aging has occurred, the effects of freezing sequence are unlikely to shift consumer perception (Koohmaraie & Geesink, 2006; Grayson et al., 2014). Together, these explanations support the marginal differences in consumer perceptions observed within the freezing sequence.

Muscle differences have a long-established influence on palatability, and the current study reaffirmed this pattern. This study found that the LL was consistently rated higher than the ST and BF for tenderness, flavor, overall liking, and overall acceptability. This mirrors decades of research documenting muscle-specific variation in consumer acceptability (O'Quinn et al., 2018; Gruber et al., 2006; King et al., 2009). These differences stem from structural and biochemical factors, including connective tissue content, fiber type orientation, and proteolytic capacity, which collectively affect tenderness potential (Koohmaraie & Geesink, 2006; Huffman et al., 1996). Thus, muscle type continues to serve as a reliable predictor of consumer sensory ratings, both in the present study and across the literature.

Postmortem aging is also a well-understood mechanism for improving tenderness through enzymatic degradation of myofibrillar and cytoskeletal proteins (Koohmaraie, 1992; Koohmaraie

& Geesink, 2006; Kemp et al., 2010). Numerous studies have demonstrated that extending aging periods increases tenderness up to a point, after which additional improvements plateau (Grayson et al., 2014). However, within the current study, no differences were found in consumer-perceived tenderness ratings. Thus, it can be concluded that since the aging periods were only 7 days apart, these differences were not strong enough to alter consumer perception of tenderness or overall liking. This finding is consistent with work showing that once muscles surpass a consumer-acceptable threshold, additional aging may not yield perceptible sensory gains (Miller et al., 2001).

Instrumental Tenderness: Shear Force

Shear force measurements remain one of the most widely accepted instrumental methods for evaluating tenderness, consistently ranked as one of the most important drivers of consumer satisfaction with beef (Killinger et al., 2004; O'Quinn et al., 2018; Gruber et al., 2006). It has been widely researched that freezing influences and impacts tenderness (Grayson et al., 2014; Wheeler et al., 1997; Colle et al., 2015). Studies have reported that freezing and thawing disrupt the muscle fiber structure, resulting in lower shear force values due to the weakening of the myofibrillar structure (Lagerstedt et al., 2008; Leygonie et al., 2012).

The current study found no differences in shear force values between the frozen then aged samples and the aged then frozen samples. Several other studies have investigated the impact of altering the freezing sequence on tenderness with differing results (Stafford et al., 2024; Setyabrata et al., 2019). Stafford et al. (2024) found that steaks that were frozen had a shear force value of 10 N less than the samples that were unfrozen. Supporting this, Greyson et al. (2014) found that the freeze then age samples did have a lower shear force value compared to the fresh and freeze only samples. Moreover, Setyabrata et al. (2019) utilized both the LL and ST

within three freezing treatments that included: Aging only, freezing first then-thaw/aged, and aging first then-frozen/thawed. The Setyabrata et al. (2019) study found there were no differences in shear force values within the freezing first then-thaw/aged samples compared to the aging first then-frozen/thawed samples between both the LL and the ST.

Numerous studies have shown that increasing aging duration consistently decreases shear force values in the LL (Grayson et al., 2014; Wheeler et al., 1997; Shackelford et al., 1991). Conversely, this study found no differences in shear force values between the two aging periods. This can be attributed to the fact that there was not a major difference in the duration of the two aging periods.

One of the main influences on tenderness variations within the present study was muscle type. Consistent with previous research, the LL was the most tender compared to the ST and BF. The LL typically demonstrates lower shear force values due to its lower collagen concentration and more oxidative muscle fiber type, which is generally smaller and can allow more degradation during postmortem aging (Koohmaraie et al., 2002; Grayson et al., 2014). In contrast, the ST and BF contain greater connective tissue and exhibit variation in calpain activity, resulting in slower structural protein breakdown and reduced tenderization potential (Colle et al., 2015). While postmortem freezing sequences adjustments have been thought to influence tenderness, the present study found that intrinsic muscle factors remain the dominant determinant of shear force values.

Color Stability Across Freezing and Aging Treatments

Color has consistently been a critical visual cue that influences beef purchasing and consumption decisions (Killinger et al., 2004; Neethling et al., 2017). Multiple studies have examined the differences in color with the altering of the freezing sequence (Colle et al., 2016;

Stafford et al., 2024). The current study found that increasing the aging period in both frozen-then-aged and aged-then-frozen samples did not change *L** values for LL and BF. This can be attributed to stable muscle pigments and minimal protein denaturation during the aging periods and freezing cycles, resulting in consistent lightness across treatments. Previous research also found that increased aging did not affect BF *L** values (Colle et al., 2016). In contrast, Stafford et al. (2024) reported that longer aging in LL increased *L** values, producing a lighter product however different aging periods were used. Additional prior studies showed that longer aging periods can increase *L** values due to more protein denaturation and water loss, altering light reflectance on the muscle (Mancini & Hunt, 2005; Bekhit et al., 2013).

There were minimal differences for *L** values for muscles of equal aging periods and different freezing treatments found in this study. This result is surprising due to the impact the freezing cycle had on water holding capacity. The only significant finding was within the ST muscle aged for 21 days. Supporting this, Setyabrata et al. (2019) found that there were no significant differences in *L** values between the freezing first then-thaw/aged and aging first then-frozen/thawed samples on initial day of display.

For *a** values, the current study had found no differences between the *a** values between the two aging periods but an interaction was found between muscle and freezing treatment. The aged then frozen LL and ST yielded a higher *a** value compared to the frozen then aged samples. This finding is supported by previous studies indicting freezing, especially, freezing and then aging, can cause damage to cell membranes, releasing prooxidants while decreasing the efficacy of reduction pathways such as metmyoglobin reducing activity (Beyer et al., 2024; Hashem et al., 2023).

No differences in *b** values were found between aging periods. Frozen then aged LL samples had higher *b** values than aged then frozen samples. In contrast, aged then frozen ST samples had higher *b** values than frozen then aged samples.

When evaluating cooked color, both the aging period and freezing sequence influenced instrumental color values. Steaks from the aged then frozen treatment showed higher *L** values than frozen then aged samples, although *a** values did not differ. Steaks aged for 21 days had higher *L** and *b** values than those aged for 28 days. This suggests that longer storage before cooking slightly darkened the product and reduced yellowness. These findings show that the postmortem processing sequence can change cooked color stability. As expected, muscle type most strongly affected cooked color. The ST steaks showed the highest *L** values, while BF was consistently the darkest after heating. These results support earlier reports that LL steaks retain higher *L** and *a** values compared to BF, which appears darker and less red after cooking (Hunt et al., 1991; King et al., 2009).

Moisture Loss: Purge, Cook Loss, and Economic Implications

Water-holding capacity, expressed as purge and cook loss, is a critical quality attribute influenced by both freezing and aging processes, and has direct implications for product yield and consumer perception (Huff-Lonergan & Lonergan, 2005; Leygonie et al., 2012). One aspect of freezing that has been under scrutiny within the industry is the increase in economic loss that is correlated to moisture loss (Kim et al., 2018; Stafford et al., 2024; Edwards et al., 2025). Additionally, it has been shown that the utilization of freezing has a negative impact on juiciness attributes, such as cook loss and purge loss (Leygonie et al 2012; Dang et al., 2021).

Previous research has shown that altering the freezing sequence can drastically impact the amount of purge loss and cook loss observed (Setyabrata et al., 2019; Stafford et al., 2024).

Setyabrata et al. (2019) reported that the freezing first, then-thaw/aged samples had a significantly higher purge loss compared to the aging first, then-frozen/thawed samples. Additionally, Stafford et al. (2024) found that the frozen samples had a significantly greater amount of purge loss compared to the unfrozen samples. The increase in purge loss found in this study was a crucial component, as the frozen then aged steaks exhibited a higher percentage of purge loss (12.49%) compared to the aged then frozen samples (8.57%). This increase in purge loss is a critical concern because it results in direct economic losses due to reduced saleable weight (Lagerstedt et al., 2008; Leygonie et al., 2012).

The aging period is another key factor in moisture loss. Colle et al. (2016) reported that within the BF, purge loss increased as aging time extended, particularly when evaluated across longer durations (2, 14, 21, 42, and 63 days). This was attributed to collagen content and differences in muscle fiber orientation, which influence water-holding capacity (Lorenzen et al., 2007; King et al., 2009). However, the present study did not observe differences in purge loss between the 21-day aged (10.39%) and 28-day aged (10.67%) samples, suggesting that moisture retention may not be significant during shorter aging periods.

Muscle type plays a crucial role in determining moisture loss due to variations in fiber orientation, collagen content, and metabolic properties. For instance, within the BF and other highly glycolytic muscles, higher purge loss is often observed due to a greater tendency for water to be displaced from the muscle structure during storage and processing (Huff-Lonergan & Lonergan, 2005; Warner et al., 2010). Additionally, differences in sarcomere length, myofibrillar spacing, and connective tissue among muscle types further influence both purge and cook loss outcomes (Honikel, 1998; Offer & Cousins, 1992). In the present study, muscle type was a significant determinant of moisture retention, with the ST and BF exhibiting greater purge and

cook losses compared to the LL. This finding is consistent with previous research demonstrating that tougher, collagen-rich muscles tend to lose more water during both storage and cooking (King et al., 2009; Colle et al., 2015). Structural characteristics such as shorter sarcomeres in the ST and higher connective tissue density in the BF contribute to increased fluid displacement during postmortem handling (Nishimura, 2010; Purslow, 2014). Conversely, the LL, which possesses an alternate fiber arrangement and lower collagen solubility, retained moisture more effectively, resulting in lower purge and cook losses across treatments (Koohmaraie et al., 2002; Wheeler et al., 2005).

Cook loss is another key indicator of water-holding capacity and is influenced by postmortem handling strategies and can affect the overall perceived juiciness (Huff-Lonergan & Lonergan, 2005; Leygonie et al., 2012). The current study found that the aged then frozen samples had a significantly higher cook loss (16.31%) compared to the frozen then aged samples (15.14%). In the present study, freezing followed by aging resulted in greater purge loss but lower cook loss. This relationship occurs because a substantial portion of water is expelled as purge during thawing, leaving less available moisture to be lost during cooking (Leygonie et al., 2012; Wheeler er al., 1990). Thus, while purge loss represents a yield disadvantage during storage and handling, it concurrently reduces the amount of fluid lost as cook loss once the product is thermally processed. This pattern is consistent with previous reports that structural damage from freezing promotes fluid displacement into extracellular spaces (Zhang et al., 2022). When combined with subsequent proteolytic changes during aging, this water is released as purge rather than being retained for later loss during cooking (Leygonie et al., 2012; Kim et al., 2015 These results contrast with Setyabrata et al. (2019), who reported no differences between freezing sequences when comparing freeze-first then thaw/aged versus age-first then

frozen/thawed samples. Likewise, Stafford et al. (2024) found no significant differences between freezing treatments or aging periods when comparing unfrozen and frozen samples. Collectively, these discrepancies highlight that the relationship between freezing sequence and cook loss remains inconsistent across studies, suggesting that outcomes may depend on subtle differences in muscle type, freezing protocol, or post-thaw handling.

Protein Degradation and the Role of Desmin

Desmin, a key intermediate filament protein, plays a central role in maintaining myofibrillar structure by linking Z-disks to the sarcolemma and anchoring myofibrils together (Huff-Lonergan & Lonergan, 2005). Its degradation during postmortem aging is closely associated with the disruption of muscle ultrastructure and the subsequent improvement in tenderness (Koohmaraie & Geesink, 2006; Melody et al., 2004). The calpastatin theory helps explain variability in postmortem proteolysis and tenderness, as calpastatin inhibits calpains and slows degradation of proteins like desmin (Koohmaraie, 1992; Goll et al., 2003; Lonergan et al., 2010). High calpastatin activity reduces proteolysis, whereas reduced activity allows greater desmin breakdown and improved tenderness (Koohmaraie et al., 1995; Morgan et al., 1993; Shackelford et al., 1994; Huff-Lonergan & Lonergan, 2005). Freezing may partially inactivate calpastatin and enhance proteolysis, however, more recent work reports inconsistent effects depending on the freezing rate, storage conditions, and muscle type (Koohmaraie et al., 1987; Leygonie et al., 2012; Kim et al., 2015).

Setyabrata et al. (2019) reported that freezing followed by aging resulted in greater desmin degradation compared to aging only, suggesting that structural disruptions caused by ice crystal formation may enhance proteolysis upon thawing. However, they found no significant differences between the freezing first then-thaw/aged and aging first then-frozen thawed samples

indicating that the sequence of freezing relative to aging may not consistently alter desmin degradation patterns (Setyabrata et al., 2019). These findings align with Warner et al. (2017), who noted that freezing can increase myofibrillar fragmentation, but the magnitude of its effect often depends on the rate of freezing and muscle type. Similarly, Leygonie et al. (2012) emphasized that while freezing generally increases proteolytic susceptibility due to physical damage to membranes, its effect on protein degradation is not uniform across studies. This conflicts with the current study, which found that the 21-day aged then frozen samples did have a greater amount of intact desmin compared to the 28-day frozen then aged samples. This then translated into the 28-day aged samples having a greater abundance of degraded desmin in bands three and four compared to the 21-day aged, then frozen samples.

Muscle type also plays an important role in desmin degradation. Setyabrata et al. (2019) observed that the LL exhibited less desmin degradation compared to the ST, reflecting inherent differences in fiber type composition and proteolytic enzyme activity. This is consistent with the current study, as the BF displayed a greater abundance of degraded desmin compared to the LL and ST. Although, no differences were observed in the amount of intact desmin among the three muscles. These variations likely stem from differences in calpain activity, calpastatin inhibition, and structural protein composition (Koohmaraie, 1992; Goll et al., 2008).

The current study demonstrates a degree of desmin degradation across muscles and treatments that exhibit biochemical changes in tenderness. However, this change in tenderness was not significant enough to elicit a difference in consumer perception of tenderness.

Conclusion

These findings demonstrate that altering the freezing sequence under standard commercial aging periods has limited sensory impacts. Although biochemical and instrumental

differences were observed, consumers were unable to distinguish between aged then frozen and frozen then aged samples. This indicates that the tenderness changes that were seen in altering the freezing sequence did not exceed the consumer sensory detection thresholds. Moisture loss was more sensitive to freezing sequence, with the frozen then aged steaks exhibiting a greater amount of purge loss, resulting in a greater economic loss for the processors. While there were differences in the degradation of desmin across muscles and treatments, these biochemical shifts did not translate into a large enough difference for consumer-perceived differences in tenderness. Collectively, these results suggest that it is not economically feasible to alter the freezing sequence at an industry level, as the added economic cost is not accompanied by improvements in consumer palatability.

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Table 2.1: Demographic characteristics of consumers (N = 192) who participated in consumer sensory panels.

Characteristic	Response	Percentage of Consumers
Gender	Male	46.88
	Female	53.13
Age	Under 20	2.08
	20-29	21.35
	30-39	27.08
	40-49	18.75

	50-59	16.15
	Over 60	14.58
Ethnicity	African American	4.69
	Asian	8.85
	Caucasian	77.08
	Latino	7.81
	Mixed Race	1.04
	Other	0.52
Marital Status	Married	61.98
	Single	38.02
Household Size	1 person	23.96
	2 people	32.81
	3 people	14.58
	4 people	22.40
	5 people	3.65
	6 people	1.04
	More than 6 people	1.56
Household Income	Under $25,000	14.06
	$25,000 - $34,999	4.69
	$35,000 - $49,999	7.29
	$50,000 - $74,999	17.19
	$75,000 - $99,999	14.58
	$100,000 - $149,999	21.35
	$150,000 - $199,999	15.63
	Over $199,999	5.21
Education Level	Non-High School Graduate	1.56
	High School Graduate	5.21
	College Graduate	39.58
	Post-College Graduate	42.19
	Some College / Technical School	11.46
Most Important Palatability Trait	Flavor	47.92
	Juiciness	8.85
	Tenderness	43.23
Weekly Beef Consumption	0	0.52
	1	6.25
	2	17.19
	3	29.17
	4	10.94
	5	11.46
	6	11.98
	7	4.7
	8	2.08
	9	3.13
	10	0
	11	1.04
	12	1.56
	13	0
	14	0.52

Table 2.2 Fresh beef purchasing motivators of consumers (N=192) who participated in consumer sensory panels.

Charatcteristic	Response
Antibiotic use in animals	51.74[gh]
Brand of product	39.34[j]
Animals fed a grain-based diet	39.76[j]
Color	71.75[ab]
Familiarity with cut	64.07[cd]
Animals fed a grass-based diet	44.31[ij]
Fresh or frozen claims	54.56[fgh]
Growth hormones used in animals	49.37[hi]
Marbling	70.08[b]
Natural/organic claims	39.29[j]
Nutrient content	60.94[de]
Packaging	45.56[i]
Price	76.81[a]
Eating Satisfaction claims	56.89[efg]
Size, weight and thickness	69.46[bc]
Animal welfare	57.99[ef]
SEM[1]	1.96
P-Value	<0.01

[abcdefghij] Means within the same column without a common superscript differ ($P < 0.05$).

[1]SE (largest) of the least squares means

Table 2.3 Least squares means of consumer sensory panelist palatability ratings1 for steaks with two freezing treatments, two aging periods, and three muscles.

Treatment	Tenderness	Flavor	Overall Liking	Juiciness Acceptability	Flavor Acceptability	Tenderness Acceptability	Overall Acceptability
Freezing Treatment[2]							
AF	58.09	64.51	61.93	89.40	91.18	84.31	88.17
FA	61.09	65.80	64.05	89.37	91.14	88.23	91.85
SEM[3]	1.69	1.06	1.41	0.02	0.02	0.03	0.03
P-Value	0.08	0.23	0.13	0.98	0.98	0.16	0.10
Aging Period							
21 d	59.02	64.07	62.50	89.71	92.79	84.68	98.88
28 d	60.17	66.24	63.40	89.06	89.53	87.86	91.07
SEM[3]	1.80	1.12	1.44	0.03	0.02	0.03	0.02
P-Value	0.49	0.06	0.58	0.78	0.07	0.28	0.86
Muscle[4]							
LL	76.35[a]	73.96[a]	75.03[a]	94.92[a]	98.42[a]	99.91[a]	99.91[a]
ST	57.66[b]	61.66[b]	61.09[b]	89.06[b]	88.73[b]	87.49[b]	90.77[b]
BF	44.77[c]	59.84[b]	52.84[c]	84.17[b]	86.33[b]	69.42[c]	78.67[c]
SEM[3]	2.07	1.35	1.84	0.03	0.03	0.03	0.03
P-Value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[abc] Means within the same column without a common superscript differ ($P < 0.05$).
[1]Sensory Scores: 0 = extremely dry/tough/dislike; 50 = neither dry nor juicy, neither tough nor tender, neither like nor dislike; 100 = extremely juicy/tender/like extremely
[2] AF: Aging and then freezing, FA: Freezing and then aging
[3]SE (largest) of the least squares means
[4]LL: *Longissimus lumborum, ST: Semitendinosus,* BF: *Biceps femoris*,

Table 2.4 Least squares means of consumer sensory panelist juiciness palatability ratings1 for steaks with two freezing treatments, two aging periods, and three muscles.

Age	Treatment[2]	Muscle[3]	Juiciness
21 d	AF	LL	70.60[ab]
		ST	54.52[e]
		BF	54.98[e]
	FA	LL	69.20[abc]
		ST	63.31[bcd]
		BF	54.76[e]
28 d	AF	LL	69.76[abc]
		ST	62.88[cd]
		BF	61.26[de]
	FA	LL	71.09[a]
		ST	59.34[de]
		BF	54.16[e]
SEM[4]			
P-Value			0.01

[abcde] Means within the same column without a common superscript differ ($P < 0.05$).
[1]Sensory Scores: 0 = extremely dry/tough/dislike; 50 = neither dry nor juicy, neither tough nor tender, neither like nor dislike; 100 = extremely juicy/tender/like extremely
[2] AF: Aging and then freezing, FA: Freezing and then aging
[3]LL: *Longissimus lumborum, ST: Semitendinosus,* BF: *Biceps femoris*
[4]SE (largest) of the least squares means

Table 2.5 Least square means of purge loss, cook loss, and Warner–Bratzler shear force (WBSF) of steaks with two freezing treatments, two aging periods, and three muscles.

Treatment	Purge Loss	Cook Loss	Shear Force[1]
Freezing Treatment[2]			
AF	8.57[b]	16.31[a]	3.93
FA	12.49[a]	15.14[b]	4.12
SEM[3]	0.35	0.51	0.51
P-Value	<0.01	0.02	0.70
Aging Period			
21 d	10.39	15.97	3.81
28 d	10.67	15.48	4.24
SEM[3]	0.35	0.51	0.51
P-Value	0.41	0.33	0.40
Muscle[4]			
LL	6.21[b]	11.51[c]	2.76[b]
ST	12.79[a]	19.56[a]	4.06[a]
BF	12.59[a]	16.10[b]	5.26[a]
SEM[3]	0.60	0.63	0.63
P-Value	<0.01	<0.01	<0.01

[abc] Means within the same column without a common superscript differ ($P < 0.05$).
[1]Warner-Bratzler shear force; kgs
[2]AF: Age and then freeze, FA: Freeze and then age
[3]SE (largest) of the least squares means
[4]LL: *Longissimus lumborum, ST: Semitendinosus,* BF: *Biceps femoris*

Table 2.6 Least square means of CIE L* for bloomed raw internal color of steaks with two freezing treatments, two aging periods, and three muscles.

Age	Treatment[1]	Muscle[2]	*L**[3]
21 d	AF	LL	44.44[bcd]
		ST	48.42[a]
		BF	46.22[ab]
21 d	FA	LL	42.87[d]
		ST	46.14[b]
		BF	46.65[ab]
28 d	AF	LL	43.51[cd]
		ST	46.45[ab]
		BF	44.94[bcd]
28 d	FA	LL	43.56[cd]
		ST	45.49[bc]
		BF	43.46[cd]
SEM[4]			1.28
P-Value			0.03

[abcd] Means within the same column without a common superscript differ ($P < 0.05$).
[1]AF: Age and then freeze, FA: Freeze and then age
[2]LL: *Longissimus lumborum, ST: Semitendinosus,* BF: *Biceps femoris*
[3]*L**: 0 = black, 100 = white
[4]SE (largest) of the least squares means

Table 2.7 Least square means of CIE a* and b* for bloomed raw internal color of steaks with two freezing treatments, two aging periods, and three muscles.

Treatment[1]	Muscle[2]	*a**[3]	*b**[4]
AF	LL	18.30[d]	15.19[d]
	ST	22.72[a]	18.52[a]
	BF	18.86[cd]	16.26[c]
FA	LL	20.40[bc]	16.60[bc]
	ST	21.04[b]	17.53[b]
	BF	18.34[d]	15.85[cd]
SEM[5]		0.98	0.48
P-Value		<0.01	<0.01

[abcd] Means within the same column without a common superscript differ ($P < 0.05$).
[1]AF: Age and then freeze, FA: Freeze and then age
[2] LL: *Longissimus lumborum, ST: Semitendinosus,* BF: *Biceps femoris*,
[3]*a**: -60 = green, 60 = red
[4]*b**: -60 = blue, 60 = yellow
[5]SE (largest) of the least squares means

Table 2.8 Least square means of relative percentages of oxymyoglobin (OMb), deoxymyoglobin (DMb), and metmyoglobin (MetMb), bloomed raw internal color of steaks with two freezing treatments, two aging periods, and three muscles.

Age	Muscle[1]	MetMb%	DMb%	OMb%
21 d	LL	39.20^{b}	11.56^{a}	49.25^{d}
	ST	32.80^{c}	8.17^{c}	59.03^{a}
	BF	44.22^{a}	9.66^{bc}	46.12^{d}
28 d	LL	36.49^{bc}	9.12^{bc}	54.39^{ab}
	ST	36.41^{bc}	10.02^{ab}	53.57^{bc}
	BF	36.86^{bc}	9.87^{abc}	53.28^{bc}
SEM[2]		2.30	0.94	2.37
P-Value		<0.01	<0.01	<0.01

^{abcd} Means within the same column without a common superscript differ ($P < 0.05$).
[1] LL: *Longissimus lumborum, ST: Semitendinosus,* BF: *Biceps femoris*,
[2]SE (largest) of the least squares means

Table 2.9 Least square means of L*, a*, b*, and relative percentages of oxymyoglobin (OMb), and metmyoglobin (MetMb), for bloomed internal cooked color of steaks with two freezing treatments, two aging periods, and three muscles.

Treatment	*L** [1]	*a** [2]	*b** [3]	MetMb%	OMb%
Freezing Treatment[4]					
AF	56.32[a]	19.69	19.01	32.03	60.30
FA	55.48[b]	19.43	18.80	33.02	59.01
SEM[5]	0.33	0.48	0.25	1.04	1.06
P-Value	0.01	0.60	0.40	0.34	0.23
Aging Period					
21 d	56.25[a]	19.63	19.17[a]	32.40	60.83[a]
28 d	55.55[b]	19.49	18.64[b]	32.64	58.48[b]
SEM[5]	0.33	0.48	0.25	1.04	1.06
P-Value	0.04	0.76	0.04	0.82	0.03
Muscle[6]					
LL	55.97[b]	19.91	19.15	32.33	60.36[ab]
ST	58.05[a]	18.98	18.82	32.03	60.80[a]
BF	53.69[c]	19.79	18.74	33.20	57.80[b]
SEM[5]	0.71	0.87	0.40	1.27	1.30
P-Value	<0.01	0.52	0.55	0.63	0.05

[abc] Means within the same column without a common superscript differ ($P < 0.05$).
[1]*L**: 0 = black, 100 = white
[2]*a**: -60 = green, 60 = red
[3]*b**: -60 = blue, 60 = yellow
[4]AF: Aging and then freezing, FA: Freezing and then aging
[5]SE (largest) of the least squares means
[6]LL: *Longissimus dorsi, ST: Semitendinosus,* BF: *Biceps femoris*,

Table 2.10 Least square means of deoxymyoglobin (DMb) for bloomed raw internal color of steaks with two freezing treatments, two aging periods, and three muscles.

Treatment[1]	Muscle[2]	DMb%
21 d	LL	4.97[b]
	ST	6.23[b]
	BF	9.12[a]
28 d	LL	9.67[a]
	ST	8.10[a]
	BF	8.87[a]
SEM[3]		0.36
P-Value		<0.01

[ab] Means within the same column without a common superscript differ ($P < 0.05$).
[1] AF: Aging and then freezing, FA: Freezing and then aging
[2]LD: *Longissimus dorsi, ST: Semitendinosus,* BF: *Biceps femoris*
[3]SE (largest) of the least squares means

Table 2.11 Least square means of relative band concentrations of desmin within steaks with two freezing treatments, two aging periods, and three muscles.

Freezing Treatment[1]	Aging period	Intact Desmin	Degraded Desmin[2]	Degraded Desmin[2]
AF	21	1.01[a]	0.12[b]	0.24[b]
	28	0.92[ab]	0.14[ab]	0.34[ab]
FA	21	0.78[bc]	0.14[ab]	0.32[ab]
	28	0.59[c]	0.17[a]	0.44[a]
SEM[3]		0.07	0.01	0.04
P-Value		<0.05	<0.05	<0.05

[abc] Means within the same column without a common superscript differ ($P < 0.05$).
[1]AF: Aged then frozen samples; FA: Frozen then aged samples
[2]Degraded Desmin represents Bands 3 & 4 on the western blot
[3]SE (largest) of the least squares means

Table 2.12 Least square means of relative band concentrations of desmin within steaks within three muscles.

Muscle[1]	Intact Desmin	Degraded Desmin[2]	Degraded Desmin[2]
BF	0.77	0.27[a]	0.74[a]
LL	0.90	0.10[b]	0.16[b]
ST	0.80	0.06[c]	0.10[b]
SEM[3]	0.06	0.01	0.04
P-Value	0.32	<0.05	<0.05

[abc] Means within the same column without a common superscript differ ($P < 0.05$).
[1]LD: *Longissimus dorsi, ST: Semitendinosus,* BF: *Biceps femoris*
[2]Degraded Desmin represents Bands 3 & 4 on the western blot
[3]SE (largest) of the least squares means