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Freezing Promotes Postmortem Proteolysis in Beef by Accelerating the Activation of Endogenous Proteolytic Systems

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Abstract: This study investigated the effect of freezing and subsequent aging on beef quality, particularly focusing on the extent of postmortem proteolysis and tenderization. The *longissimus lumborum* muscle was collected from 8 steers 24 h postmortem, sliced into 8 2.5-cm-thick steaks, and randomly allocated into 4 groups. Treatment groups consisted of 1) aging at 4°C for 24 h; 2) aging for 168 h; 3) freezing at −20°C for 24 h followed by thawing/aging for 24 h; and 4) freezing for 24 h followed by thawing/aging for 168 h. In general, freezing decreased the color intensity of the steaks, whereas aging increased it ($P < 0.05$). Freezing also increased water loss, evidenced by greater drip loss and purge loss ($P < 0.05$). On the other hand, both freezing and aging improved beef proteolysis and tenderness ($P < 0.05$). This was associated with enhanced protease activity, indicated by greater calpain-1 autolysis and cathepsin B activity ($P < 0.05$). Additionally, freezing may have accelerated the activation of caspase-3, but our sampling timing did not permit verifying this possibility. This increase in the activity of proteases is likely caused by ice crystals disrupting cellular organelles, releasing factors that trigger their activation. In support of this, frozen steaks displayed an elevated level of free calcium and mitochondrial dysfunction ($P < 0.05$). Collectively, these findings suggest that freezing enhances postmortem proteolysis and tenderness in beef, likely by compromising key cellular organelles and subsequently accentuating the activity of several endogenous protease systems during aging.

Key words: freezing, tenderness, proteolysis, calpain-1, cathepsin B, caspase-3
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Introduction

Fresh meat is vulnerable to microbial spoilage and chemical reactions that lead to its deterioration. Both the meat industry and consumers utilize freezing to extend meat's shelf life and mitigate the loss of its quality characteristics. Although freezing induces physical and biochemical alterations in meat and may lead to undesirable quality traits like reduced color intensity and poor juiciness (Farouk et al., 2004; Jeong et al., 2011; Jia et al., 2022), its associated benefits ultimately outweigh its drawbacks. In addition, several studies have demonstrated that freezing positively affects meat tenderness (Lagerstedt et al., 2008; Qi et al., 2012). This has been attributed to the mechanical damage inflicted by ice crystals on the meat's myofibrillar structure (Leygonie et al., 2012a) and improved proteolysis (Crouse and Koohmaraie, 1990; Grayson et al., 2014; Setyabrata and Kim, 2019).

The calpains, cathepsins, and caspases are the major protease families that can potentially contribute to postmortem proteolysis (Sentandreu et al., 2002). Calpain-1 is a calcium-dependent, self-degrading protease located in the sarcoplasm and is considered the primary enzyme involved in postmortem proteolysis (Koohmaraie, 1992). It requires a calcium threshold of 3–50 μM to elicit half-maximal activity

(Goll et al., 2003). Upon activation, calpain-1 targets several key myofibrillar proteins (e.g., titin, nebulin, and desmin), thereby weakening the muscle structure and improving tenderness (Huff-Lonergan et al., 1996; Koohmaraie and Geesink, 2006). Cathepsins are a family of lysosomal proteases generally exhibiting optimal activity in acidic conditions (pH 3–6.5) (López-Bote, 2017). The cathepsin family comprises 15 members (Patel et al., 2018), with cathepsins B, D, and L as the main isoforms thought to be involved in postmortem meat tenderization (Sentandreu et al., 2002). However, the relatively high ultimate pH of fresh meat and the limited postmortem degradation of the lysosome can limit the cathepsins' contribution to postmortem proteolysis (Koohmaraie, 1994; Robert et al., 1999). Lastly, the caspases are a group of proteases known for their essential role in programmed cell death (Ouali et al., 2006; Sentandreu et al., 2002). The initiation of apoptosis is primarily governed by 2 key signaling pathways: the extrinsic (death receptor pathway) and the intrinsic pathway (mitochondrial pathway). Regardless of the initiating mechanism, a cascade of events is triggered, ultimately resulting in the activation of initiator caspases (such as caspase-2, -8, -9, and -10), which then activate downstream effector caspases (such as caspase-3, -6, and -7). However, the mitochondrial pathway has been deemed the predominant apoptotic pathway contributing to postmortem proteolysis in skeletal muscle (Kemp and Parr, 2012).

Enhanced postmortem proteolysis in frozen/thawed meat is probably associated with disrupting key cellular organelles, leading to increased protease activity. For instance, the disruption of the sarcoplasmic reticulum and lysosomes could potentially contribute to an increase in calpain-1 and cathepsin activities, respectively (Bahuaud et al., 2008; Lee et al., 2021; Zhang and Ertbjerg, 2018). The mitochondria have also been found to disrupt following freezing/thawing (Kuznetsov et al., 2003), which promotes the release of pro-apoptotic proteins into the cytosol and, eventually, the activation of effector caspase-3 (Denecker et al., 2000). However, to the best of our knowledge, no previous studies have thoroughly investigated the proteolytic enzymes involved in postmortem proteolysis following freezing/thawing. Therefore, this study aims to evaluate the impact of freezing/thawing and subsequent aging on the extent of postmortem proteolysis in beef. We hypothesize that ice crystal formation accelerates postmortem proteolysis by disrupting key cellular organelles and increasing endogenous protease activity during aging.

Materials and Methods

Experimental design

Eight steers of similar weight, feeding regimen, and genetics were humanely harvested at the Utah State University animal harvest facility. The *longissimus lumborum* (*LL*) muscle was collected from one side of all carcasses 24 h postmortem. Each muscle was fabricated into eight 2.5-cm-thick steaks. Steaks were weighed, individually vacuum packaged, and randomly divided into 4 experimental groups (2 steaks per group). The first group was aged at 4°C for 24 h (48 h postmortem), whereas the second group was held at the same temperature and aged for 168 h (192 h postmortem). The third and fourth groups were frozen at −20°C for 24 h and subsequently thawed/aged for 24 and 168 h at 4°C, respectively. The thawing period (~4 h) was considered part of the aging period. Once the aging period had concluded, all steaks were removed from their packages, blotted dry, and reweighed to determine purge loss during storage. Purge loss was determined by calculating the percentage of weight lost relative to the initial weight of the steak. Then, 1 of the 2 steaks from each experiment group was cooked and used for Warner-Bratzler shear force (WBSF) and cook loss determination. The remaining steak was subjected to color evaluation before being split into 2 portions. One portion was utilized to assess pH, drip loss, and mitochondrial oxygen consumption rate (OCR), while the second portion was snap-frozen in liquid nitrogen and stored at −80°C for subsequent evaluation of proteolytic enzyme activities, free calcium concentration, and proteolysis.

Warner-Bratzler shear force

Beef tenderness was evaluated in accordance with the guidelines established by the American Meat Science Association (Belk et al., 2015) using a WBSF V-notch blade attached to TMS-Pro Texture Analyzer (Food Technology Co.; Sterling, VA, USA). In brief, steaks ($n = 8$) were weighed and cooked on a clamshell grill until an internal temperature of 71°C was reached. After cooking, steaks were allowed to equilibrate to room temperature, blotted dry, and reweighed before being refrigerated overnight. Cook loss was calculated as a percentage of moisture loss from the steak's initial weight. On the following day, six 1.27-cm cylindrical cores were removed parallel to the muscle fiber orientation using a handheld coring device. Cores were subsequently sheared perpendicular to the longitudinal direction of the muscle fibers. The shear force was

expressed as the average maximal force in Newtons (N) of the 6 cores.

Drip loss

Drip loss was evaluated according to the procedure detailed by Rasmussen and Andersson, (1996). Two cores (~10 g each) were cut from each steak with a 2.5-cm-diameter coring device, blotted dry, and weighed. Each core was then placed in a drip loss tube and stored at 4°C for 48 h. Following the storage period, samples were removed from the tubes, blotted again, and weighed a second time. Drip loss was calculated as a percentage of weight lost from the initial weight.

Color analysis

The assessment of beef color was carried out using a Konica Minolta chromameter (CR-400, Konica Minolta Sensing Inc.; Osaka, Japan) with a 2° observer angle, illuminant D65, and an 8 mm aperture port. Steaks were removed from their packages at the end of each aging period and allowed to bloom for 20 min at room temperature. Four subsequent scans were taken across random locations on each steak, averaged, and expressed as Commission Internationale de l'Éclairage (CIE) L^* (lightness), a^* (redness), and b^* (yellowness). The chromameter was calibrated with a white calibration plate provided by the manufacturer before each use.

pH determination

Meat pH determination followed a procedure previously described by Bendall (1973). Frozen muscle samples were powdered under liquid nitrogen and homogenized 1:8 (*w/v*) in a cold buffer (150 mM KCl and 5 mM iodoacetic acid, pH 7.0) using a bead-beating homogenizer (TissueLyser LT, Qiagen; Hilden, Germany). Samples were centrifuged (17,000 × *g* for 5 min at room temperature) and equilibrated to 25°C for 10 min. Sample pH was measured with a pH electrode attached to an Orion Star A214 pH/ISE benchtop meter (Thermo Fisher Scientific, Pittsburgh, PA, USA).

Sample preparation for SDS-PAGE

Sample preparation for the degree of calpain-1 autolysis and calpastatin abundance was done as described by Dang et al. (2020). Frozen muscle samples were homogenized in 10 volumes of a buffer solution consisting of 100 mM Tris-base (pH 8.3), 10 mM EDTA, 10% (*v/v*) glycerol, 0.1% (*v/v*) 2-mercaptoethanol, and 2% (*v/v*) protease inhibitor cocktail (Cat# P8340, MilliporeSigma; St. Louis, MO, USA). After incubation on ice for 20 min, the samples were subjected to centrifugation at 20,000 × *g* for 20 min at 4°C. The resulting supernatants were collected, and protein concentration was determined using a Pierce BCA protein assay kit following the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL, USA). The samples were then diluted with 5 × Laemmli buffer (final concentration: 40 mM Tris-base, 100 mM dithiothreitol, 2% [*w/v*] SDS, 0.05% [*w/v*] bromophenol blue, and 2% [*v/v*] glycerol) to yield equal protein concentrations (3 mg/ml). Subsequently, the samples were heated at 60°C for 10 min, allowed to equilibrate to room temperature, and stored at −80°C until loaded in gels.

For analysis of desmin and troponin-T proteolysis, muscle tissue was powdered under liquid nitrogen and solubilized in a buffer containing 8 M urea, 2 M thiourea, 3% (*w/v*) SDS, 75 mM dithiothreitol, and 50 mM Tris-HCl (pH 6.8) using a bead-beating homogenizer (Warren et al., 2003). The samples were heated at 60°C for 10 min, centrifuged at 17,000 × *g* at room temperature, and the supernatants were transferred to new tubes. The protein concentration of the supernatant was subsequently measured with an RC DC assay kit (Bio-Rad Laboratories; Hercules, CA, USA). Samples were diluted with the solubilization buffer described above stained blue with 0.05% (*w/v*) bromophenol blue to a protein concentration of 3 mg/ml. Samples were stored at −80°C until loaded in gels.

SDS-PAGE and immunoblotting

Frozen protein samples were thawed at room temperature and subsequently heated at 60°C for 5 min before being loaded into their respective polyacrylamide gels. A reference sample collected from the *LL* 30 min postmortem was prepared as previously mentioned and included along with a protein molecular weight standard in each gel. Samples from 2 animals were loaded onto one gel, resulting in the utilization of 4 gels for each protein. Proteins were separated electrophoretically and transferred to nitrocellulose membranes. Membranes were stained with Ponceau S to quantify the total protein using the UVP Chemstudio Imaging System and software (Analytik Jena, Upland, CA, USA). Subsequently, membranes were destained and blocked with 1.5% (*w/v*) casein in phosphate-buffered saline containing 0.1% (*v/v*) tween-20 (PBS-T) for 1 h at room temperature. The membranes were immunoblotted overnight at 4°C with primary antibodies diluted in PBS-T. The primary antibody dilutions were as follows: anti-desmin, 1:5,000 (Cat# D1033, MilliporeSigma; St. Louis, MO, USA); anti-troponin-T, 1:20,000 (Cat# T6277,

MilliporeSigma; St. Louis, MO, USA); calpastatin, 1:1,000 (Cat# MA3-944, Thermo Fisher Scientific; Rockford, IL, USA); and anti-calpain-1, 1:2,000 (Cat# MA3–940, Thermo Fisher Scientific; Rockford, IL, USA). Afterward, the membranes were washed thrice with PBS-T (5 min each) and incubated with secondary antibodies at room temperature for 1 h. Calpain and calpastatin membranes were incubated with an HRP-conjugated secondary antibody (Cat# 20401, Biotium Inc., Fremont, CA, USA) at a dilution of 1:10,000. After washing, membranes were incubated with Immobilon Western Chemiluminescent HRP Substrate (Cat# WBKLS0100, MilliporeSigma; Burlington, MA, USA) for 5 min at room temperature. On the other hand, desmin and troponin-T blots were incubated with a fluorescent secondary antibody (1:20,000; Cat# CF680, Biotium Inc.; Fremont, CA, USA) and subsequently washed. Visualization of bands was performed using the previously mentioned imaging system and software. Protein band intensities were then quantified and normalized to the intensity of total protein within each lane. Calpain-1 autolysis was computed by dividing the intensity of the 76 kDa band by the total signal intensity (sum of 80, 78, and 76 kDa bands). Desmin and troponin-T proteolysis was evaluated by calculating the ratio of the intact band intensity over the total signal intensity (intact band + degradation products). The intensity of the 135 kDa bands was analyzed to quantify intact calpastatin abundance.

Free calcium determination

Cytosolic calcium concentration was evaluated according to the method of Hopkins and Thompson (2001) modified by Hwang et al. (2004). Approximately 4 g of muscle samples from each treatment was removed from the −80°C freezer and transferred to a −20°C freezer 14 d before the measurement. Muscle samples were placed on ice for 10 min, finely diced with razors, and centrifuged at 40,000 × *g* for 45 min at 4°C. In a new test tube, ~1 ml of supernatant was added to 20 µl of 4 M KCl and incubated in a dry heating block at 20°C for 10 min. Calcium concentration was measured with an Orion calcium-selective electrode connected to an Orion Star A214 pH/ISE benchtop meter (Thermo Fisher Scientific, Pittsburgh, PA, USA). Obtained millivolt values were converted to µM calcium concentrations using a calcium standard of 0.1 to 10 ppm.

Mitochondrial isolation and respiration

The differential centrifugation method, as outlined by Matarneh et al. (2017), was utilized to isolate mitochondria from the *LL* muscle. Muscle samples weighing approximately 0.5 g were placed in beakers containing 5 ml of ice-cold homogenization buffer (100 mM sucrose, 180 mM KCl, 50 mM Tris-base, 5 mM $MgCl_2$, 1 mM K-ATP, and 10 mM EDTA, pH 7.4) and finely minced with scissors. The serine protease Subtilisin A was added to each sample at 0.4 mg/ml prior to homogenization in a Dounce homogenizer. The resulting homogenate underwent filtration through cheesecloth and centrifugation at 1,000 × *g* for 10 min at 4°C. The supernatant was collected and subjected to another round of filtration before a second centrifugation at 8,000 × *g* for 10 min at 4°C. The supernatant was decanted, and the mitochondrial pellet was resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 220 mM mannitol, 70 mM sucrose, and 1 mM EGTA. Mitochondrial protein concentration was determined using a Pierce BCA protein assay kit.

Equal amounts of mitochondrial protein were aliquoted into reaction plates before being subjected to a Seahorse XFp flux analyzer (Seahorse Bioscience, North Billerica, MA, USA) to assess mitochondrial OCR following the procedure of England et al. (2018). The basal respiration rate was determined following the addition of 10 mM pyruvate. Then, 5 mM ADP was added to measure ADP-mediated respiration (state 3), while 2 µM oligomycin was added to assess maximal non-phosphorylating respiration (state 4). Lastly, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) at a concentration of 0.3 µM was added to determine maximal uncoupled respiration. The respiratory control ratio (RCR) was calculated as the ratio of state 3/state 4 respiration.

Caspase-3 activity

Caspase-3 activity was assessed with EnzChek Caspase-3 assay kit #1 (Molecular Probes, Invitrogen; Carlsbad, CA, USA). Muscle tissue was homogenized at a 1:5 (*w*/*v*) in an ice-cold lysis buffer consisting of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 0.01% (*v*/*v*) Triton X-100. The resulting tissue homogenate was centrifuged at 12,000 × *g* for 5 min at 4°C. Subsequently, 100 µl of the supernatant was transferred in triplicate to a 96-well opaque microplate, with or without the addition of 1 µl of 1 M Ac-DEVD-CHO (caspase-3 inhibitor). After a 10-min incubation period at room temperature, 50 µl of the fluorescent caspase-3 substrate (10 µM Z-Devd-AMC) was added to each well and incubated for an additional 30 min. Fluorescence measurements were collected at 5 min intervals for 2 h with a fluorescent microplate reader (Ex = 342 nm and Em = 441 nm). Total caspase-3

activity was expressed as µM of substrate cleaved/min/mg tissue.

Cathepsin B activity

Powdered muscle tissue was homogenized (1:4) in ice-cold lysis buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 0.01% (*v*/*v*) Triton X-100 with bead-beating homogenizer. Tissue homogenate was centrifugated at 12,000 × *g* for 5 min at 4°C. In triplicate, 100 µl of supernatant was transferred to 96-well opaque plates, followed by the addition of 100 µl of a working solution (lysis buffer adjusted to a pH of 6 and maintained at 40°C) supplemented with 1 mM Z-Arg-Arg-AMC (Cat# C5429, MilliporeSigma; St. Louis, MO, USA). The fluorescence of AMC was measured using a fluorescence microplate reader (Ex = 348 nm and Em = 440 nm) at 40°C. Activity values were expressed as intensity/min/mg tissue.

Statistical analysis

Data were analyzed using a mixed model of JMP (SAS Institute Inc., Cary, NC, USA) with steak as the experimental unit. The statistical model included the fixed effect of treatment (frozen or unfrozen), time (24 or 168 h), and their interaction and the random effect of steak. Only the interaction effect was presented when a significant interaction was detected; otherwise, only the main effects were presented. If neither the main effects nor the interaction effect was found to be significant, the interaction effect was nevertheless presented. Differences between means were evaluated using a student's t-test with $P \leq 0.05$ considered statistically significant. All data are expressed as least-squares means ± SE.

Results and Discussion

pH, color, and water loss

No treatment, time, or interaction effects were observed for pH (Figure 1A). The mean pH value across the different treatments and time points was ~5.6, a value that lies within the typical ultimate pH range of beef *longissimus* muscle (Buhler et al., 2021). This lack of pH difference between treatments

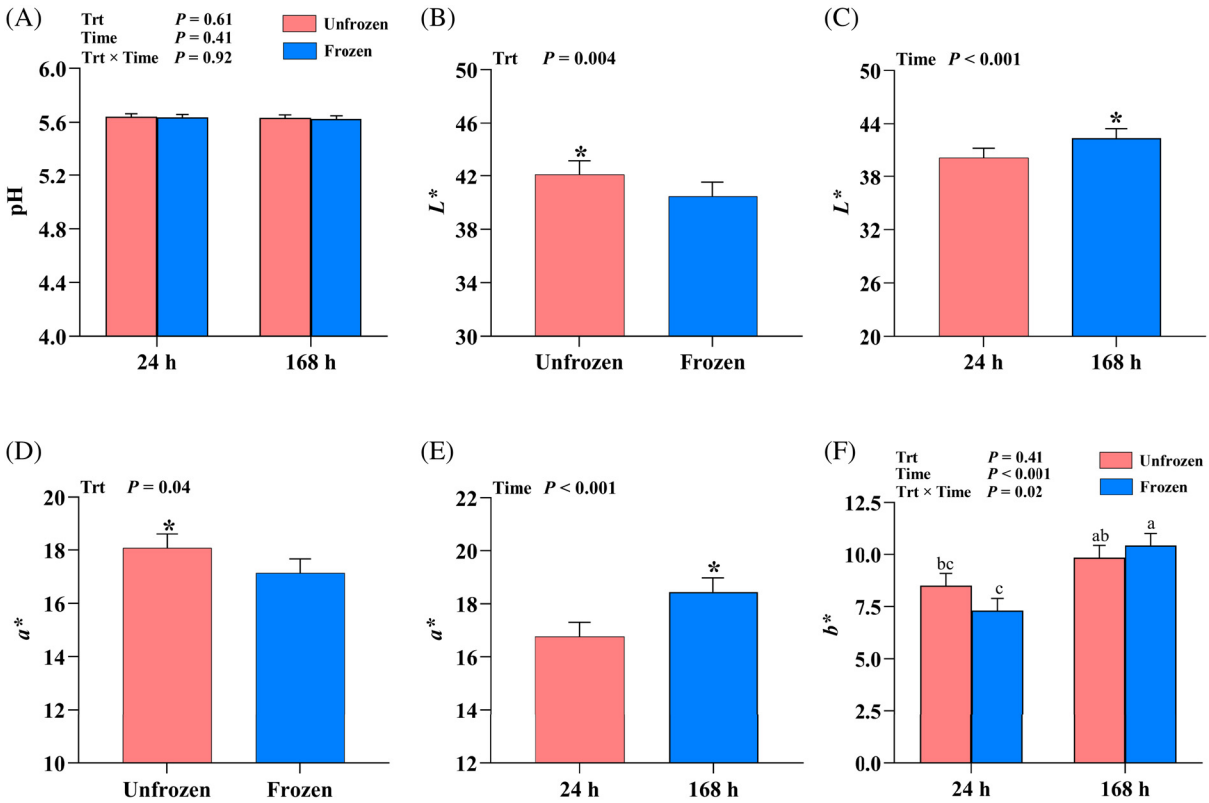

Figure 1. A) pH values of unfrozen and frozen steaks after 24 and 168 h of aging; B) *L** values of unfrozen and frozen steaks; C) *L** values of steaks after 24 and 168 h of aging; D) *a** values of unfrozen and frozen steaks; E) *a** values of steaks after 24 and 168 h of aging; and F) *b** values of unfrozen and frozen steaks after 24 and 168 h of aging. Data are least-squares means ± SE. *Indicates a significant difference ($P \leq 0.05$). [a–c]Means lacking a common letter differ significantly ($P \leq 0.05$). Trt = treatment.

was anticipated given that samples were collected 24 h postmortem, where a significant decline in pH beyond this point is not expected.

A treatment effect was detected for L^* ($P = 0.004$, Figure 1B) and a^* ($P = 0.04$, Figure 1D), with frozen samples exhibiting lower L^* and a^* than their unfrozen counterparts. Time also influenced both L^* ($P < 0.001$, Figure 1C) and a^* ($P < 0.001$, Figure 1E), resulting in greater L^* and a^* values at 168 h than at 24 h. Yellowness, on the other hand, was affected by the interaction between treatment and time ($P = 0.02$, Figure 1F). No differences between the two treatments at either time point were observed. Yet frozen samples displayed an increase in b^* from 24 to 168 h, while unfrozen samples did not exhibit a similar effect.

Freezing differentially influenced drip loss over time (treatment × time, $P = 0.05$, Figure 2A). Frozen samples experienced greater drip loss at 24 h compared to those unfrozen, but no difference between the two treatments was seen at 168 h. Purge loss was affected by treatment ($P < 0.001$, Figure 2B) and aging time ($P < 0.001$, Figure 2C), with frozen samples and those aged 168 h having greater purge loss than their unfrozen and 24 h counterparts. Cook loss was not different regardless of treatment, aging time, or their interaction (Figure 2D).

The results of the current study indicate that freezing and subsequent thawing increase the water loss of beef, which corresponds to findings observed in previous studies (Balan et al., 2019; Gonzalez-Sanguinetti et al., 1985; Lagerstedt et al., 2008). This outcome is likely due to the disruption of cellular structures by ice crystal formation and expansion during freezing, facilitating the release of water upon thawing (Dang et al., 2021). The decrease in a^* of the frozen samples compared to that of the unfrozen (Figure 1D) may have arisen from the increased water loss. Typically, as water is lost from meat, a^* decreases due to the concomitant loss of myoglobin, which can lead to an increase in L^* (Jeong et al., 2011). Surprisingly, however, frozen samples exhibited lower L^* than those unfrozen (Figure 1B). While unexpected, this could be attributed to reduced light reflectance from the meat's surface. Regardless, similar results have been previously obtained by Aroeira et al.

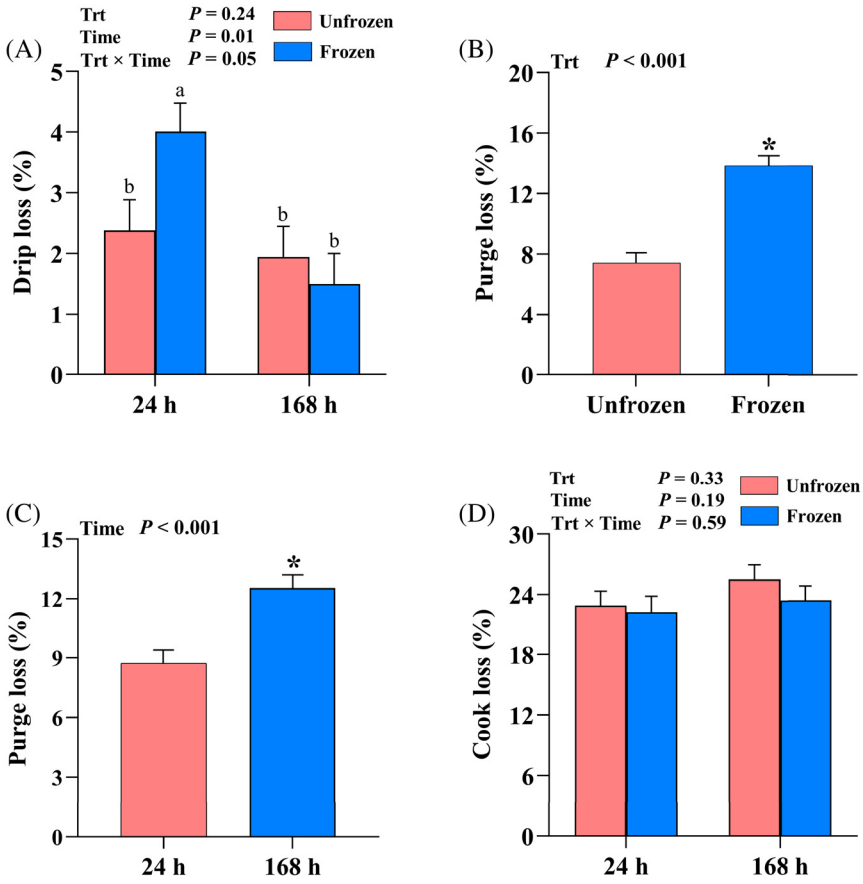

Figure 2. A) Drip loss percentages of unfrozen and frozen steaks after 24 and 168 h of aging; B) purge loss percentages of unfrozen and frozen steaks; C) purge loss percentages of steaks after 24 and 168 h of aging; and D) cook loss percentages of unfrozen and frozen steaks after 24 and 168 h of aging. Data are least-squares means ± SE. *Indicates a significant difference ($P \leq 0.05$). [a,b]Means lacking a common letter differ significantly ($P \leq 0.05$). Trt = treatment.

(2017) and Leygonie et al. (2012b). Another interesting observation is the increase in color values over time. The increase in color intensity during aging has been linked to enhanced blooming capacity, coinciding with the gradual decrease in mitochondrial capacity to utilize oxygen (Bekhit and Faustman, 2005; Henriott et al., 2020).

Tenderness and proteolysis

Tenderness is arguably the most valued characteristic of cooked beef (Miller et al., 2001; Troy and Kerry, 2010). Although findings from studies like those by Balan et al. (2019) and Muela et al. (2012) have shown that freezing can have a detrimental effect on meat tenderness, freezing typically has a positive impact on this quality trait (Cho et al., 2017; Hergenreder et al., 2013; Winger and Fennema, 1976). Both treatment and time effects were noted for tenderness ($P < 0.001$, Figure 3). Shear force values were lower in steaks subjected to freezing/thawing in comparison to the unfrozen steaks (Figure 3A). This was also the case in steaks aged for 168 h compared to 24 h (Figure 3B). The average shear force value was ~10 N lower in the frozen group than in the unfrozen. It has been reported that consumers can detect a shear force difference of about 1 kg (9.8 N) (Huffman et al., 1996; Miller et al., 1995), suggesting that freezing can lead to a perceivable improvement in meat tenderness regardless of aging.

The formation of ice crystals during meat freezing mechanically damages the myofibrils and corresponding tissue matrix, thereby weakening the muscle's overall structure (Hiner et al., 1945; Petrović et al., 1993). A study conducted by Aroeira et al. (2020) revealed that beef steaks that had undergone freezing showed an increase in myofibrillar fragmentation and tenderness immediately after thawing. However, it is unlikely that mechanical damage caused by ice crystals is responsible for the decline in WBSF observed in this study from 24 to 168 h (Figure 3B). Instead, this increase in tenderness is likely a result of the degradation of myofibrillar proteins by endogenous proteases. Therefore, evaluating proteolysis and endogenous protease activity would offer additional insight into how freezing improves beef tenderness following aging.

The extent of degradation of desmin and troponin-T has been utilized as a marker of proteolytic activity during meat aging (Huff-Lonergan et al., 1996; Koohmaraie and Geesink, 2006). Desmin is a structural protein that connects adjacent sarcomeres and helps maintain the structural integrity of the myofibril, whereas troponin-T functions as a regulatory protein involved in skeletal muscle contraction. Herein, proteolysis of desmin and troponin-T was evaluated by monitoring the disappearance of the intact protein band (Figure 4). We observed greater desmin and troponin-T degradation in the frozen samples than in their unfrozen counterparts ($P < 0.001$, Figure 4C and 4D, respectively). Greater desmin and troponin-T proteolysis was also observed at 168 h than at 24 h ($P < 0.001$, Figure 4E and 4F, respectively). The increased proteolysis in the frozen steaks is likely a consequence of an increase in endogenous protease activity triggered by the disruption of key cellular organelles. However, chemical modifications and changes in cytosolic solute concentrations due to dehydration could also contribute to protein breakdown in frozen/thawed meat (Lee et al., 2022).

Calpain-1 autolysis, calpastatin abundance, and free calcium concentration

There are 3 calpain isoforms in mammalian skeletal muscle: calpain-1 (μ-calpain), calpain-2 (m-calpain), and calpain-3 (p94) (Goll et al., 2003). Yet

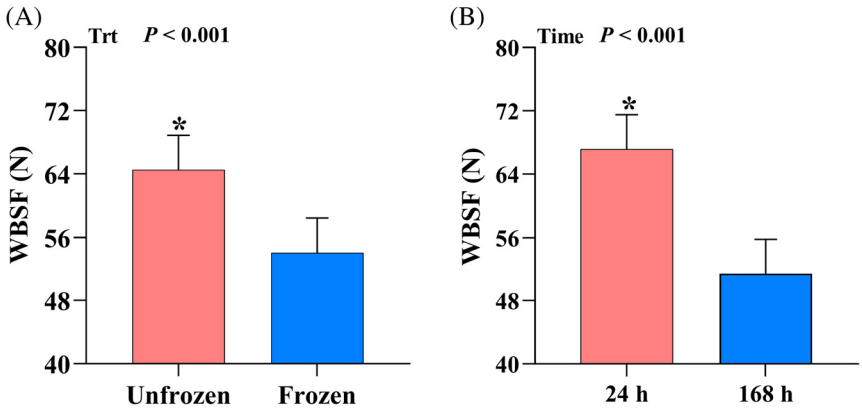

Figure 3. A) WBSF values of unfrozen and frozen steaks and B) WBSF values of steaks after 24 and 168 h of aging. Data are least-squares means ± SE. *Indicates a significant difference ($P \leq 0.05$). Trt = treatment.

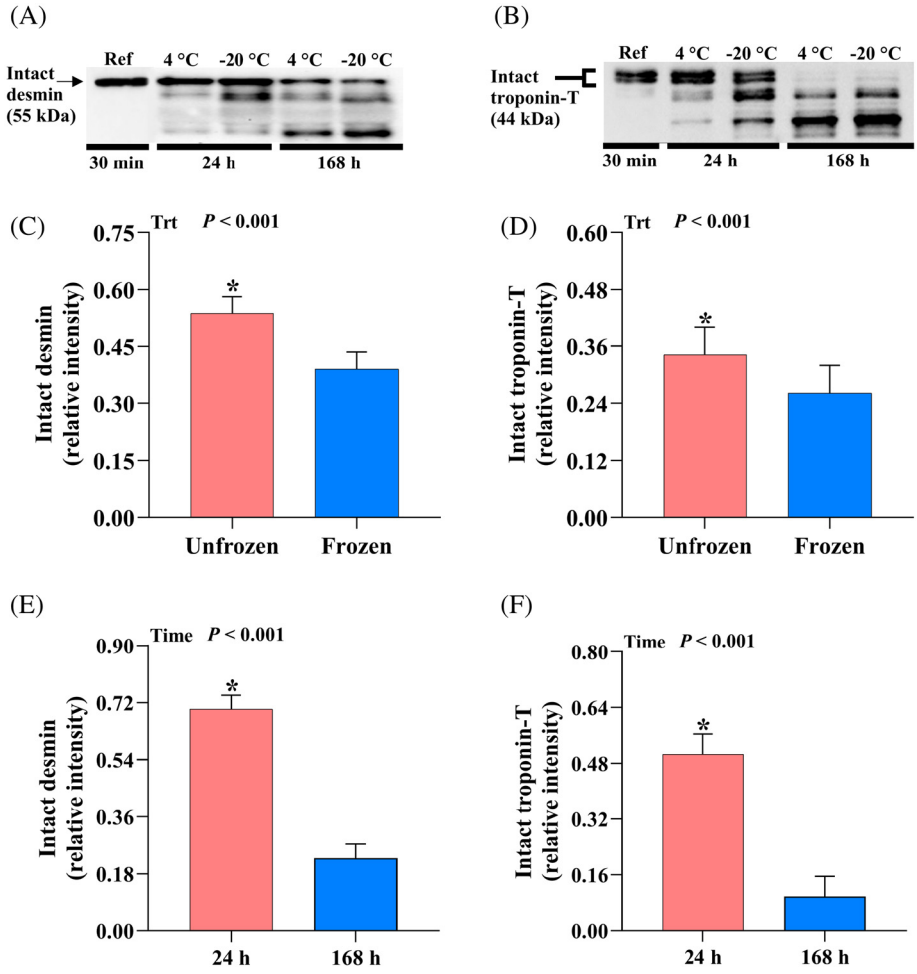

Figure 4. A) Representative Western blot showing desmin proteolysis of unfrozen and frozen steaks after 24 and 168 h of aging; B) representative Western blot showing troponin-T proteolysis of unfrozen and frozen steaks after 24 and 168 h of aging; C) relative band intensity of intact desmin protein of unfrozen and frozen steaks; D) relative band intensity of intact troponin-T protein of unfrozen and frozen steaks; E) relative band intensity of intact desmin protein of steaks after 24 and 168 h of aging; and F) relative band intensity of intact troponin-T protein of steaks after 24 and 168 h of aging. Data are least-squares means ± SE. *Indicates a significant difference ($P \leq 0.05$). Trt = treatment; Ref = reference.

calpain-1 is the primary contributor to postmortem proteolysis due to the relatively low calcium threshold needed to elicit its proteolytic activity (Huff-Lonergan et al., 1996; Koohmaraie, 1992). In the presence of calcium, autolysis of the 80 kDa catalytic subunit of calpain-1 occurs, converting it into the active 76 kDa form through a 78 kDa intermediate (Zimmerman and Schlaepfer, 1991). Therefore, the degree of autolysis is frequently utilized as an indicator of calpain-1 activation. We evaluated calpain-1 autolysis in this study by examining the intensity of the 76 kDa band (Figure 5A). An interaction between treatment and time was detected for calpain-1 autolysis ($P = 0.01$). Frozen steaks had greater calpain-1 autolysis than unfrozen steaks at 24 h ($P < 0.001$). This could be due to increased calcium availability (Zhang and Ertbjerg, 2018), as ice crystal formation likely compromised the sarcoplasmic reticulum. However, no difference between the treatments was seen at 168 h, as the protease seemed to be completely autolyzed. Veiseth et al. (2001) observed a 42% and 95% reduction in calpain-1 activity in ovine *longissimus* muscle 24 and 72 h postmortem, respectively, suggesting calpain-1 activity diminishes as time progresses postmortem. Given that our initial sample was evaluated 48 h postmortem, it is likely that samples from both treatments experienced calpain-1-mediated proteolysis prior to our assessment. However, the increase in calpain-1 autolysis in the frozen samples at 24 h compared to the unfrozen ones, in conjunction with the lack of difference in autolysis between treatments at 168 h, suggests that autolysis was accelerated by freezing/thawing.

Calpastatin, the endogenous inhibitor of calpain-1, binds to calpain-1 and prevents its autolytic activation (Goll et al., 2003). Calpastatin was only affected by time, with greater abundance observed at 24 h than at 168 h

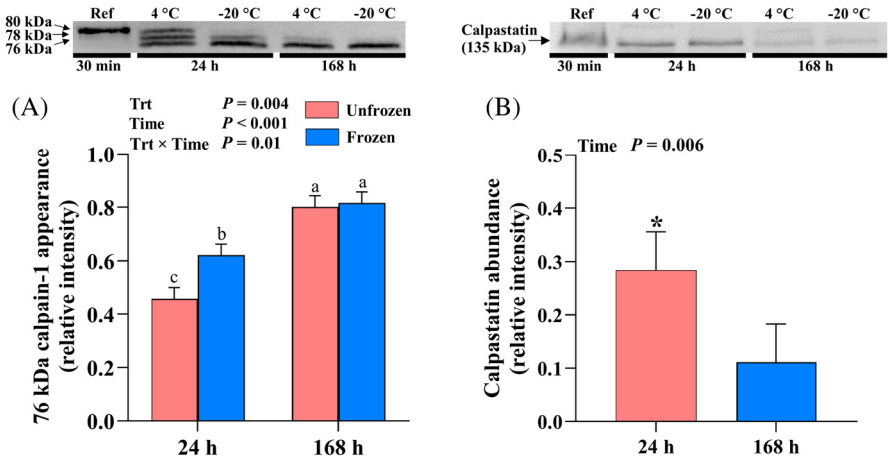

Figure 5. A) Representative Western blot showing calpain-1 autolysis (top) and relative band intensity of the 76 kDa subunit of calpain-1 (bottom) of unfrozen and frozen steaks after 24 and 168 h of aging and B) representative Western blot of calpastatin (top) and relative band intensity of calpastatin (bottom) of steaks after 24 and 168 h of aging. Data are least-squares means ± SE. *Indicates a significant difference ($P \leq 0.05$). [a–c]Means lacking a common letter differ significantly ($P \leq 0.05$). Trt = treatment; Ref = reference.

($P = 0.006$, Figure 5B). The decrease in calpastatin abundance in postmortem muscle results mainly from its degradation by calpain-1 when the cytosolic calcium concentration reaches the threshold necessary for calpain-1 activation (Doumit and Koohmaraie, 1999). On the other hand, the lack of freezing effect on calpastatin could be because our 24 h samples were collected 48 h postmortem, a time frame at which the majority of calpastatin would be degraded (Huang et al., 2014).

Calcium is crucial in initiating the proteolytic action of calpain-1. Thus, strategies such as $CaCl_2$ injection (Wheeler et al., 1992), ultrasonication (Dang et al., 2022), and electrical stimulation (Hwang and Thompson, 2001) have been employed to increase cytosolic calcium concentration with the goal of improving meat tenderness. There were no time or treatment × time effects for calcium concentration. However, a treatment effect ($P < 0.001$, Figure 6) was observed, where the frozen samples had greater free calcium than their unfrozen counterparts. Similar results were obtained by Zhang and Ertbjerg (2018), who observed an increase in free calcium concentrations in pork samples that underwent a freezing/thawing cycle. This effect is likely due to the disruption of the sarcoplasmic reticulum and mitochondrial membranes during the freezing/thawing process, allowing the release of calcium (Dang et al., 2022; Finkel et al., 2015). These data confirm that freezing and subsequent thawing increase cytosolic calcium concentration and contribute to accelerated calpain-1 activation. Although calpain-1 is commonly regarded as the primary protease involved in postmortem proteolysis, several other endogenous proteases exist in skeletal muscle (e.g., caspases and cathepsins) and could potentially contribute to postmortem proteolysis (Bahuaud et al., 2008, Dang et al., 2022).

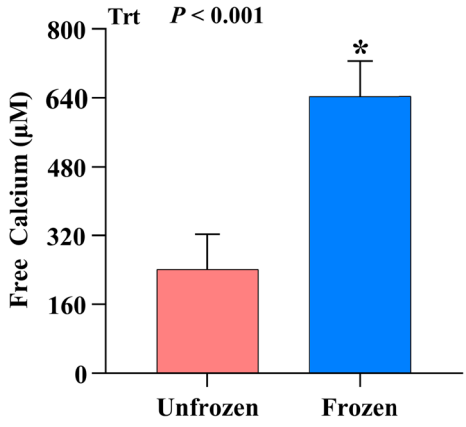

Figure 6. Free calcium concentrations (μM) of unfrozen and frozen steaks. Data are least-squares means ± SE. *Indicates a significant difference ($P \leq 0.05$). Trt = treatment.

Cathepsin B activity

Several cathepsins have been evaluated for their impact on postmortem proteolysis, particularly cathepsin B, D, N, and L (Geesink and Veiseth, 2008; Kaur et al., 2020; Sentandreu et al., 2002). Upon their release from the lysosome, cathepsin B and D favor the degradation of myofibrillar protein substrates such as myosin, actin, desmin, troponin-T, and α-actinin, whereas cathepsins N and L are primarily collagenolytic (Agarwal, 1990; Baron et al., 2004; Dransfield and Etherington, 1981). Cathepsin D functions effectively within a pH range of 3.0–4.5 (Minarowska et al., 2009); thus, its role in postmortem proteolysis may be negligible. Conversely, cathepsin B exhibits optimal activity within a pH range similar to the ultimate pH of meat

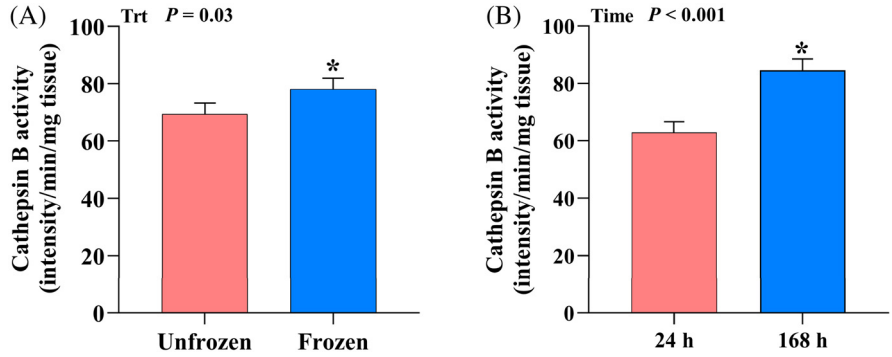

Figure 7. A) Cathepsin B activity (intensity/min/mg tissue) of unfrozen and frozen steaks and B) Cathepsin B activity (intensity/min/mg tissue) after 24 and 168 h of aging. Data are least-squares means ± SE. *Indicates a significant difference ($P \leq 0.05$). Trt = treatment.

(pH ~5.6) (Kianifard et al., 2020), suggesting it may contribute to postmortem proteolysis (Huang et al., 2019; Kaur et al., 2020). In the present study, cathepsin B activity was evaluated and found to be affected by treatment ($P = 0.03$, Figure 7A) and time ($P < 0.001$, Figure 7B). Specifically, greater cathepsin B activity was seen in the frozen samples than the unfrozen, and at 168 h compared to 24 h. The enhancement in cathepsin B activity resulting from freezing and aging likely stems from increased lysosome deterioration. Similar to our results, cathepsin B activity was higher in beef *semitendinosus* muscle samples subjected to freezing at −20°C for 24 h than in those unfrozen (Lee et al., 2021). In a different study, Tian et al. (2013) found that cathepsin B increased significantly over a 192 h aging period in yak meat.

The contribution of cathepsins to postmortem proteolysis has been debated and often suggested to be limited due to their preference for low pH conditions and their confinement within the lysosome (Kaur et al., 2021; Koohmaraie et al., 1991). However, freezing/thawing procedures can compromise the lysosomal membrane (Kaur et al., 2020), releasing the cathepsins into the cytosol and giving them access to their substrates. Bahuaud et al. (2008) observed that the formation of ice crystals in fish fillets ruptures the lysosomal membrane, subsequently increasing the activity of cathepsin B. Therefore, cathepsin B could be one of the proteases contributing to enhanced proteolysis in the current study.

Mitochondrial respiration and caspase-3 activity

Mitochondria are commonly referred to as the "powerhouses of the cell" because of their essential role in cellular ATP production. However, they also play key roles in regulating cytosolic calcium levels and triggering apoptosis (Orrenius et al., 2007; Zou et al., 2023). While the former requires intact mitochondria, the latter occurs when they lose their integrity. Mitochondrial disruption in postmortem muscle can arise from intramitochondrial calcium overload and the subsequent increase in reactive oxygen species (Brookes et al., 2004; Finkel et al., 2015). This allows the release of cytochrome c into the cytosol, which eventually triggers the activation of the mitochondrial apoptotic pathway and the effector protease caspase-3 (Loeffler and Kroemer, 2000). Upon activation, caspase-3 is capable of degrading myofibrillar proteins and contributing to postmortem proteolysis (Huang et al., 2011; Wang et al., 2017). In previous research, we showed that ultrasonication-induced mitochondrial dysfunction enhances caspase-3 activity, proteolysis, and beef tenderness (Dang et al., 2022)

To evaluate the effect of freezing on the mitochondrial apoptotic pathway, mitochondrial respiration efficiency and caspase-3 activity were assessed. An interaction between time and treatment was noted for baseline, state 3, state 4, and FCCP respirations ($P \leq 0.05$, Figure 8), whereas RCR was not influenced by treatment, time, or treatment × time. At 24 h, no variation was observed between the 2 treatments for baseline respiration (Figure 8A); however, at 168 h, frozen samples exhibited reduced baseline respiration compared to the unfrozen ($P = 0.002$). This pattern was also reflected in state 4 respiration (Figure 8C). In contrast, both state 3 respiration (Figure 8B) and FCCP respiration (Figure 8D) displayed the same pattern, where unfrozen samples had greater respiration than that of the frozen at 24 h ($P < 0.001$), with no difference between the 2 treatments at 168 h. Collectively, these data indicate that freezing and aging negatively impact mitochondrial efficiency, as assessed by several respiration parameters.

Baseline respiration refers to the OCR of the isolated mitochondria before the addition of ADP, while

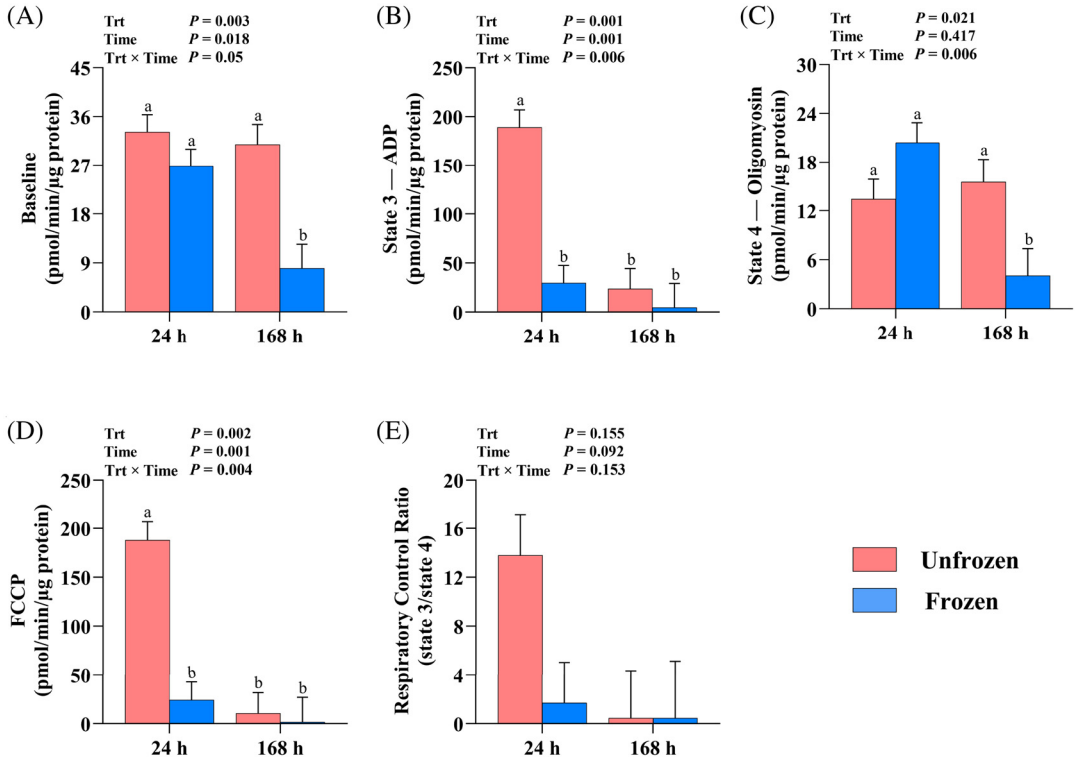

Figure 8. A) Baseline respiration (pmol/min/µg mitochondrial protein); B) state 3 respiration (pmol/min/µg mitochondrial protein); C) state 4 respiration (pmol/min/µg mitochondrial protein); D) uncoupled carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) maximal respiration rate (pmol/min/µg mitochondrial protein); and E) respiratory control ratio (state 3/state 4) of isolated mitochondria from unfrozen and frozen steaks after 24 and 168 h of aging. Data are least-squares means ± SE. [a,b]Means lacking a common letter differ significantly ($P \leq 0.05$). Trt = treatment.

state 3 is ADP-stimulated respiration and reflects the mitochondrial capacity to synthesize ATP. Decreased state 3 respiration is indicative of impaired mitochondrial phosphorylation efficiency, which signals mitochondrial dysfunction (Brand and Nicholls, 2011). State 4 refers to nonphosphorylating respiration. In state 4 respiration, oligomycin, an inhibitor of ATP synthase, is utilized to evaluate proton leakage through the inner mitochondrial membrane (Hill et al., 2012). Herein, we observed greater state 4 respiration in the frozen samples than in the unfrozen ones (Figure 8C), providing further evidence of diminished mitochondrial integrity. Finally, FCCP uncouples oxygen consumption from ATP synthesis by dissipating protons across the inner mitochondrial membrane, enabling the measurement of maximal OCR (Djafarzadeh and Jakob, 2017). The RCR (state 3/state 4) provides a suitable measurement of overall respiration efficiency, and it is positively correlated with mitochondrial function and efficiency (Brand and Nicholls, 2011; Divakaruni and Jastroch, 2022). Despite the absence of treatment, time, or interaction effects for RCR (Figure 8E), notably higher numerical RCR was seen at 24 h in the unfrozen samples compared to their frozen counterparts.

Our mitochondrial OCR data demonstrate that freezing elicits greater mitochondrial dysfunction, which is similar to the results observed by Tang et al. (2006). Because mitochondrial dysfunction is the main trigger for caspase-3 activation (Wang et al., 2017), caspase-3 activity was also examined in this study. An interaction effect between treatment and time was observed for caspase-3 activity ($P < 0.001$, Figure 9). Caspase-3 activity was lower in the frozen samples at 24 and 168 h when compared to those unfrozen ($P \leq 0.024$). Caspases have been shown to be activated ~10 min after apoptosis is induced by mitochondrial dysfunction and the subsequent release of cytochrome c (Green, 2005). However, it has also been demonstrated that caspase-3 activity peaks within 24 h postmortem and declines rapidly afterward (Chen et al., 2011; Huang et al., 2014). Therefore, it seems that caspase-3 activity peaked faster in the frozen samples because of their augmented mitochondrial dysfunction (Figure 8), which may have occurred before our initial sample was obtained (48 h postmortem). Although not verified in this investigation, mitochondrial dysfunction may accelerate caspase-3 activation, potentially contributing to the improved proteolysis and tenderness usually observed in previously frozen steaks.

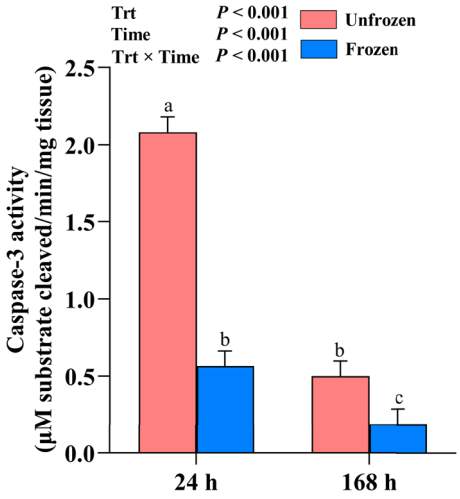

Figure 9. Caspase-3 activity (µM of cleaved substrate/min/mg tissue) of unfrozen and frozen steaks after 24 and 168 h of aging. Data are least-squares means ± SE. [a–c]Means lacking a common letter differ significantly ($P \leq 0.05$). Trt = treatment.

Conclusions

The collective findings of this study demonstrate that freezing enhances beef tenderness by accentuating postmortem proteolysis. This is likely due to the increase in calpain-1 autolysis and cathepsin B activity. Freezing/thawing may have also accelerated caspase-3 activation, an effect that we could not verify due to our sampling timing. The increase in the activity of these proteases is likely a consequence of ice crystals disrupting cellular organelles, leading to the release of factors that initiate protease activation. This is demonstrated by increased free calcium concentration and mitochondrial dysfunction. While freezing improved tenderness, it also increased water loss and reduced color intensity. Overall, this research furthers our understanding of the effects of freezing storage on beef quality, particularly tenderness. However, further research should be dedicated to optimizing the freezing/thawing process in order to mitigate the loss of product quality.

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