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Abstract

This study aimed to determine the effect of frozen-then-chilled storage on free Ca2+, proteolytic enzyme activity of calpains and the proteasome, water-holding capacity and shear force of porcine longissimus thoracis et lumborum muscle. Pork loins were subjected to either chilled storage at 2 ± 1 °C for 1, 2, 4, 6 and 9 days, or frozen-then chilled storage (-20 ± 1 °C for 1 week followed by thawing overnight). Free Ca2+ increased with chilled storage in the non-frozen group. Frozen-then-chilled storage increased free Ca2+ concentration, followed by a faster decrease of calpain-1 activity and activation of around 50% of calpain-2. Proteasome activity was reduced by around 40% following freezing-thawing. Purge loss increased and water-holding capacity of myofibrils decreased in the frozen-thawed group, suggesting considerable denaturation of myofibrillar proteins. Shear force was not affected by freezing-thawing, and we speculate that the tenderizing effect of calpain activation was counteracted by loss of proteasome activity and substantial exudate loss.

Keywords	Freezing; Calpains; Proteasome; Purge loss; Shear force

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Cover letter

July 11th 2018

Dear Editor

Please find attached a minor revision of the manuscript entitled: Effects of frozen-then-chilled storage on proteolytic enzyme activity and water-holding capacity of pork loin.

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the publication of this manuscript and, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

We upload in separate files the manuscript, four Figures and two Tables and a response to the reviewers.

Yours sincerely

Per Ertbjerg
Associate Professor, Meat Technology
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All in all, the comments were addressed very well. I believe most major concerns have been alleviated, however I still have some minor comments regarding structure at specific points of the article.

Highlights:

- Add “storage” after frozen-then-chilled in second line

Answer: Done

Introduction:

L62 – add “an” before effective

Answer: Done

L63-64 – This is an improved justification, but do you have a reference for frozen-then-chilled storage offering more flexibility in the production process?

Answer: This is our own suggestion and hence we have no reference

L63 – remove comma after Coombs in reference

L65 – remove comma after Crouse in reference

L66 – add in comma after pork

L73 – remove space between “activity” and “,”

Answer: Changes were done as suggested.

Materials and Methods:

L78-82 – I would move “from the 5th thoracic vertebrae to the last lumbar vertebrae” to the first line after “(M. longissimus thoracis et lumborum),” I would also put “from a local slaughterhouse in Finland at 6 h…” and the rest of its sentence immediately after “Danish Landrace)” and remove “the loins were obtained”

L78 – please spell out PSE and DFD

L87 – I would suggest starting a new paragraph with “A full factorial design..”

L97 – I would put “, aperture diameter 8 mm, and calibrated using a white tile…” after “D65 illuminant”)

L114 – add “the” before previous

L129 – add in “(100%)” after reference standard

L158 – consider starting with “Following treatment (chilled or frozen-then-chilled storage),”

L160 – replace “frozen-then-chilled storage” with “treaytment (expressed as a percentage)”

L165 – add in “previously” after described

Answer: Changes were done as suggested.

Results:

L209 – it may be worth adding “No autolyzed calpain-1 activity was detected” at the end as this appears to be correct looking at Fig. 2A and its absence in 2C

L215 – replace “at” with “on”

L216 – remove “of two”

L235 – add in “chilled” before storage

L236 – add in “chilled” before storage

L240 – put comma after found, and replace “and” with “where” and delete “was”

L241 – replace “whereas” with “while”

Answer: Changes were done as suggested.

Discussion:

L254 – it may be worth mentioning that no difference in free Ca2+ was found in the Colle study regardless of freezing or not

L258 – add in “the” before leaking

L257-259 – would this phenomenon only exist only in frozen-thawed meat?

L261 – start sentence with “However,” before Colle

L286 - replace “at” with on

L313 – remove comma after Houbak

L334 – replace “at” with on

L352 – remove comma after Houbak

L353 – remove comma after Thomas

Answer: Changes were done as suggested.

Conclusion:

I'd suggest adding a small section (1-2 sentences) at the end giving a reason why freeze-thawing prior to chilled storage/ageing is beneficial based on your data.

Answer: A sentence was added at the end of the conclusion justifying why freeze-thawing prior to chilled storage/ageing can be beneficial.

Figure captions:

Fig. 4 L490 – place A) before water-holding capacity and B) before purge loss, and A) needs to be before B) here

Answer: Changes were done as suggested.

- Frozen-then-chilled storage of pork increased the free Ca^{2+} concentration
- Frozen-then-chilled storage had faster loss of calpain-1 and -2 activity than fresh
- The proteasome activity was reduced by around 40% following freezing-thawing
- Water-holding capacity of myofibrils decreased and purge loss increased
- Shear force was not affected by freezing-thawing

Effects of frozen-then-chilled storage on proteolytic enzyme activity and water-holding capacity of pork loin

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Abstract

This study aimed to determine the effect of frozen-then-chilled storage on free Ca^{2+}, proteolytic enzyme activity of calpains and the proteasome, water-holding capacity and shear force of porcine *longissimus thoracis et lumborum* muscle. Pork loins were subjected to either chilled storage at 2 ± 1 °C for 1, 2, 4, 6 and 9 days, or frozen-then chilled storage (-20 ± 1 °C for 1 week followed by thawing overnight). Free Ca^{2+} increased with chilled storage in the non-frozen group. Frozen-then-chilled storage increased free Ca^{2+} concentration, followed by a faster decrease of calpain-1 activity and activation of around 50% of calpain-2. Proteasome activity was reduced by around 40% following freezing-thawing. Purge loss increased and water-holding capacity of myofibrils decreased in the frozen-thawed group, suggesting considerable denaturation of myofibrillar proteins. Shear force was not affected by freezing-thawing, and we speculate that the tenderizing effect of calpain activation was counteracted by loss of proteasome activity and substantial exudate loss.

Keywords: Freezing, Calpains, Proteasome, Purge loss, Shear force

1. Introduction

Freezing is currently playing an essential role in extending the shelf-life of meat and meat products by preventing microbial spoilage and retarding oxidative deterioration. Often no major changes are observed

in sensory properties of frozen-thawed beef (Holman, Coombs, van de Ven, & Hopkins, 2017; Vieira, Diaz, Martínez, & García-Cachán, 2009) and lamb (Muela, Monge, Sañudo, Campo, & Beltrán, 2016). Lagerstedt, Enfält, Johansson, & Lundström (2008) reported decreased sensory tenderness and juiciness in the frozen-thawed beef compared to chilled meat, but no significant difference of acceptability between chilled and frozen-thawed beef was observed by consumers. However, some quality defects regarding biochemical and physicochemical changes could still occur by the freezing and thawing process and prolonged frozen storage periods (Coombs, Holman, Friend, & Hopkins, 2017). Ice crystallization during freezing damages muscle integrity and results in an increase of ionic strength in the liquid water outside the crystals, possibly leading to decreased quality of thawed meat (Leygonie, Britz, & Hoffman, 2012). Myofibrillar proteins, accounting for 60% to 70% of the total protein, play an important role in the muscle structure as the majority of water in the muscle fiber is trapped within and between the myofibrils (Huff-Lonergan & Lonergan, 2005). The damage of muscle fibers caused by ice crystallization and protein denaturation due to freezing and thawing might both contribute to movement of water from inside to outside of the muscle fibers and an increased exudate loss after thawing (Calvelo, 1981). Ice crystal formation is regarded as the main driving force of thaw loss. However, several studies have reported denaturation of myofibrillar proteins during frozen storage by observing an increased surface hydrophobicity, decreased protein solubility and decreased ATPase activity (Chan, Omana, & Betti, 2011; Xia, Kong, Xiong, & Ren, 2010), while frozen-thawed beef exhibited a decreased total endotherm of myosin (Wagner & Anon, 1985).

The calpains are believed to be the most important proteolytic enzymes with regard to degradation of myofibrillar proteins and meat tenderization (Lonergan, Zhang, & Lonergan, 2010). The activity of calpain-1 and calpain-2 is regulated by the free Ca^{2+} concentration in the sarcoplasm and also by the inhibitor calpastatin. The free Ca^{2+} concentration required for half maximal activity of calpain-1 and -2

are in the range of 3-50 µM and 400-800 µM, respectively (Goll, Thompson, Li, Wei, & Cong, 2003). Geesink, Taylor, Bekhit, & Bickerstaffe (2001) observed 106 µM of free Ca^{2+} concentration in 1 day *post-mortem* ovine muscle, which could activate calpain-1. Many studies have shown that the autolysis of calpain-1 coincided with increased myofibrils fragmentation during aging (Koohmaraie & Geesink, 2006; Veiseth, Shackelford, Wheeler, & Koohmaraie, 2001). Few studies have suggested a role for calpain-2 in proteolysis of pork (Pomponio et al., 2008; Pomponio & Ertbjerg, 2012) and also of beef during prolonged aging (Camou, Marchello, Thompson, Mares, & Goll, 2007; Colle & Doumit, 2017). The proteasome (26S) dissociates into the regulatory subunit (19S) and the multi-catalytic core (20S) after the depletion of ATP in muscle (Kemp, Sensky, Bardsley, Buttery, & Parr, 2010). There are some indications that the 20S proteasome is involved in proteolysis and in improving water-holding of meat during cold storage (Houbak, Ertbjerg, & Therkildsen, 2008; Zeng, Li, & Ertbjerg, 2017).

Chilled-then-frozen storage has been regarded as an effective method to produce frozen meat with relatively high quality attributes (Coombs et al., 2017). However, frozen-then-chilled storage may be an alternative for the industry offering more flexibility in the production process. Frozen-then-chilled storage has by Crouse & Koohmaraie (1990) been reported to increase meat tenderness compared to chilled storage in beef and by Kim, Kim, Seo, Setyabrata, & Kim (2018) in pork, possibly through altered *post-mortem* proteolysis. Some studies have indicated greater proteolysis measured by fragmentation index (Aroeira et al., 2016), autolysis of calpain-2 (Colle et al., 2018), and desmin degradation (Grayson, King, Shackelford, Koohmaraie, & Wheeler, 2014) in frozen-thawed beef during subsequent chilled storage, compared to the non-frozen beef. Knowledge on the effect of the freezing-thawing process in combination with chilled storage on the proteolytic enzymes of pork is lacking. Therefore, this study aimed to evaluate the effect of frozen-then-chilled storage on 1) free Ca^{2+}, the activity of calpain-1 and

-2, and the proteasome activity, and on 2) water-holding (purge loss, cooking loss, total loss and water-holding capacity of myofibrils) and shear force of porcine *longissimus thoracis et lumborum* muscle.

2. Materials and methods

2.1. Sample processing

Eight loins (*M. longissimus thoracis et lumborum*) from the 5th thoracic vertebrae to the last lumbar vertebrae showing no visible signs of being PSE (pale, soft, exudative) or DFD (dark, firm, dry) were obtained from eight different pigs (age 160-165 days, the cross Norwegian Landrace × Swedish Yorkshire × Danish Landrace) from a local slaughterhouse in Finland at 6 h *post-mortem* and were then transported to the meat laboratory at University of Helsinki. After trimming of visible connective tissue and external fat, at 12 h *post-mortem,* a small proportion (around 30 g) of each muscle was collected and frozen in liquid nitrogen and used as 12 h *post-mortem* reference samples for calpain analysis. The rest of each loin was first cut into five pieces (along the muscle length) and each piece was thereafter divided into two and individually vacuum packaged, resulting in 10 pieces of approximately 200 g.

A full factorial design with two treatments (non-frozen and frozen-thawed) and five chilled storage periods (1, 2, 4, 6 and 9 days) was used. Each animal was represented at every treatment and chilled storage period resulting in 8 replicates per measurement. The pieces were either stored (non-frozen, n = 40) at 2 ± 1 °C until 1 (24 h), 2, 4, 6 and 9 days *post-mortem*; or frozen at 12 h *post-mortem* at -20 ± 1 °C for 1 week and then thawed (frozen-thawed, n = 40) at 2 ± 1 °C overnight (defined as day 1), followed by chilled storage for the same days used in the non-frozen group. Chilled storage was in a cold room (Huurre, Vantaa, Finland) and freezing and frozen storage was in a walk-in freezer (Huurre). pH and color were measured on non-frozen samples at 24 h *post-mortem*. The average pH was 5.6 ± 0.1 measured by an insertion electrode (Mettler-Toleda Inlab 427). L*, a* and b* average values were 52.5 ± 2.6, 7.9

± 1.5 and 5.2 ± 1.6, respectively, measured by a Minolta Chroma meter CR-400 (Minolta Camera Co. Ltd., Osaka, Japan) set at D65 illuminant, aperture diameter 8 mm and calibrated using a white tile (C: Y = 93.6, x = 0.3130, y = 0.3193). Following chilled storage, samples for measuring calpains and the proteasome and water-holding capacity of myofibrils were frozen in liquid nitrogen and then stored at -80 ° C; samples for purge, cooking loss, shear force and free Ca^{2+} concentration were measured fresh.

2.2. Extraction procedure for proteolytic enzymes and isolation of myofibrils

Frozen samples (1.5 g) in 9 mL of cold extraction buffer (100 mM Tris-HCl buffer, 5 mM EDTA, and 10 mM monothioglycerol, pH 8.3) were homogenized using an IKA Ultra-Turrax T25 homogenizer (Labortechnik, Staufen, Germany, 3 ×10 s at 13,500 rpm, 10 s cooling between bursts), and were then centrifuged at 15,000 × g for 30 min at 4 °C. Glycerol was added to aliquots (500 µL) of the supernatant at a final concentration of 30% in order to protect calpain and proteasome activity during freezing and thawing. Myofibrils in the pellet were washed three times using washing buffer (75 mM KCl, 100 mM MES (2-(N-Morpholino) ethanesulfonic acid hydrate), 2 mM $MgCl_2$, 2 mM EGTA (ethylene glycol tetraacetic acid), pH 5.5) for measuring water-holding capacity of myofibrils.

2.3. Casein zymography

Calpains were determined according to the previous method described by Pomponio & Ertbjerg (2012) with minor modifications. Separating gels (containing 30% acrylamide, 3 M Tris-HCl, pH 8.8, 0.2% casein, 10% ammonium persulfate (APS), and tetramethylethylenediamine (TEMED)) were prepared and then covered by stacking gels (30% acrylamide, 1 M Tris-HCl (pH 6.8), 10% APS, and TEMED). Three volumes (75 µL) of sample were mixed with 1 volume (25 µL) of sample buffer (300 mM Tris, 40% glycerol, 0.02% bromophenol blue, and 100 mM DTT, pH 6.8). Samples (15 µL) in duplicate were loaded onto the gel; the electrophoresis was subsequently performed at 80 V for 3 h at 0 °C in running

buffer (25 mM Tris, 192 mM glycine, and 1 mM EDTA, pH 8.3). The incubation buffer (50 mM Tris-HCl, 10 mM monothioglycerol, and 4 mM $CaCl_2$, pH 7.5) was used to activate calpain and digest casein in the gels at room temperature with shaking for 1 hour (2 changes of buffer). After stopping the calpain activity by shaking gels for 30 min in buffer (20 mM Tris-HCl and 10 mM EDTA, pH 7.0), gels were stained in Coomassie Brilliant Blue G-250 overnight and then destained in deionized water for 5 h (5 changes of water). Clear bands in the blue gel indicated the calpain activity. Quantification of the bands was carried out using an AlphaImager® HP Versatile gel imaging system (ProteinSimple, Santa Clara, CA). The supernatants of samples from all eight muscles at 12 h *post-mortem* were mixed and collected and used as a reference standard (100%). The results of native and autolyzed calpain activity were expressed as relative intensity (%), and the native calpain-1 and -2 activity at 12 h in the non-frozen group were taken as 100%.

2.4. Free Ca^{2+} concentration

Free Ca^{2+} concentration was measured as described previously by Pomponio & Ertbjerg (2012) with some modifications. Meat samples (20 g) were finely chopped with a sharp knife and centrifuged at 20,000 × g for 30 min at 4 °C for collecting the supernatant. Then the free Ca^{2+} concentration in 4 mL aliquots of the supernatant following mixing with 80 µL of KCl (4 M) was detected by a Ca^{2+} ion selective electrode equipped with a reference electrode (perfectION™ Combination Calcium Electrode, Mettler Toledo AG, Greifensee, Switzerland). The measurement range of the electrode was from 0.5 µM to 1.0 M. The Ca^{2+} concentration and millivolts were correlated by establishing a calibration curve before each run. For each sample, the method of Ca^{2+} standard addition was used in which we established a relationship between the added $CaCl_2$ concentration and the measured Ca^{2+} concentration in sarcoplasm. All measurements were performed in duplicate at room temperature.

2.5. Proteasome activity measurement

The chymotrypsin-like activity of the proteasome was determined using carbobenzoxy-Gly-Gly-Leu-7-amido-4-methylcoumarin (Z-GGL-AMC) (Sigma, Saint Louis, MO) as a substrate. An aliquot (50 µL) of the supernatant (from section 2.2) was incubated with 12 µM Z-GGL-AMC for 6 mins at 30 °C in the activity buffer (20 mM Tris-HCl, 5 mM EDTA, and 10 mM monothioglycerol, pH 7.7) at a final volume of 250 µL. The AMC was subsequently released, and fluorescence was determined every 2 min for a total of 30 min to obtain the slope (changes in fluorescence per min) using a spectrofluorometer Infinite M200 scalable microplate reader (Tecan, Mannedorf, Germany). The excitation and emission wavelength were 360 and 450 nm, respectively. A standard curve containing 0.06 to 0.16 µM of AMC was made in order to calculate the amount of released AMC ($\mu M \times min^{-1}$). The activity was expressed in µM / (min × g meat).

2.6. Purge loss, cooking loss and Allo-Kramer shear force

Following treatment (chilled or frozen-then-chilled storage), the bags were opened and the meat blotted dry using filter paper. Purge loss was measured by calculating the difference between the initial weight and the weight after treatment (expressed as percentage) and thus included the thaw loss of frozen-thawed samples. Cooking loss was measured by cooking the steaks (approximately 130 g) in a water bath (72 °C) for 60 min (the center temperature of samples reached around 71.5 °C) followed by cooling in cold water for 30 min; then the weight loss of the steak before and after cooking was calculated, and the result of cooking loss was expressed as a percentage. The total loss was expressed as the sum of the purge and cooking loss. Allo-Kramer shear force was measured as described previously (Liu, Ruusunen, Puolanne, & Ertbjerg, 2014). The same steaks after cooking were cut into 6-8 small slices (20 × 20 × 6 mm, fiber axis along 20 mm direction), and shear force (N/g) was measured using an Allo-Kramer shear cell using

an Instron Model 6625 (Instron Co, Canton, MA). The average value of the small slices (6-8 replicates) was calculated and recorded.

2.7. Water-holding capacity of myofibrils

Water-holding capacity of myofibrils was determined according to the method described by Bao, Boeren, & Ertbjerg (2018). Myofibrils (around 1g) were mixed with 9 mL of the washing buffer (see section 2.2), and 1 mL of homogenate was transferred into an Eppendorf tube and then centrifuged at 2400 g for 10 min, and the supernatant was discarded. The amount of water held by the myofibrils was calculated by taking the weight difference of the myofibril pellet before and after drying overnight in an oven at 100 °C. The weight of the dry myofibril protein pellets were corrected for the residual salt originating from the washing buffer. The relative water-holding capacity of myofibrils was calculated as the amount of water held by one gram of protein.

2.8. Statistical analysis

The general linear model was used to analyze data by the IBM SPSS Statistics 24 software. Treatment (frozen-thawed and non-frozen) and chilled storage durations and their interaction were arranged as fixed effects, and animal number was arranged as a random factor. The significant differences between means (significance was defined at $P < 0.05$) were evaluated by the Tukey HSD (honest significant difference) test.

3. Results

3.1. Free Ca^{2+}

Frozen-then-chilled storage treatment significantly affected the concentration of free Ca^{2+} in pork *longissimus thoracis et lumborum* muscle (Table 1). On day 1, the frozen-thawed group showed a

significant increase of free Ca^{2+} concentration (from 140 to 420 μM) compared to the non-frozen group (Fig. 1). There was a clear effect ($P < 0.01$) toward an increase of free Ca^{2+} concentration with chilled storage time (Table 1, Fig. 1). The free Ca^{2+} concentration increased to 400 μM during chilled storage of non-frozen samples, whereas the free Ca^{2+} concentration in the frozen-thawed samples reached 510 μM on day 9. An interaction of chilled storage time × freezing-thawing treatment was also observed ($P < 0.01$, Table 1).

3.2. Calpain activity

Calpain-1 and -2 activities in *longissimus thoracis et lumborum*, sampled during aging at days 1, 2, 4, 6 and 9, were measured by casein zymography. Fig. 2A illustrates how the calpain activity was affected by storage. A faster decrease of both calpain-1 and calpain-2 activities during chilled storage was observed in the frozen-thawed group (Fig. 2B, Table 1). Native calpain-2 activity in the non-frozen group decreased ($P < 0.05$) from 106% at day 1 to 87% at day 9, whereas calpain-2 activity of the frozen-thawed group decreased ($P < 0.05$) to 47% after 9 days. The interaction between chilled storage and freezing-thawing treatment was significant for all calpain activities (Table 1). Activity of autolyzed calpain-2 was observed on day 4 for the non-frozen samples, and on day 2 for the frozen-thawed samples (Fig. 2C). An additional increase ($P < 0.05$) of autolyzed calpain-2 activity was found in the frozen-thawed group on day 6. No autolyzed calpain-1 activity was detected.

3.3. Proteasome activity

The effect of chilled storage on chymotrypsin-like activity of the proteasome in both the non-frozen and frozen-thawed groups is shown in Fig. 3. The frozen-thawed samples had lower proteasome activity compared to the non-frozen samples throughout the storage period ($P < 0.01$, Table 1). The proteasome activity on day 1 was thus 40% lower due to freezing-thawing. During storage, the proteasome activities

in both groups declined ($P < 0.05$) within the first 4 days, and thereafter remained relatively stable, with the values after 9 days being 24% lower in the frozen-thawed group compared with the non-frozen group.

3.4. Water-holding capacity of myofibrils

The water-holding capacity of myofibrils was greater ($P < 0.01$) in the non-frozen samples in comparison with the frozen-thawed samples stored at the same period (Fig. 4A, Table 1). A significant effect of chilled storage on the water-holding capacity of myofibrils was observed in both groups (Table 1); the water-holding capacity in the non-frozen group decreased from 10.8 g H_2O/g protein (day 1) to 9.24 (day 9), whereas the water-holding capacity in the frozen-thawed group remained slightly more stable and decreased from 9.14 g H_2O/g protein (day 1) to 8.03 (day 9).

3.5. Exudate loss

In this study, purge loss was defined as the sum of water loss during thawing and the subsequent chilled storage. Purge loss in both the non-frozen and frozen-thawed samples increased significantly during chilled storage (Fig. 4B). The frozen-thawed samples had greater purge loss ($P < 0.01$) compared to the non-frozen samples throughout the storage period (Fig. 4B, Table 1), and no significant interaction between freezing-thawing and chilled storage on purge loss was observed. During chilled storage, the cooking loss in the non-frozen group increased ($P < 0.05$) from 22.8% (day 1) to 26.3% (day 6), whereas cooking loss was unaffected by chilled storage in the frozen-thawed group (Table 1 and 2). The frozen-thawed samples showed greater total exudate loss ($P < 0.05$) during chilled storage compared to non-frozen samples (Table 2).

3.6. Shear force

A decreasing effect of chilled storage time on shear force was found, where Allo-Kramer shear force decreased over 9 days of storage, while there were no effect of freezing-thawing (Table 1 and 2).

4. Discussion

4.1. Free Ca^{2+} and calpains

The level of free Ca^{2+} influences the activation of calpains (Goll et al., 2003). The free Ca^{2+} concentration in *post-rigor* meat has previously been studied by various methods. Ji & Takahashi (2006) used atomic absorption spectrophotometry and observed around 75 µM and 50 µM of free Ca^{2+} in 1 day *post-mortem* pork and beef, respectively. However, several studies used a calcium selective electrode and around 100 µM of free Ca^{2+} has been reported in lamb (Hopkins & Thompson, 2001) and beef 1 day *post-mortem* (Hwang, Park, Cho, & Lee, 2004). In the present study on pork, a similar value of free Ca^{2+} after 1 day of storage in the non-frozen group was observed and a significant increase of free Ca^{2+} to 400 µM after 9 days of chilled storage was observed. Also using a calcium selective electrode, Colle et al. (2018) reported 120 µM of free Ca^{2+} in beef 1 day *post-mortem*, however, they did not observe an increase of free Ca^{2+} during 14 days of storage. No difference in free Ca^{2+} was found in the Colle study regardless of freezing or not. Zhang, Ma, Guo, Yu, & Han et al. (2018) found that free Ca^{2+} in beef gradually increased from 70 to 120 µM over a 7 day storage period. In ovine muscle the free Ca^{2+} was reported to increase almost twofold to 190 µM from day 1 to day 7 measured by atomic absorption spectrophotometry (Geesink et al., 2001). We speculate that the increase of free Ca^{2+} *post-mortem* in chilled stored as well as in frozen-thawed meat may be related to the leaking of membranes in sarcoplasmic reticulum, mitochondria and sarcolemma in combination with cessation of the ATP-requiring calcium pumps in the same membranes. The freezing-thawing process induced a large increase in free Ca^{2+} (Fig. 1), possibly due to accelerated leakage of membranes caused by ice crystals. However, Colle et al. (2018) did not observe any increase in free Ca^{2+} due to freezing-thawing. One reason that we

obtained a relative larger increase and also a larger final value of free Ca^{2+} is likely attributable to the use of an internal standard of Ca^{2+} added to the sarcoplasm of each sample in order to correct for considerable interference effects. In addition, the free Ca^{2+} may increase more in pork compared to sheep and beef.

Casein zymography has been used to determine the activity of native and autolyzed calpains (Veiseth et al., 2001). In the present study, the extractable activity of calpain-1 decreased faster than that of calpain-2 in both fresh and frozen-thawed pork, similar to several previous studies regarding calpain autolysis (Camou et al., 2007; Pomponio & Ertbjerg, 2012). Calpain-1 activity was not detectable on day 4 of chilled storage in the non-frozen group. The freezing-thawing process accelerated the disappearance of calpain-1 and it was not detectable on day 2. This result might be attributed to the observation that the free Ca^{2+} on day 1 was threefold greater in the sarcoplasm following freezing-thawing, and also based on the findings that both calpains and calpastatin are stable during frozen storage (Kristensen, Christensen, & Ertbjerg, 2006). In agreement, a decrease in calpain-1 activity was also observed in lamb following a freezing-thawing process (Ingólfsson & Dransfield, 1991).

Calpain-1 is activated in early *post-mortem* proteolysis, while calpain-2 in some studies has been reported to be partially activated later *post-mortem* (Boehm, Kendall, Thompson, & Goll, 1998; Dransfield, 1993; Pomponio et al., 2008). In this study, a decrease of calpain-2 activity by around 20% during 9 days of chilled storage in the non-frozen group, paralleled with the appearance of autolysis products, suggests a role for calpain-2 in proteolysis. In agreement, Pomponio et al. (2008) found that calpain-2 activity was 61% of its initial activity in pork on day 6 *post-mortem*. The increase of free Ca^{2+} found in the sarcoplasm of non-frozen pork during chilled storage could be an explanation for this activation as it approaches the level of half maximal activity of calpain-2 of 400-800 μM (Goll et al., 2003). Interestingly, an effect of

the freezing-thawing process on calpain-2 activity on day 1 was not observed in the present study, although the free sarcoplasmic Ca^{2+} was found to increase to 420 μM which appear to be sufficient to partly activate calpain-2 (Goll et al., 2003). In addition, the observation (Fig. 1 and 2B), that the frozen-thawed group showed an additional smaller increase in the free Ca^{2+} concentration and a substantial faster decrease of calpain-2 activity during subsequent chilled storage, suggests that a delay occurred between the increase in the free Ca^{2+} level and the activation of calpain-2. The binding of free sarcoplasmic Ca^{2+} to EF-hand structures and other Ca^{2+}-binding sites in calpain-2 molecules might need some time since some of these sites have low affinity for Ca^{2+} and also calpain-2 requires binding of more Ca^{2+} ions for activation compared to calpain-1 (Goll et al., 2003). In agreement, Colle et al. (2018), found that the calpain-2 activity in 2 day *post-mortem* beef had decreased by around 40% after a freezing-thawing process followed by aging for 1 day. Moreover, the interaction of phospholipids and calpain-2, presumably due to the disruption of muscle fibers and increased solute concentration caused by thawing, might potentially reduce the requirement of Ca^{2+} and contribute to the activation of calpain-2.

4.2. Proteasome activity

This is the first study showing the effect of frozen-then-chilled storage on the proteasome activity in pork. Our results suggest a role of the freezing-thawing process in decreasing the proteasome activity, which possibly results in decreased proteasome-related proteolysis during subsequent chilled storage. The chymotrypsin-like activity of proteasome decreased significantly by around 40% during chilled storage of 9 days in the non-frozen group. In agreement, Lamare, Taylor, Farout, Briand, & Briand (2002), observed a substantial decrease of chymotrypsin-like activity in beef *Rectus abdominis* muscle after 7 days of storage, whereas no changes of proteasome levels were found when they measured the α-1 subunit of the proteasome by western blot analysis; therefore, they attributed this decreased activity to structural changes during aging. Thomas, Gondoza, Hoffman, Oosthuizen, & Naudé (2004) found 26%

decreased chymotrypsin-like proteasome activity in ostrich *iliofibularis* muscle after 12 days of aging, and suggested a possible role of the proteasome in the tenderization process. Robert, Briand, Taylor, & Briand (1999) observed loss of Z disc material and disruption of I band in the myofibrils followed by incubating the pure 20S proteasomes for 4 hours. Houbak et al. (2008) found less degradation of structural proteins following inhibition of the proteasome chymotrypsin-like activity, suggesting involvement of the proteasome in proteolysis of *post-mortem* beef.

4.3. Water-holding capacity and exudate losses

Generally, decreased water-holding capacity of myofibrils and greater water loss was observed in the frozen-thawed group (Fig. 4 and Table 2). In agreement, freezing and thawing is well known to increase the water loss of meat, presumably due to mechanical damage caused by ice crystallization and protein denaturation (Calvelo, 1981). The mechanical damage from shrinkage and distortion of muscle fibers due to the formation of large ice crystals in the intra- and extracellular areas affect the reabsorption of water by muscle fibers during thawing. The importance of protein denaturation for formation of thaw loss is currently not well understood. Protein denaturation, shown as decreased solubility of myofibrillar protein and Ca^{2+}-ATPase activity (Qi et al., 2012; Wagner & Anon, 1985), and the formation of aggregates (Zhang, Li, Diao, Kong, & Xia, 2017), might be attributed to the increased concentration of solutes, dehydration of proteins, reaction of free fatty acids and proteins especially in fish muscle (Sikorski, Olley, Kostuch, & Olcott, 1976). In this study, the decreased water-holding capacity of myofibrils in the frozen-thawed group (Fig. 4A) further indicates that considerable denaturation of myofibrillar proteins is taking place due to freezing and thawing. On day 1, the water-holding capacity of myofibrils was reduced by 15% in the frozen-thawed group compared to the non-frozen group, and therefore, the water loss of meat would be expected to increase considerably due to freezing and thawing. In accord, the purge (or thaw) loss for frozen-thawed meat was around 9% points greater than that of the

non-frozen meat on day 1, suggesting that increased exudate loss of meat upon thawing is mainly because of the denaturation of myofibrillar proteins rather than a direct effect of mechanical damage of muscle fibers by ice crystals.

The freezing-thawing process slightly increased cooking loss on day 1. Likewise, Grayson et al. (2014) found a greater cooking loss when beef *longissimus lumborum* muscle was subjected to freezing-thawing treatments. In our study, the cooking loss of the non-frozen group increased during 9 days of chilled storage, while no significant difference in the frozen-thawed group was observed during chilled storage, presumably due to the substantial increase of purge loss caused by freezing and thawing.

4.4. Shear force

In beef, freezing and thawing or a combination of freezing, thawing and ageing has been reported either to increase the tenderness (Grayson et al., 2014) or not to affect shear force or sensory tenderness (Colle et al., 2018). In pork, a decreased shear force value was observed in samples subjected to frozen-then-chilled storage compared to frozen/thawed samples (Kim, et al., 2018). The freezing-thawing process did not affect the Allo-Kramer shear force in the present study. The increased activation of calpain-2, as shown in Fig. 2C, on one hand suggested increased proteolysis (Pomponio & Ertbjerg, 2012). On the other hand, the decrease of proteasome activity during frozen-then-chilled storage (Fig. 3) may negatively affect the degradation of myofibrillar proteins and hence the tenderization (Houbak et al., 2008; Thomas et al., 2004). The combined effect of increased calpain-2 activity and decreased proteasome activity following freezing and thawing may thus explain the lack of effect on shear force. In addition, the substantial exudate loss upon thawing could be another factor affecting meat toughness (Leygonie et al., 2012).

5. Conclusion

This study observed a significant increase of free Ca^{2+} in non-frozen pork during 9 days of chilled storage. Freezing-thawing of pork *longissimus* muscle resulted in a substantial release of free Ca^{2+}, and subsequent chilled storage accelerated the decrease of extractable activity of calpain-1 and activated about 50% of calpain-2. About 40% of the proteasome activity was initially lost in the frozen-thawed group and the activity remained lower during chilled storage compared to that of the non-frozen group. The freezing-thawing process increased total exudate loss, and reduced the water-holding capacity of myofibrils suggesting considerable denaturation of myofibrillar proteins. Freezing-thawing did not affect Allo-Kramer shear force; this might be attributed to activation of calpains together with a counteractive effect of loss of the proteasome activity and of a substantial exudate loss upon thawing. Frozen-then-chilled storage can be a method of activating calpain-1 and -2 of pork, possibly contributing to the meat tenderization process without excessive water loss.

Acknowledgments

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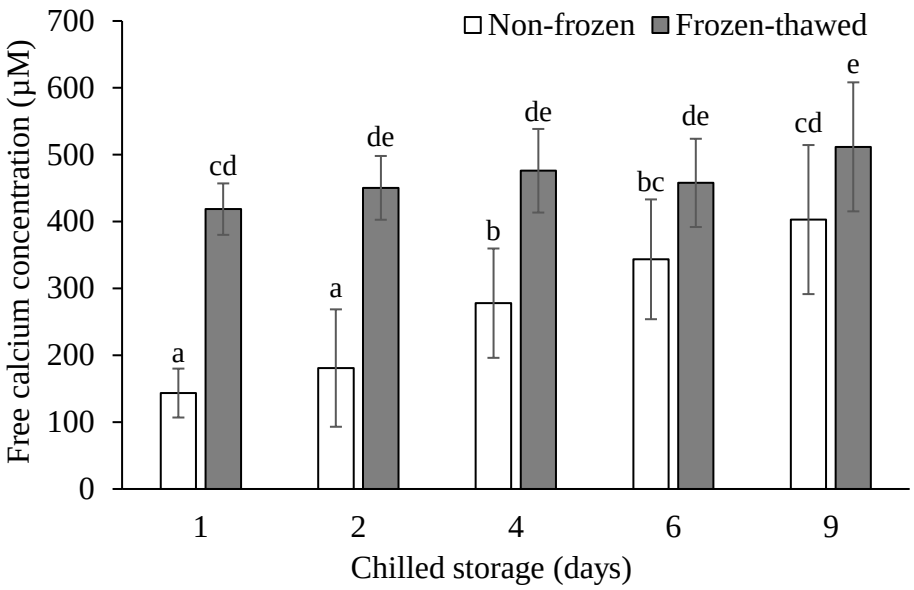
Figure captions:

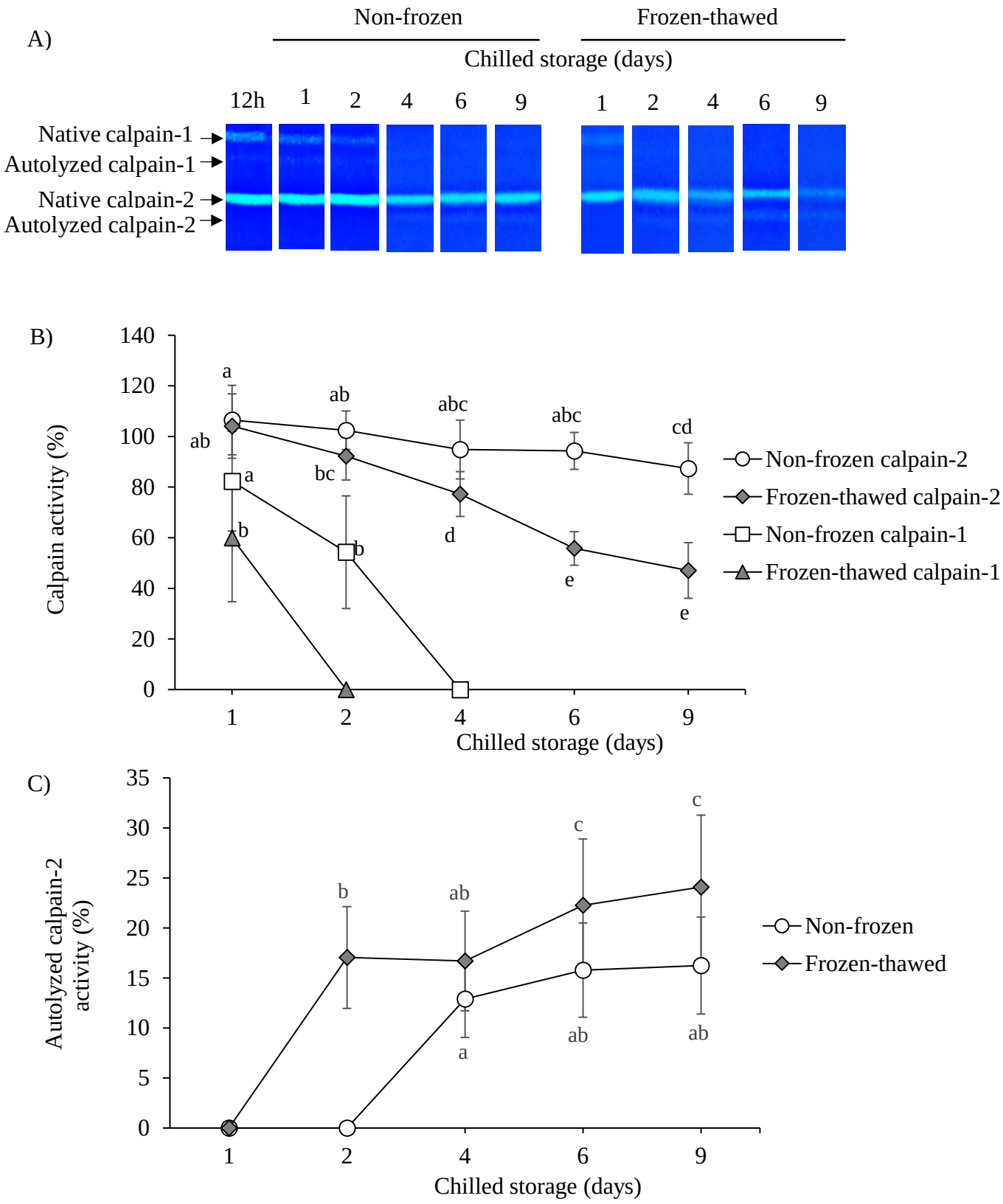
Fig.1. The effect of frozen-then-chilled storage on the free Ca^{2+} concentration of porcine *longissimus thoracis et lumborum* muscle. Means ± standard deviations are shown. a-e: Mean values with the same letter do not differ ($P > 0.05$).

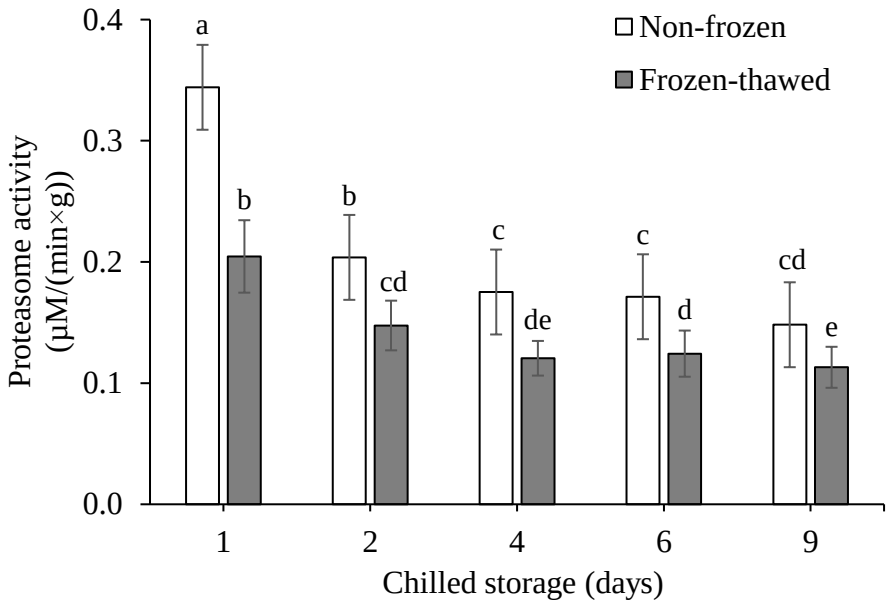
Fig. 2. Effect of frozen-then-chilled storage on A) native and autolyzed calpain-1 and -2 on the zymography gel and B) the activity of native calpain-1 and -2, and C) autolyzed calpain-2. Calpain activity at 12 h in the non-frozen samples was taken as 100%. a-e: Within each trait, mean values with the same letter do not differ ($P > 0.05$).

Fig. 3. The effect of frozen-then-chilled storage on the proteasome activity of porcine *longissimus thoracis et lumborum* muscle. Means ± standard deviations are shown. a-e: Mean values with the same letter do not differ ($P > 0.05$).

Fig. 4. Effect of frozen-then-chilled storage on A) water-holding capacity of myofibrils and B) purge loss. Means ± standard deviations are shown. a-f: Mean values with the same letter do not differ ($P > 0.05$).


Non-frozen
Frozen-thawed
Free calcium concentration (µM)
700
600
500
400
300
200
100
0
a
cd
a
de
b
de
bc
de
cd
e
1
2
4
6
9
Chilled storage (days)


A)
Non-frozen
Frozen-thawed
Chilled storage (days)
12h
1
2
4
6
9
1
2
4
6
9
Native calpain-1
Autolyzed calpain-1
Native calpain-2
Autolyzed calpain-2
B)
Calpain activity (%)
140
120
100
80
60
40
20
0
a
ab
abc
abc
cd
ab
a
bc
b
b
d
e
e
1
2
4
6
9
Chilled storage (days)
Non-frozen calpain-2
Frozen-thawed calpain-2
Non-frozen calpain-1
Frozen-thawed calpain-1
C)
Autolyzed calpain-2 activity (%)
35
30
25
20
15
10
5
0
b
ab
c
c
a
ab
ab
1
2
4
6
9
Chilled storage (days)
Non-frozen
Frozen-thawed


0.4
0.3
0.2
0.1
0.0
Proteasome activity
(μM/(min×g))
Non-frozen
Frozen-thawed
a
b
b
cd
c
de
c
d
cd
e
1
2
4
6
9
Chilled storage (days)

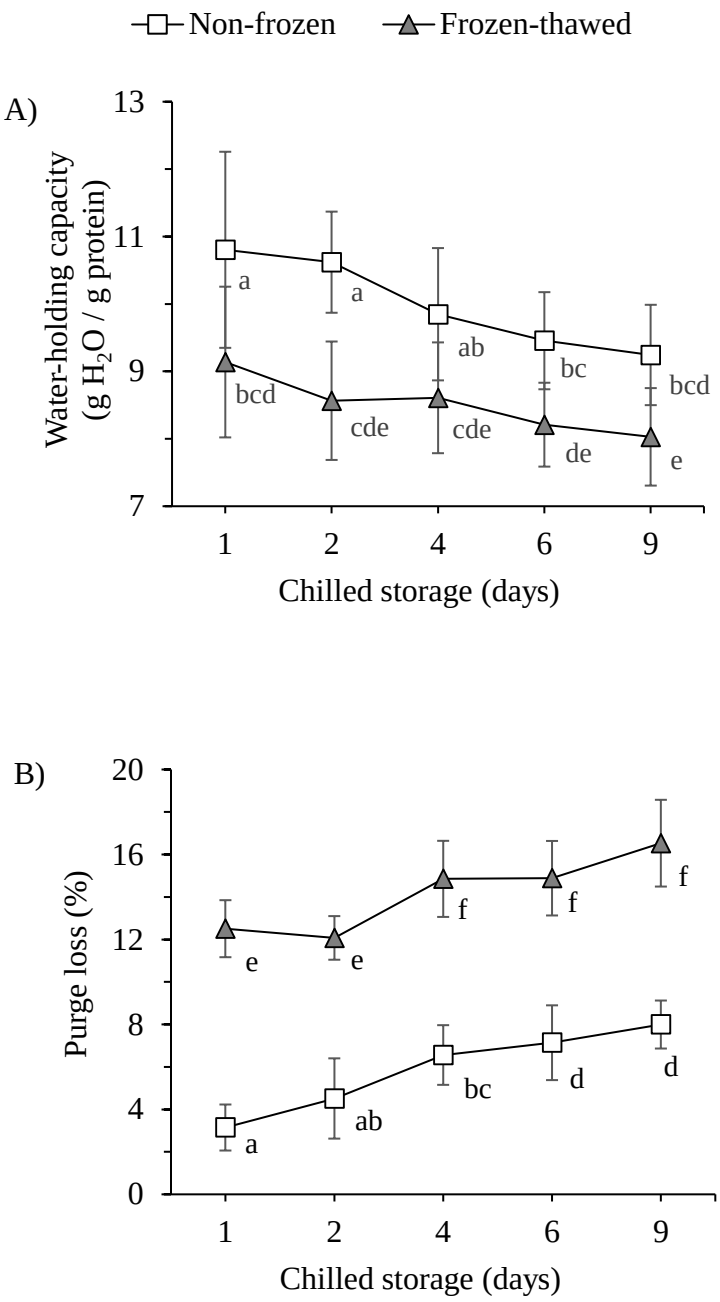

Non-frozen
Frozen-thawed
A)
Water-holding capacity
(g H_2O / g protein)
13
11
9
7
a
a
ab
bc
bcd
bcd
cde
cde
de
e
1
2
4
6
9
Chilled storage (days)
B)
Purge loss (%)
20
16
12
8
4
0
e
e
f
f
f
a
ab
bc
d
d
1
2
4
6
9
Chilled storage (days)

Table 1 Effect of chilled storage, treatment (control vs freezing-thawing) and their interaction on free Ca^{2+}, the activities of calpain-1, -2, autolyzed calpain-2 and the proteasome, water-holding capacity, water losses and shear force.

Effects	Free Ca^{2+}	Calpain-1 activity	Calpain-2 activity	Autolyzed calpain-2 activity	Proteasome activity	Water-holding of myofibrils	Purge loss	Cooking loss	Total loss	Shear force
Treatment	**	**	**	**	**	**	**	*	**	NS
Chilled storage	**	**	**	**	**	**	**	NS	**	*
Treatment × Chilled storage	**	**	**	**	**	NS	NS	**	**	NS

NS: not significant. * ($P < 0.05$), ** ($P < 0.01$).

Table 2 Effect of frozen-then-chilled storage on cooking loss, total loss and shear force of porcine *longissimus thoracis et lumborum* muscle.

	Treatment	Chilled storage (days)					
		1	2	4	6	9	SEM
Cooking loss (%)	Non-frozen	22.8^{a}	25.3^{bc}	25.0^{bc}	26.3^{bcd}	27.8^{cd}	0.63
	Frozen-thawed	26.5^{bcd}	25.1^{bcd}	27.6^{d}	26.4^{bcd}	24.7^{b}	
Total loss (%)	Non-frozen	26.4^{a}	29.3^{ab}	31.5^{bc}	33.1^{cd}	35.0^{de}	0.63
	Frozen-thawed	39.0^{fg}	37.1^{ef}	41.3^{g}	41.7^{g}	41.2^{g}	
Allo-Kramer shear force (N/g)	Non-frozen	152^{a}	149^{abc}	145^{bcd}	144^{cde}	145^{bcd}	3.8
	Frozen-thawed	153^{a}	151^{ab}	139^{e}	143 ^{de}	142de	

SEM: Standard error of the mean. Within traits, superscripts with the same letter do not differ ($P > 0.05$).