# The Effect of Aging/Freezing Sequence and Freezing Rate on Quality Attributes of Beef Loins (*M. longissimus lumborum*)

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**Abstract:** The objective of this study was to determine the effect of different aging/freezing sequences combined with different freezing rates on quality attributes of beef loins (*M. longissimus lumborum*). Loins from 1 side of 8 beef carcasses (USDA Low Choice grade) were obtained at 2 d postmortem, cut into 10 steaks (2.54 cm), and randomly assigned into 5 treatments, where factorial combinations of 2 aging/freezing sequences (aging first then freezing/thawing [AFT] and freezing first then thawing/aging [FTA]) and 2 freezing rates (fast freezing [FF] and slow freezing [SF]) were used and compared to an aged only (AO) treatment as a control. Samples frozen using the FF condition were found to have a critical freezing time ($Tc$) of 21.5 min while the SF counterpart had a $Tc$ of 175 min. Histological observation showed more visually enlarged gaps between muscle fibers in the SF samples, regardless of aging/freezing sequence. The SF-FTA samples had a significantly higher purge/thaw loss followed by FF-FTA, while the loss from FF-AFT, SF-AFT, and AO were not different from each other ($P > 0.05$), suggesting that freezing rate effect might be more profound when applied in FTA. Cook loss was higher in AO compared to both FTA treatments ($P < 0.05$); however, it was not different when compared to AFT ($P > 0.05$). A lower shear force value and higher troponin-T protein degradation were found in both AFT and FTA compared to AO ($P < 0.05$), which would be attributed to the ice-crystal damage due to freezing. These results suggest that aging/freezing sequence could be an overriding factor in determining major meat quality attributes of frozen/thawed meat over freezing rate. However, fast freezing could negate some quality defects associated with the FTA process.

## Introduction

Freezing is one of the most effective and efficient methods used to prolong the shelf life of meat products (Kim et al., 2015). However, frozen meat is typically considered as being inferior in quality to fresh, never frozen meat. Impaired water-holding capacity (WHC) and oxidative stability, as well as textural changes, are some quality defects frequently found in frozen/thawed products. These quality defects are mainly attributed to the cryo-damage within the muscle structure by the formation of ice crystals (Leygonie et al., 2012a).

Several intrinsic and extrinsic factors influence the quality of frozen/thawed meat products. One of the most well-known of these factors is rate of freezing, as it plays a critical role in determining the final meat quality attributes, affecting the size, shape and location (intra- or extracellular) of ice crystals formation within the frozen meat (Leygonie et al., 2012a; Kim et al., 2015, 2017). Slow freezing rates generate large extracellular ice crystals, resulting in a considerable cryo-damage to muscle proteins and cell membranes (Grujić et al., 1993; Hergenreder et al., 2013). Inversely, fast freezing results in smaller intracellular ice crystals, distributed more uniformly throughout the frozen muscle tissue compared to slow freezing (Grujić et al., 1993). As a result, it is generally accepted that a lesser extent of cryo-dam-

age occurs through fast freezing, which in turn reduces adverse impacts of freezing on some quality traits (WHC and oxidative stability, in particular) of frozen/thawed meat compared with slow freezing (Leygonie et al., 2012a; Kim et al., 2017, 2018)

In addition to freezing rates, importance of postmortem aging for the quality of frozen/thawed meat products has been reported (Kim et al., 2018). Considerable fragmentation of cytoskeletal myofibrillar proteins occurs through the action of endogenous proteolytic enzymes in muscle tissue during postmortem aging. As a consequence, swelling of muscle cells occurs due to increased water retention in the intercellular space, resulting from the degradation of intermediate filaments and costamere linkages (Pearce et al., 2011). Aging first then freezing/thawing (AFT) typically results in improvements in tenderness, WHC, and/or juiciness in the meat product. For frozen/thawed meat, aging provides its "buffering nature" through the swollen muscle structure, which in turn reduces the extent of cryo-damages induced by the physical impacts of freezing (Kim et al., 2018; Setyabrata and Kim, 2019). In this respect, the positive impacts of aging prior to freezing on meat quality attributes have been reported in several studies (Kim et al., 2011, 2018; Choe et al., 2016; Coombs et al., 2017)

Additionally, freezing first then thawing/aging (FTA) has also been suggested as a potential way of processing frozen/thawed meat products with some quality improvements. Upon thawing of frozen meat products and subsequent aging process, improvement in tenderness (shown by a decrease in shear force values) has been reported in some studies (Crouse and Koohmaraie, 1990; Grayson et al., 2014; Kim et al., 2018). It was postulated that the observed improvement in instrumental tenderness would be attributed to the increase in protein degradation through the suppression of the activity of calpastatin (inhibitor of calpains) by freezing at a relatively early stage of the postmortem period (Koohmaraie, 1990; Whipple and Koohmaraie, 1992). However, considerable increases in purge/thaw loss of meat products having undergone FTA have been also reported in pork (Kim et al., 2018) and beef (Setyabrata and Kim, 2019) studies, which could be a major drawback of the FTA process.

Taken together, as fast-freezing could reduce the extent of cryo-damage (minimizing purge/thawing loss in particular), it would be reasonable to hypothesize that combining FTA with fast freezing could improve the final meat quality attributes of frozen/thawed meat by decreasing purge/thawing loss associated with FTA. While numerous studies have been conducted to determine the impacts of aging, freezing and/or thawing, respectively, on meat quality, there are currently little published literatures regarding the combined impacts of aging/freezing sequence with different freezing rates on meat quality characteristics (FTA with fast freezing, in particular). Therefore, the objective of this study was to determine the effect of aging/freezing sequence coupled with different freezing rates on meat quality and biochemical attributes of beef loins.

## Materials and Methods

### Sample collection, preparation and processing

At 2 d postmortem, beef loins (*M. longissimus lumborum*) from 1 side of 8 beef carcasses (USDA Low Choice grade; USDA, 2017) were obtained from the Purdue University Meat Laboratory harvest facility. Loins were collected from 2 different slaughter periods (1 mo apart), where 6 loins were collected from the first harvest and 2 loins from the second harvest. One thin cut (0.5 cm thick, ~50 g) from each loin was collected as an initial biochemical sample at 2 d postmortem. A total of 10 steaks (2.54 cm thick/steak) were cut from each beef loin and vacuum packaged individually using a vacuum pouch (3 mil; BUNZL Processor Division, Riverside, MO). Then, the 10 steaks were randomly assigned to 5 different treatments (yielding 2 steaks per treatment), consisting of aged only control (AO) and 4 aging/freezing treatments, where factorial combinations of 2 freezing rates (fast freezing [FF] and slow freezing [SF]) and 2 aging/freezing sequences (FTA and AFT) were applied, as illustrated in Fig. 1A. Additional steaks (one from each loin, $n = 8$, 2.54 cm thick) were also collected and used for monitoring the temperature changes during the different aging/freezing process.

The samples were separated according to their treatments and placed in a single layer inside different boxes prior to the treatment application. Aging was conducted at 3°C for 14 d. Fast freezing (FF-FTA and FF-AFT) was conducted using a cryogenic freezing cabinet (CF Cabinet Freezer, RS Cyro Equipment, Inc., Manteno, IL) set at an ambient temperature of –75°C. Slow freezing (SF-FTA and SF-AFT) was performed using a conventional blast freezer with an ambient temperature of –20°C. Freezing processes for both fast and slow freezing were monitored using a T type thermocouple (Copper/Constantan; Omega Engineering, Stamford, CT) inserted into the geometrical center of the extra steak samples and connected to an OctTemp 2000 data logger (Madge Tech, Inc., Warner, NH).
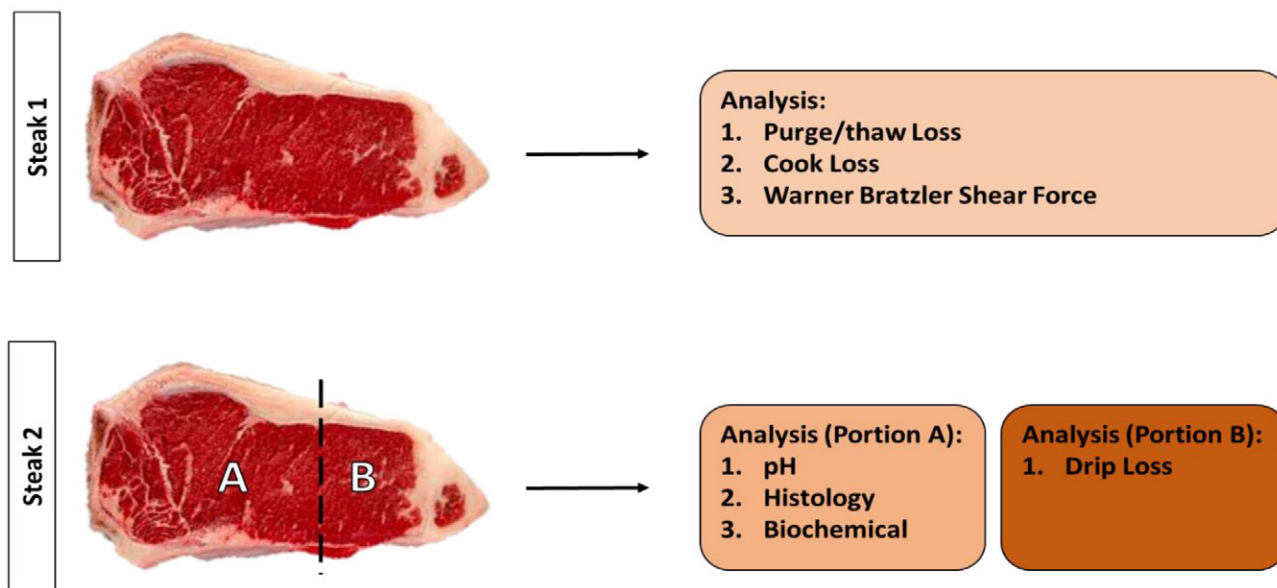
(A)
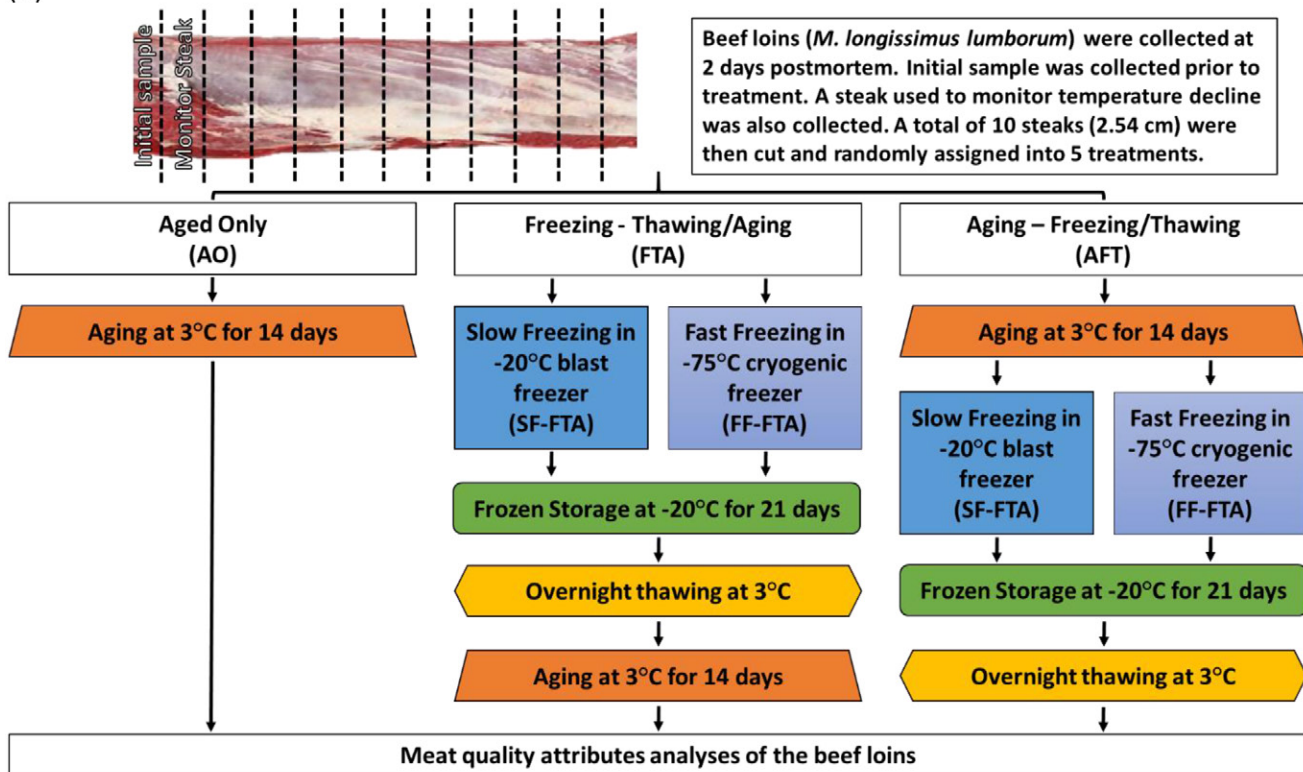


(B)



**Figure 1.** Schematic illustration of sample assignment, aging/freezing treatment procedure and analysis distribution.

The freezing process was considered complete for all treatments when the internal temperature of the steaks reached –20°C. All frozen samples were stored for 21 d at –20°C prior to overnight thawing at 3°C. Following the aging/freezing treatments, 1 steak sample from each treatment was used to measure purge/thaw loss, cook

loss, and shear force (Fig. 1B). The other one was used to determine other meat quality attributes (pH changes and drip loss), histology, and biochemical analyses.

## pH measurement

The pH of each steak was measured before and after treatments using a meat pH probe (Hanna HI 99163, Hanna Instrument, Inc., Warner, NH), calibrated with pH 4 and 7 buffer. The pH value was measured by directly inserting the probe into 2 random locations, avoiding any visible fat and connective tissue.

## Histological analysis

Two types of histological analyses were performed for the qualitative muscle structure evaluation. Regular histology slides were prepared by cutting a perpendicular slice (5 mm thickness) from 3 randomly selected steak samples from each treatment. The sliced samples were placed in a plastic cassette and fixed using a 10% neutral buffered formalin for 24 h. Fixed samples were then transported to the Purdue Histology Research Laboratory for slide preparation. All samples were embedded on paraffin and sectioned to a thickness of 0.4 mm (Leica semi-automatic rotary microtome, Leica Co., Wetzlar, Germany).

Cryo-histology was also conducted, where samples (cubes; $1.5 \times 1.5 \times 1.5$ cm) were collected from 2 randomly selected frozen steaks from each of the aging/freezing treatments prior to thawing (aged/frozen for AFT and frozen only for FTA). The frozen cubes were embedded on an optimum cutting temperature media. The samples were sectioned using an ultramicrotome (10 μm thickness Leica semi-automatic rotary microtome, Leica Co.) and fixed in a 95% ethanol for 1 min. All slides from both regular and cryo-histology were stained using hematoxylin and eosin Y slide autostainer and were observed using at 100× magnification (Carl Zeiss, Oberkochen, Germany).

## Water-holding capacity measurement

Several measurements, including purge/thaw loss, drip loss, and cook loss were conducted to assess the WHC of steaks from each treatment. All samples were gently blotted dry using a paper towel prior to any weighing.

Purge/thaw loss was determined by weighing the steaks prior to and after each treatment. Weight differences between the initial (at 2 d postmortem) and final weight (after each aging/freezing treatment) were then divided by the initial weight to obtain the percent purge/thaw loss from each sample.

Cook loss was determined by cooking the samples assigned for the shear force measurement on an open electric griddle set at 135°C (Farberware, Walter Idde and Co., Bronx, NY). When the internal temperature of the samples reached 41°C, samples were then turned and cooked until the internal temperature reached 71°C. Temperature increase was monitored using type T thermocouples (Copper/Constantan; Omega Engineering) paired to an OctTemp 2000 data logger (Madge Tech, Inc.). Cook loss was expressed as a percentage and determined by calculating the weight difference of the sample prior to and after cooking.

Drip loss was conducted using the Honikel drip loss method (Honikel, 1998) with modification described by Kim et al. (2017). Approximately 40 g of samples, roughly cube shaped, were collected and trimmed from any visible fat or connective tissue. Samples were suspended using netting in an airtight container for 48 h. All samples were weighed before and after the suspension for the drip loss (%) calculation.

## Warner-Bratzler shear force measurement

The cooked steak samples for WBSF were cooled overnight at 4°C prior to the shear force analysis. Six samples within each steak were collected (1.4 cm in diameter) by coring parallel to the fiber direction. The cores were sheared perpendicular to the fiber direction, using TA-XT Plus Texture Analyzer (Stable Micro System Ltd., UK) prepared for WBSF measurement. The average peak shear force from the cores was calculated and expressed in Newtons ($N$).

## Proteolysis analysis

*Whole muscle protein extraction.* Protein extraction was conducted following the methods previously described by Kim et al. (2010) with modification. The muscle samples were snap frozen using liquid nitrogen and immediately pulverized into powder using a commercial blender (Waring Commercial, Torrington, CT) and 1 g of the powdered meat sample was homogenized in 10 mL whole muscle protein extraction buffer (10 m$M$ sodium phosphate, 2% sodium dodecyl sulfate [SDS], pH 7.0). The homogenate was then centrifuged at $1500 \times g$ for 15 min at 25°C. The protein concentration was determined using Epoch Spectrophotometer System (BioTek Instruments, Inc., Winooski, VT) set for protein quantification and adjusted to a concentration of 6.4 mg/mL protein with the extraction buffer.

The gel samples were then prepared by adding 1 mL of the protein extract with 500 ML tracking dye (3m$M$ EDTA, 3% [wt/vol] SDS, 20% [vol/vol] glycerol, 0.003% [wt/vol] bromophenol blue, and 30 m$M$ Tris-HCl; pH 8.0) and 100 ML 2-mercaptoethanol, creating a final concentration of 4 mg/mL protein. Gel samples were heated in a 50°C heating block for 20 min prior to storage at –80°C for subsequent analysis.

**SDS-PAGE and Western blot.** Immunoblotting assays were conducted to observe desmin and troponin-T degradation. SDS-PAGE and Western blot were performed in accordance with the method described by Kim et al. (2010) with appropriate modifications. For SDS-PAGE, polyacrylamide separating gels (12%) were created using 100:1 acrylamide:bisacrylamide, 15 mL 2 M Tris-HCl buffer (pH 8.8), 300 μL ammonium sulfate (10% wt/vol), and 30 μL tetramethylethylenediamine (TEMED). The polyacrylamide stacking gels (5%) were made using 100:1 acrylamide:bisacrylamide, 4 mL 1 M Tris-HCl buffer (pH 6.8), 120 μL ammonium sulfate (10% wt/vol), and 20 μL TEMED. Gels were loaded with 40 mg of protein; with the initial beef sample collected at 2 d postmortem (INI) was used as the internal reference. Electrophoresis was conducted for 3 h at 100 V (Hoefer Inc., Richmond, CA), continued with protein transfer to polyvinylidene fluoride membranes (Millipore Corporation, Billerica, MA) at 90 V for 90 min.

The membranes were blocked using 5% nonfat dry milk powder dissolved in PBS-Tween solution for 1.5 h prior to incubation with primary antibodies. The incubation was conducted for 16 h at 4°C using primary antibodies diluted in PBS-Tween solution containing 3% nonfat dry milk (1:40,000 dilution for both monoclonal mouse anti desmin and monoclonal mouse anti troponin-T; Sigma Aldrich, St. Louis, MO). Following the incubation, membranes were washed 3 times with PBS-Tween solution for 10 min each. All membranes were incubated for the second time using secondary antibody (goat anti-mouse IgG [H + L] horseradish peroxidase conjugate; Bio-Rad, Hercules, CA) diluted in 3% nonfat dry milk PBS-Tween solution with 1:15,000 dilution for 1 h. Membranes were washed using the same washing step previously mentioned. Protein bands in the membranes were developed using Pierce ECL Western blotting reagents (ThermoFisher Scientific, Waltham, MA). The bands were visualized and quantified using UVP GelDoc-It imagers (UVP LLC, Upland, CA). The intensity ratio was then calculated by comparing the quantified band of the sample to the quantified band of the initial beef collected at 2 d postmortem as the internal standard (INI).
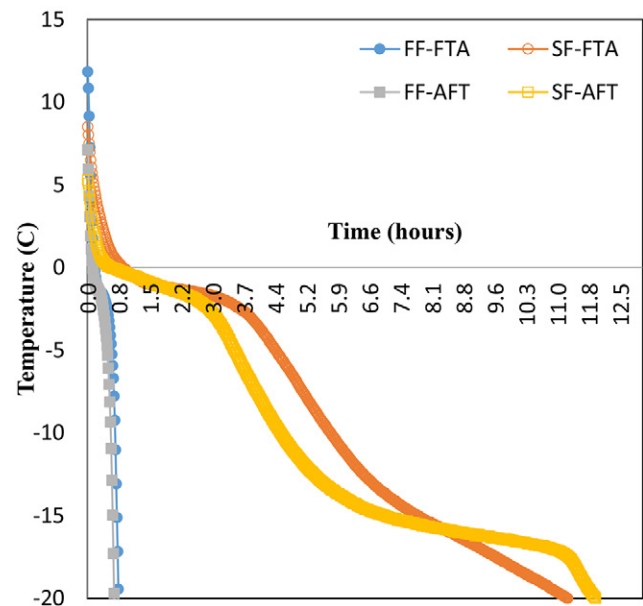


**Figure 2.** Temperature decline rate of *M. longissimus lumborum* (LL) in -20°C blast freezer (slow freezing-thawing/aging [SF-FTA] and aging-slow freezing/thawing [SF-AFT]) and –75°C nitrogen chamber (fast freezing-thawing/aging [FF-FTA] and aging-fast freezing/thawing [FF-AFT]).

### Statistical analysis

The experimental design was a randomized block design with having 2 factors in a factorial structure (combining the 2 aging/freezing sequences [FTA and AFT] and 2 freezing rates [FF and SF] with non-frozen, AO as the control). The PROC MIXED procedure from SAS 9.4 software (SAS Institute Inc., Cary, NC) was used to analyze the data. The aging/freezing sequence and freezing rate were analyzed as the fixed effect, and loins and their interactions with the main effects were analyzed as random effects. Each loin served as a block. Least square means were separated for all traits of interest using PDIFF option (F test, $P \leq 0.05$). No impacts of different harvest periods were found on meat quality attributes ($P > 0.05$), and thus data were analyzed without including harvest day as a factor.

## Results and Discussion

### Temperature decline and pH

The rate and extent of internal temperature decline of the samples treated with different aging/freezing sequence and freezing rate is displayed in Fig. 2. Regardless of aging/freezing sequence (FTA or AFT), the internal temperature was rapidly decreased when FF was applied compared to SF. Both FF-FTA and

**Table 1.** Least square means of pH value and water holding capacity (WHC) of *M. longissimus lumborum* (LL) treated with different aging/freezing sequence and freezing rate combination

| Treatment | pH | WHC, % | | |
| | | Purge/thaw loss | Cooking loss | Drip loss |
|---|---|---|---|---|
| Aging/freezing sequence effect | | | | |
| Aged only (AO) | 5.63 | 4.14[a] | 19.42[a] | 1.01[a] |
| Freezing-thawing/aging (FTA) | 5.63 | 7.93[b] | 13.56[c] | 1.58[ab] |
| Aging-freezing/thawing (AFT) | 5.63 | 4.65[a] | 16.49[b] | 1.85[b] |
| SEM[1] | 0.04 | 0.64 | 0.92 | 0.31 |
| *P*-value | 0.6454 | < 0.0001 | 0.0003 | 0.0187 |
| Freezing rate effect | | | | |
| Fast freezing (FF) | 5.64 | 5.83 | 15.18 | 1.78 |
| Slow freezing (SF) | 5.63 | 5.77 | 14.88 | 1.65 |
| SEM | 0.04 | 0.57 | 0.72 | 0.30 |
| *P*-value | 0.6519 | 0.2327 | 0.7324 | 0.5671 |
| Aging/freezing sequence and freezing rate effect | | | | |
| Aged only (AO) | 5.64 | 4.14[A] | 19.42[A] | 1.01 |
| FTA with fast freezing (FF-FTA) | 5.64 | 7.24[B] | 14.18[BC] | 1.77 |
| FTA with slow freezing (SF-FTA) | 5.63 | 8.64[C] | 12.95[C] | 1.39 |
| AFT with fast freezing (FF-AFT) | 5.63 | 4.42[A] | 16.18[ABC] | 1.79 |
| AFT with slow freezing (SF-AFT) | 5.64 | 4.90[A] | 16.80[AB] | 1.90 |
| SEM | 0.04 | 0.48 | 1.04 | 0.32 |
| *P*-value | 0.8122 | < 0.0001 | < 0.0001 | 0.0562 |

[1]SEM, standard error of the mean.

[a–c]Least square means with different letters are different in the same column (aging/freezing sequence effect; *P* < 0.05).

[A–C]Least square means with different letters are different in the same column (treatment effect; *P* < 0.05).

FF-AFT reached –20°C within 50 min, while their SF counterparts (SF-FTA and SF-AFT) took up to 10 h to reach the final internal temperature.

The rate of temperature decline has been considered as one of the main factors that affects the characteristics of ice crystals formed and, therefore, would lead to a different degree of damage in the muscle structure. It was proposed that the time required to pass –1 to –7°C, known as the initial characteristic freezing time (*Tc*), is a critical factor that determines the characteristics of ice crystal, because the majority of the ice crystals are formed during this period (Grujić et al., 1993; Kiani and Sun, 2011; Kim et al., 2017, 2018). In the present study, the samples assigned to FF had *Tc* of 21.5 min, whereas SF samples had *Tc* of 175 min on average to pass the critical temperature range.

Previous studies found that samples frozen in a slow freezing system (–20°C ambient temperature) had a *Tc* > 150 min and developed larger, more irregularly shaped ice crystals mostly in extracellular regions of the muscle (Rahelić et al., 1985; Grujić et al., 1993). Conversely,

these studies also identified that only smaller intracellular ice crystals were formed and observed in samples frozen in a fast freezing system (–78°C ambient temperature), which had a *Tc value* of less than 25 min. As the difference in *Tc* between the different freezing rates in the present study was more than 160 min, it would likely affect the shape, extent, and distribution region of ice crystal formation within muscle cells (Grujić et al., 1993), which is discussed in more detail in the next section.

The final pH was not affected by any of main effects (aging/freezing sequences and freezing rates) and their interactions (*P* > 0.05; Table 1). This observation is in agreement with previous studies, where no impact of freezing rate on the pH of beef (Añón and Calvelo, 1980; Muela et al., 2010) and pork (Kim et al., 2018) was reported. Similar to the current study, several studies also found no significant aging/freezing sequence effect on the final pH of frozen/thawed meat compared to fresh (never frozen) meat products (Choe et al., 2016; Kim et al., 2017; Bogdanowicz et al., 2018). However, other studies reported a different final pH value between frozen/thawed and non-frozen meat products (Leygonie et al., 2012b; Kim et al., 2015, 2018). Although significant, however, the magnitude of difference observed in those studies was less than 0.1, which was potentially too small to have any practical effect on meat quality (Kim et al., 2015).

### Histological analysis

The representative histological images of the steak samples are shown in Fig. 3. The beef samples assigned to slow freezing (both SF-FTA and SF-AFT) exhibited more visually enlarged gaps compared to the samples assigned fast freezing, regardless of sequence of aging/freezing. Conversely, the AO (never frozen) samples showed less noticeable separation between the muscle fibers.

When comparing images of beef samples assigned to different sequence of aging/freezing, in general, more enlarged gaps between muscle fibers were appeared in the FTA samples compared to AFT. This observation may highlight the importance of aging in negating the damage generated from ice crystals when aging is applied prior to the freezing process. It was previously suggested that muscle swelling would occur during aging due to proteolysis, weakening the muscle structure and entrapping more water within the cell (Melody et al., 2004; Huff-Lonergan and Lonergan, 2005). It could then be suggested that more fragmented/weakened structure of the AFT samples through proteolysis would be more flexible and toler-
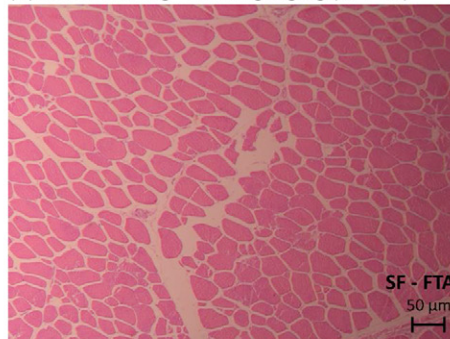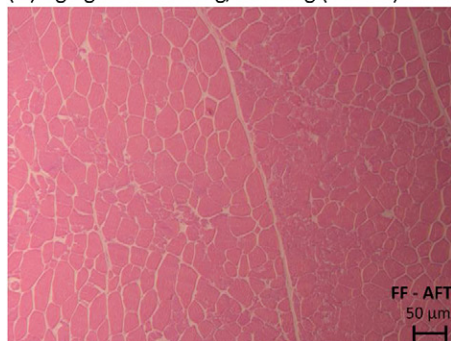
(A) Aged Only (AO)
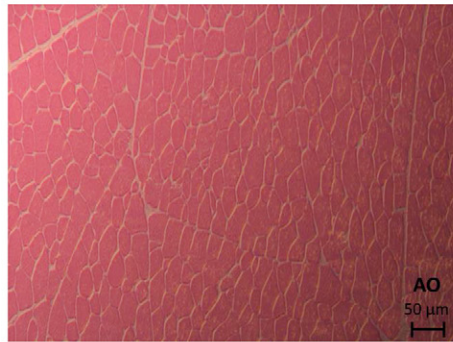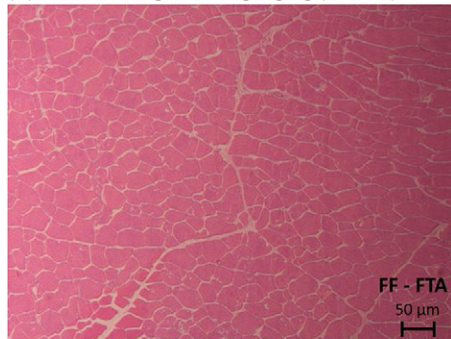


(B) Fast Freezing-Thawing/Aging (FF-FTA)          (C) Slow Freezing- Thawing/Aging (SF-FTA)

(D) Aging-Fast Freezing/Thawing (FF-AFT)          (E) Aging-Slow Freezing/Thawing (SF-AFT)
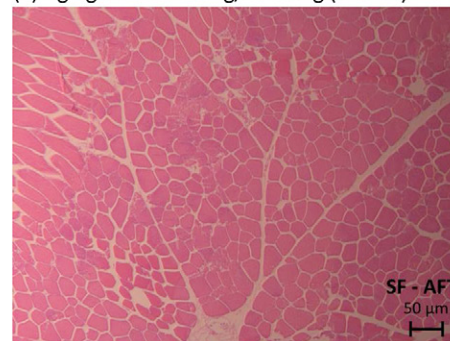
**Figure 3.** Histology slides observation using light microscope at 100× magnification of *M. longissimus lumborum* (LL) treated with different aging/freezing sequence and freezing rate combinations. AO (aged only); FF-FTA (fast freezing-thawing/aging); SF-FTA (slow freezing-thawing/aging); FF-AFT (aging-fast freezing/thawing); SF-AFT (aging-slow freezing/thawing).

ant to the damage generated by the large ice crystals during freezing compared to the FTA samples. On the other hand, the intact muscle structure prior to postmortem proteolysis in FTA would then be more susceptible to the large ice-crystal formation on freezing. It was previously postulated that enlarged muscle fibers gaps in FTA would be less likely affected by further proteolysis (Setyabrata and Kim, 2019), and thus, produce and sustain a higher degree of damage in the final product. The magnitude of freezing-induced damage, however, could potentially be further diminished by applying fast-freezing, as the muscle structure of FF-FTA exhibited relatively smaller gaps

between the muscle fibers compared to SF-FTA, and comparable to both AO and FF-AFT (Fig. 3).

Cryo-histology was also conducted to observe the location and size of ice crystal formation within the frozen muscle samples (Fig. 4). Larger extracellular ice crystals were clearly identified in the cryo-histology results from the SF samples (SF-FTA and SF-AFT), potentially leading to the formation of enlarged muscle fiber gaps after thawing, as observed in regular histology for both SF-FTA and SF-AFT. In contrast, smaller intracellular ice crystals were observed in both cryo-histology results of FF-FTA and
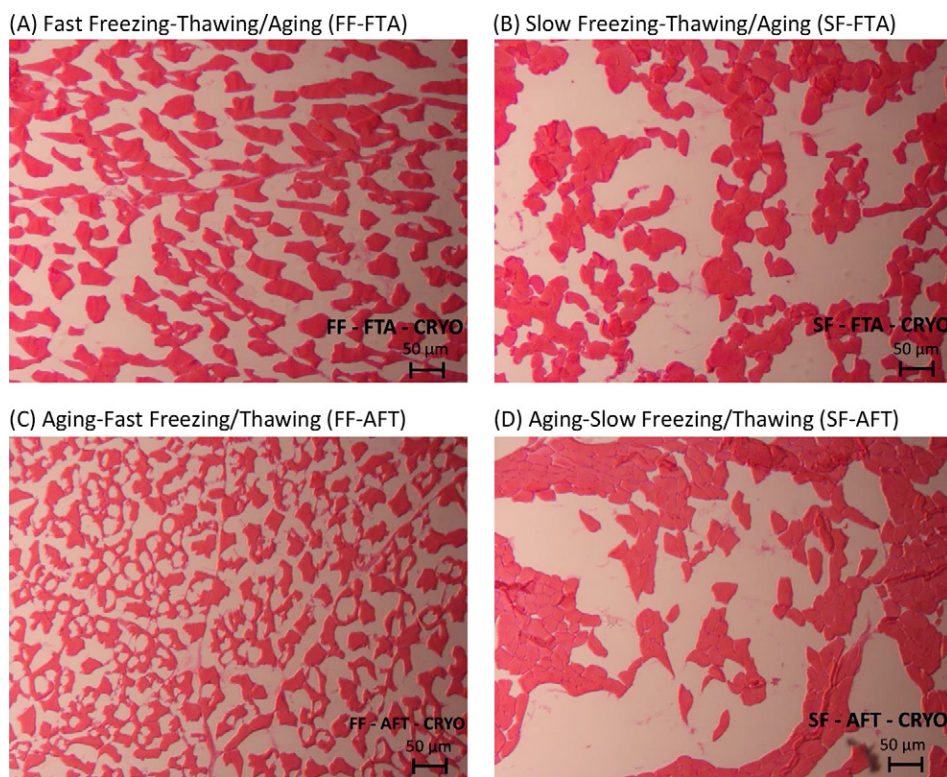
**Figure 4.** Cryohistology slides observation using light microscope at 100× magnification of *M. longissimus lumborum* (LL) treated with different aging/freezing sequence and freezing rate combinations. FF-FTA (fast freezing-thawing/aging); SF-FTA (slow freezing-thawing/aging); FF-AFT (aging-fast freezing/thawing); SF-AFT (aging-slow freezing/thawing).

FF-AFT, although more were apparent in the FF-AFT beef samples compared to the FF-FTA samples.

Numerous studies have previously reported that slow freezing would cause a noticeable damage due to the large extracellular ice crystals formed between muscle fibers (Rahelić et al., 1985; Grujić et al., 1993; Alizadeh et al., 2007; Kim et al., 2018), which was also found in the current study. Within the fast-frozen samples, extracellular ice crystals were also observed, although the extracellular ice crystals were more abundant in FF-FTA compared to FF-AFT. This observation could be attributed to the extent of proteolysis prior to freezing. Potentially, due to higher muscle integrity prior to aging in FTA samples compared to AFT, intracellular water was not able to be distributed equally throughout the muscle cells during freezing process and thus was entrapped within the extracellular space in the muscle.

### Water-holding capacity

The WHC of the beef samples was evaluated using multiple assays including purge/thaw loss, drip loss, and cook loss (Table 1). A significant interaction between freezing rate and sequence was found

in purge/thaw loss, where SF-FTA had a higher loss ($P < 0.0001$) compared to all other treatment combinations. Samples from FF-FTA had a higher purge/thaw loss compared to FF-AFT, SF-AFT and AO ($P < 0.05$). However, no differences in purge/thaw loss were found between FF-AFT, SF-AFT and AO ($P > 0.05$).

Interestingly, the aging/freezing sequence and freezing rate interaction was more pronounced in the purge/thaw loss of FTA when compared to AFT. Both FF-AFT and SF-AFT were found to have similar purge/thaw loss and were comparable to AO ($P > 0.05$). Aging prior to freezing was suggested to improve the WHC of frozen/thawed meat (Kim et al., 2011, 2015). Farouk et al. (2012) suggested that aging prior to freezing would disrupt the drip channel and thus entrapped more free water within the muscle, mitigating damage from freezing. It is also possible that due to this disruption, water is then localized within the already disrupted muscle structure and thus help to minimize the dispersion of ice crystal in the muscle.

It is worth noting that although severe purge/thaw loss was observed in both FTA treatments, freezing rate appeared to play a critical role in determining the

final frozen/thawed product quality for the FTA treated samples. Between the FTA treatments, FF-FTA had a lower purge/thaw loss when compared to SF-FTA ($P <$ 0.0001). The improved WHC of the FF-FTA might be attributed to smaller ice crystal formation, distributed in both intra- and extracellular level, when compared to SF-FTA, as previously discussed in the histology images. The current observation indicated that damage to drip channels prior to aging in FTA is likely to produce irreversible damage to the postmortem muscle and prevent entrapment of water when proteolysis occurs. However, the magnitude of the damage would be considerably reduced by applying fast freezing, minimizing the muscle gaps produced.

For cook loss, a significant interaction between aging/freezing sequence and freezing rate was found. The SF-FTA exhibited a lower cook loss compared to SF-AFT ($P < 0.05$), while the steaks from FF-FTA were not different from FF-AFT ($P > 0.05$). This result indicated that for cooking loss, the aging/freezing sequence was not a major contributing factor if FF was applied. On the other hand, when SF was applied, aging/freezing sequence would become more important, producing a lower cook loss when combined with FTA. A decrease in cook loss in SF-FTA was likely due to the increase in purge/thaw loss, leaving relatively less exudate available during cooking. In fact, steaks from AO exhibited a higher cook loss compared to FTA, irrespective of freezing rate ($P < 0.05$), but were not different compared to steaks from AFT ($P > 0.05$). This supported the previous speculation, as AO had a higher cooking loss, but had a lower purge/thaw loss compared to FTA treatments.

Only aging/freezing sequence affected drip loss ($P = 0.0187$), showing that AO had a lower drip loss compared to AFT, while FTA was not different from either AO or AFT ($P > 0.05$). The higher drip loss in AFT, compared to AO, was due to the freezing impacts on the muscle structure and subsequent release of water on thawing. Hiner et al. (1945) also suggested that ice crystal formation, regardless of location, resulted in muscle fiber damage, reducing its ability to hold and retain water. Kim et al. (2017) also reported a similar trend, where frozen/thawed beef loins had a higher drip loss compared to their non-frozen counterparts.

### Instrumental tenderness

The AO sample had a greater shear force compared to all other treatments ($P < 0.0001$, Fig. 5). No differences among the frozen samples were found ($P > 0.05$), irrespective of the aging/freezing sequences and freezing rates. Similar to the current results, numerous
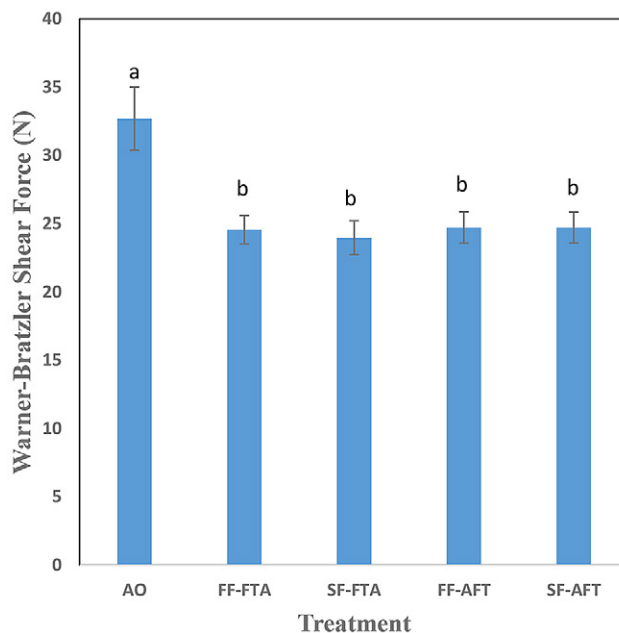


**Figure 5.** Warner-Bratzler Shear Force of *M. longissimus lumborum* (LL) treated with different aging/freezing sequence and freezing rate combinations. AO (aged only); FF-FTA (fast freezing-thawing/aging); SF-FTA (slow freezing-thawing/aging); FF-AFT (aging-fast freezing/thawing); SF-AFT (aging-slow freezing/thawing). [a,b]Least square means with different letters indicate significant ($P < 0.05$); Error bars indicate standard error of the mean.

previous reports also indicated that aged only (never frozen) samples had a higher shear force value when compared to their frozen counterpart, regardless of aging/freezing sequence (Crouse and Koohmaraie, 1990; Shanks et al., 2002; Lagerstedt et al., 2008; Grayson et al., 2014; Aroeira et al., 2016; Kim and Kim, 2017; Kim et al., 2017).

It is generally accepted that the reduction of instrumental tenderness value in the frozen/thawed products could be attributed to the ice crystal formation in the muscle (Crouse and Koohmaraie, 1990; Leygonie et al., 2012a). The weakened structure due to ice crystal damage would then be further fragmented through the postmortem proteolysis decreasing shear force values, which likely would improve tenderness (Koohmaraie, 1990; Grayson et al., 2014). The current results, however, showed no impacts of different combinations of aging/freezing sequence coupled with freezing rate on shear force values. This demonstrates that ice crystal formation, regardless of its size and location, would generate damage and disrupt muscle structure of the products.

### Proteolysis analysis

Both desmin and troponin-T have been known to be related to meat tenderness and are commonly used

## (A) Western Blot - Desmin
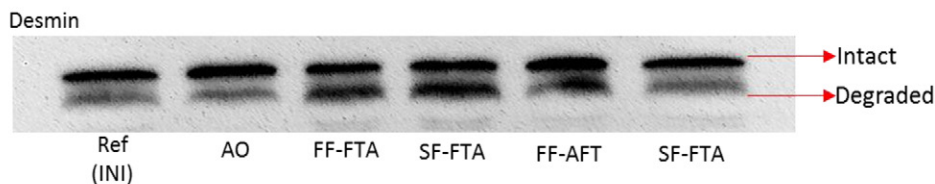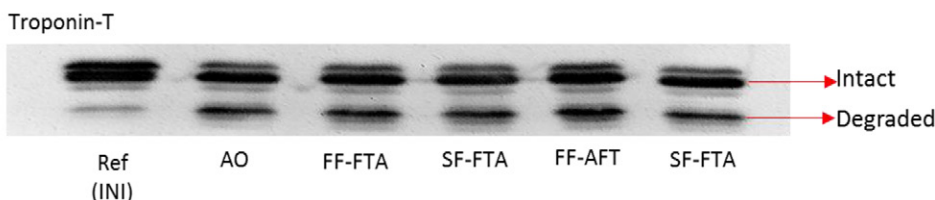


## (B) Western Blot – Troponin-T



**Figure 6.** Representative western blot of both Desmin and Troponin-T from *M. longissimus lumborum* (LL). AO (aged only); FF-FTA (fast freezing-thawing/aging); SF-FTA (slow freezing-thawing/aging); FF-AFT (aging-fast freezing/thawing); SF-AFT (aging-slow freezing/thawing); Ref (initial sample, INI).

as an indication of tenderness development (Zhang et al., 2006). The Western blot results of troponin-T revealed that a higher abundance of degraded products was found in both freezing treatments, where both FTA and AFT had a significantly higher troponin-T degradation compared to AO ($P = 0.044$, Table 2 and Fig. 6). No significant freezing rate and interaction effect was found in troponin-T ($P > 0.05$). This result indicates more fragmentation occurred in both frozen/thawed treatments. In fact, this observation was in agreement with the result of WBSF values in the present study (Fig. 5), where AO had a higher WBSF value compared to the frozen/thawed beef samples (AFT and FTA). Numerous studies have reported a positive impact of freezing on meat tenderization/muscle fragmentation (Petrović et al., 1993; Shanks et al., 2002; Vieira et al., 2009; Leygonie et al., 2012a), which could be primarily due to the loss of structural weakening induced by ice crystal formation coupled with endogenous proteolytic degradation (Leygonie et al., 2012a; Kim et al., 2015; Setyabrata and Kim, 2019).

A significant interaction in desmin degradation was identified in the current study, where SF-AFT had a lower degradation compared to all other treatment ($P < 0.05$), except FF-AFT ($P > 0.05$). Although not significant, a strong trend ($P = 0.063$) of greater desmin degradations were found in FF-AFT compared to SF-AFT. The greater formation of small intracellular ice crystals within the muscle fiber could potentially contribute to the higher desmin degradation in FF-AFT, perhaps from inducing immediate damage to intermediate filament connecting the myofibrils.

## Conclusions

The results in the current study showed that aging/freezing sequence and freezing rate would affect the final quality attributes of frozen/thawed beef steaks. The impact of freezing rate was more noticeable on FTA compared to AFT. The combination of fast freezing with FTA significantly reduced the purge/thaw loss of the product compared to its slow frozen counterpart. The improvements, however, did not fully mitigate the negative freezing effect, when compared to both aged only and AFT. While freezing/thawing improved instrumental tenderness of beef *M. longissimus lumborum* steaks compared to non-frozen control, no freezing rate impact was found for instrumental tenderness, regardless of aging/freezing sequence. In conclusion, fast freezing could improve meat quality attributes of products from the FTA process, but the considerable increase in purge/thaw loss by freezing first prior to aging would still be a major drawback. Thus, further research on evaluating different aging/freezing/thawing conditions (e.g., beef from different breeds, freezing whole muscle subprimals (sections) vs. steak, or different freezing and thawing rates) would be beneficial for the meat industry to produce frozen/thawed beef products with improved quality.

## Acknowledgments

**Table 2.** Least square means of protein abundance for desmin and troponin-T from *M. longissimus lumborum* (LL) treated with different aging/freezing sequence and freezing rate combination

| Treatment | Desmin | | Troponin-T | |
|---|---|---|---|---|
| | Intact | Degradation | Intact | Degradation |
| Aging/freezing sequence effect | | | | |
| Aged only (AO) | 0.74 | 1.12 | 0.75 | 1.93[a] |
| Freezing-thawing/aging (FTA) | 0.65 | 1.09 | 0.75 | 2.21[b] |
| Aging-freezing/thawing (AFT) | 0.65 | 0.89 | 0.80 | 2.19[b] |
| SEM[1] | 0.08 | 0.11 | 0.07 | 0.14 |
| *P*-value | 0.0625 | 0.0691 | 0.3214 | 0.0440 |
| Freezing rate effect | | | | |
| Fast freezing (FF) | 0.68 | 1.06 | 0.77 | 2.24 |
| Slow freezing (SF) | 0.63 | 0.92 | 0.78 | 2.16 |
| SEM | 0.07 | 0.10 | 0.08 | 0.15 |
| *P*-value | 0.2370 | 0.0915 | 0.9595 | 0.3920 |
| Aging/freezing sequence and freezing rate effect | | | | |
| Aged only (AO) | 0.74 | 1.12[a] | 0.75 | 1.93 |
| FTA with fast freezing (FF-FTA) | 0.67 | 1.11[a] | 0.74 | 2.25 |
| FTA with slow freezing (SF-FTA) | 0.63 | 1.07[a] | 0.76 | 2.18 |
| AFT with fast freezing (FF-AFT) | 0.67 | 1.01[ab] | 0.81 | 2.24 |
| AFT with slow freezing (SF-AFT) | 0.63 | 0.77[b] | 0.80 | 2.13 |
| SEM | 0.05 | 0.102 | 0.05 | 0.12 |
| *P*-value | 0.1051 | 0.0114 | 0.5523 | 0.0646 |

[1]SEM, standard error of the mean.

[a,b]Least square means with different letters are different in the same column ($P < 0.05$).

# Literature Cited

Alizadeh, E., N. Chapleau, M. de Lamballerie, and A. Le-Bail. 2007. Effect of different freezing processes on the microstructure of Atlantic salmon (*Salmo salar*) fillets. Innov. Food Sci. Emerg. Technol. 8:493–499. doi:10.1016/j.ifset.2006.12.003

Añón, M. C., and A. Calvelo. 1980. Freezing rate effects on the drip loss of frozen beef. Meat Sci. 4:1–14. doi:10.1016/0309-1740(80)90018-2

Aroeira, C. N., R. A. Torres Filho, P. R. Fontes, L. A. M. Gomide, A. L. S. Ramos, M. M. Ladeira, and E. M. Ramos. 2016. Freezing, thawing and aging effects on beef tenderness from *Bos indicus* and *Bos taurus* cattle. Meat Sci. 116:118–125. doi:10.1016/j.meatsci.2016.02.006

Bogdanowicz, J., M. Cierach, and T. Żmijewski. 2018. Effects of aging treatment and freezing/thawing methods on the quality attributes of beef from Limousin × Holstein-Friesian and Hereford × Holstein-Friesian crossbreeds. Meat Sci. 137:71–76. doi:10.1016/j.meatsci.2017.10.015

Choe, J. H., A. Stuart, and Y. H. B. Kim. 2016. Effect of different aging temperatures prior to freezing on meat quality attributes of frozen/thawed lamb loins. Meat Sci. 116:158–164. doi:10.1016/j.meatsci.2016.02.014

Coombs, C. E. O., B. W. B. Holman, M. A. Friend, and D. L. Hopkins. 2017. Long-term red meat preservation using chilled and frozen storage combinations : A review. Meat Sci. 125:84–94. doi:10.1016/j.meatsci.2016.11.025

Crouse, J. D., and M. Koohmaraie. 1990. Effect of freezing of beef on subsequent postmortem aging and shear force. J. Food Sci. 55:573–574. doi:10.1111/j.1365-2621.1990.tb06819.x

Farouk, M. M. M., N. M. M. Mustafa, G. Wu, and G. Krsinic. 2012. The "sponge effect" hypothesis: An alternative explanation of the improvement in the waterholding capacity of meat with aging. Meat Sci. 90:670–677. doi:10.1016/j.meatsci.2011.10.012

Grayson, A. L., D. A. King, S. D. Shackelford, M. Koohmaraie, and T. L. Wheeler. 2014. Freezing and thawing or freezing, thawing, and aging effects on beef tenderness. J. Anim. Sci. 92:2735–2740. doi:10.2527/jas.2014-7613

Grujić, R., L. Petrović, B. Pikula, and L. Amidžić. 1993. Definition of the optimum freezing rate—1. Investigation of structure and ultrastructure of beef *M. longissimus dorsi* frozen at different freezing rates. Meat Sci. 33:301–318. doi:10.1016/0309-1740(93)90003-Z

Hergenreder, J. E., J. J. Hosch, K. A. Varnold, A. L. Haack, L. S. Senaratne, S. Pokharel, C. Beauchamp, B. Lobaugh, and C. R. Calkins. 2013. The effects of freezing and thawing rates on tenderness, sensory quality, and retail display of beef subprimals. J. Anim. Sci. 91:483–490. doi:10.2527/jas.2012-5223

Hiner, L., L. L. Madsen, and O. G. Hankins. 1945. Histological characteristics, tenderness, and drip losses of beef in relation to temperature of freezing. Food Res. 10:312–324. doi:10.1111/j.1365-2621.1945.tb16173.x

Honikel, K. O. 1998. Reference methods for the assessment of physical characteristics of meat. Meat Sci. 49:447–457. doi:10.1016/S0309-1740(98)00034-5

Huff-Lonergan, E., and S. M. Lonergan. 2005. Mechanisms of waterholding capacity of meat: The role of postmortem biochemical and structural changes. Meat Sci. 71:194–204. doi:10.1016/j.meatsci.2005.04.022

Kiani, H., and D. W. Sun. 2011. Water crystallization and its importance to freezing of foods: A review. Trends Food Sci. Technol. 22:407–426. doi:10.1016/j.tifs.2011.04.011

Kim, H. W., J. H. Kim, J. K. Seo, D. Setyabrata, and Y. H. B. Kim. 2018. Effects of aging/freezing sequence and freezing rate on meat quality and oxidative stability of pork loins. Meat Sci. 139:162–170. doi:10.1016/j.meatsci.2018.01.024

Kim, H. W., and Y. H. B. Kim. 2017. Effects of aging and freezing/thawing sequence on quality attributes of bovine *Mm. gluteus medius* and *biceps femoris*. Asian-Australas. J. Anim. Sci. 30:254–261. doi:10.5713/ajas.16.0279

Kim, Y. H. B., M. Frandsen, and K. Rosenvold. 2011. Effect of ageing prior to freezing on colour stability of ovine *longissimus* muscle. Meat Sci. 88:332–337. doi:10.1016/j.meatsci.2010.12.020

Kim, Y. H. B., E. Huff-Lonergan, J. G. Sebranek, and S. M. Lonergan. 2010. High-oxygen modified atmosphere packaging system induces lipid and myoglobin oxidation and protein polymerization. Meat Sci. 85:759–767. doi:10.1016/j.meatsci.2010.04.001

Kim, Y. H. B., C. Liesse, R. Kemp, and P. Balan. 2015. Evaluation of combined effects of ageing period and freezing rate on quality attributes of beef loins. Meat Sci. 110:40–45. doi:10.1016/j.meatsci.2015.06.015

Kim, Y. H. B., B. Meyers, H. W. Kim, A. M. Liceaga, and R. P. Lemenager. 2017. Effects of stepwise dry/wet-aging and freezing on meat quality of beef loins. Meat Sci. 123:57–63. doi:10.1016/j.meatsci.2016.09.002

Koohmaraie, M. 1990. Quantification of Ca2(+)-dependent protease activities by hydrophobic and ion-exchange chromatography. J. Anim. Sci. 68:659–665. doi:10.2527/1990.683659x

Lagerstedt, Å., L. Enfält, L. Johansson, and K. Lundström. 2008. Effect of freezing on sensory quality, shear force and water loss in beef *M. longissimus dorsi*. Meat Sci. 80:457–461. doi:10.1016/j.meatsci.2008.01.009

Leygonie, C., T. J. Britz, and L. C. Hoffman. 2012a. Review Impact of freezing and thawing on the quality of meat. Meat Sci. 91:93–98. doi:10.1016/j.meatsci.2012.01.013

Leygonie, C., T. J. Britz, and L. C. Hoffman. 2012b. Meat quality comparison between fresh and frozen/thawed ostrich M. iliofibularis. Meat Sci. 91:364–368. doi:10.1016/j.meatsci.2012.02.020

Melody, J. L., S. M. Lonergan, L. J. Rowe, T. W. Huiatt, M. S. Mayes, and E. Huff-Lonergan. 2004. Early postmortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles. J. Anim. Sci. 82:1195–1205. doi:10.2527/2004.8241195x

Muela, E., C. Sañudo, M. M. Campo, I. Medel, and J. A. Beltrán. 2010. Effect of freezing method and frozen storage duration on instrumental quality of lamb throughout display. Meat Sci. 84:662–669. doi:10.1016/j.meatsci.2009.10.028

Pearce, K. L., K. Rosenvold, H. J. Andersen, and D. L. Hopkins. 2011. Water distribution and mobility in meat during the conversion of muscle to meat and ageing and the impacts on fresh meat quality attributes— A review. Meat Sci. 89:111–124. doi:10.1016/j.meatsci.2011.04.007

Petrović, L., R. Grujić, and M. Petrović. 1993. Definition of the optimal freezing rate—2. Investigation of the physico-chemical properties of beef *M. longissimus dorsi* frozen at different freezing rates. Meat Sci. 33:319–331. doi:10.1016/0309-1740(93)90004-2

Rahelić, S., A. H. Gawwad, and S. Puač. 1985. Structure of beef *Longissimus dorsi* muscle frozen at various temperatures: Part 2—ultrastructure of muscles frozen at -10, -22, -33, -78 and -115°C. Meat Sci. 14:73–81. doi:10.1016/0309-1740(85)90083-X

Setyabrata, D., and Y. H. B. Kim. 2019. Impacts of aging/freezing sequence on microstructure, protein degradation and physico-chemical properties of beef muscles. Meat Sci. 151:64–74. doi:10.1016/j.meatsci.2019.01.007

Shanks, B. C., D. M. Wulf, and R. J. Maddock. 2002. Technical note: The effect of freezing on Warner-Bratzler shear force values of beef *longissimus* steaks across several postmortem aging periods. J. Anim. Sci. 80:2122–2125. doi:10.1093/ansci/80.8.2122

USDA. 2017. United States standards for grades of carcass beef. Agricultural Marketing Service, Washington, DC

Vieira, C., M. T. Diaz, B. Martínez, and M. D. García-Cachán. 2009. Effect of frozen storage conditions (temperature and length of storage) on microbiological and sensory quality of rustic crossbred beef at different states of ageing. Meat Sci. 83:398–404. doi:10.1016/j.meatsci.2009.06.013

Whipple, G., and M. Koohmaraie. 1992. Freezing and calcium chloride marination effects on beef tenderness and calpastatin activity. J. Anim. Sci. 70:3081–3085. doi:10.2527/1992.70103081x

Zhang, W. G., S. M. Lonergan, M. A. Gardner, and E. Huff-Lonergan. 2006. Contribution of postmortem changes of integrin, desmin and m-calpain to variation in water holding capacity of pork. Meat Sci. 74:578–585. doi:10.1016/j.meatsci.2006.05.008