Can postmortem proteolysis explain tenderness differences in various bovine muscles?

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Abstract

This study investigated the relationship between postmortem proteolysis, muscle pH decline, sarcomere length (SL), intramuscular fat (IMF) and Warner-Bratzler shear force (WBSF) in four bovine muscles (*biceps femoris* (BF), *infraspinatus* (IS), *longissimus lumborum* (LL), *psoas major* (PM). The WBSF was low in BF, IS and PM, while LL had a higher value ($P<0.001$), but still considered as tender. The PM had fastest pH decline ($P<0.001$), ultimate pH was lowest in LL and PM and highest for IS ($P<0.001$), sarcomeres were longest for PM and shortest for BF and LL ($P<0.001$), while IS and PM had more IMF than BF and LL ($P=0.038$). Troponin T degradation was similar in all muscles after 2d postmortem, however after 13d LL had more degradation than IS ($P=0.003$). The MMP-2 activity increased during storage ($P=0.001$), while IS had less activity than the other muscles ($P=0.022$). Although the variation in proteolytic activity could not explain the variation in WBSF, the study provides useful knowledge for the meat industry for optimising processing and storage procedures for different beef muscles.

Keywords

muscle; MMP-2; troponin T; connective tissue; microstructure; Warner-Bratzler shear force

1. Introduction

Tenderness is a critical factor determining the consumer's acceptance of meat, and considerable variation in tenderness exists between different muscles (Belew, Brooks, McKenna, & Savell, 2003; Ramsbottom, Strandine, & Koonz, 1945; Shackelford, Wheeler, & Koohmaraie, 1995;). The ultimate tenderness of meat is thought to mainly be determined by three factors: the background toughness, the toughening phase and the tenderisation phase, which again can be influenced by e.g. animal genetics, feeding, handling and the slaughter process (for review, see Warner, Greenwood, Pethick, & Ferguson, 2010). While background toughness is a constant value established at slaughter, the opposing toughening and tenderisation phases occur during postmortem storage of meat. The background toughness is mainly determined by the organisation and amount of connective tissue in the muscle. Early studies by Strandine, Koonz, and Ramsbottom (1949), where 50 beef and 12 chicken muscles were compared, revealed a general correlation between perimysium organisation and tenderness of the muscles. In addition, the background toughness can be influenced by the level of intramuscular fat (IMF) (Dikeman et al., 1986), which is known to vary between muscles (Highfill, Esquivel-Font, Dikeman, & Kropf, 2012; Von Seggern, Calkins, Johnson, Brickler, & Gwartney, 2005). The toughening phase is a result of sarcomere shortening during rigor development (Wheeler & Koohmaraie, 1994). A strong negative relationship is seen between sarcomere length and meat toughness when sarcomeres are shorter than 2µm, while the relationship diminishes when sarcomeres are longer than 2 µm (Herring, Cassens, Suess, Brungardt, & Briskey, 1967). Moreover, the degree of contraction in which a muscle enters the state of rigor mortis is highly variable among different muscles within the carcass (Locker, 1960; Rhee, Wheeler, Shackelford, & Koohmaraie, 2004).

The tenderisation phase is mainly thought to be a result of calpain-mediated proteolysis of key myofibrillar and associated proteins during postmortem storage of meat (Lonergan, Zhang, & Lonergan, 2010). These proteins maintain the structural integrity of the myofibrils, and once

66 degraded, the rigid structure of the myofibrils weakens leading to muscle fibre breakage and more
67 tender meat (Veiseth-Kent, Hollung, Ofstad, Aass, & Hildrum, 2010). In addition, a possible role
68 of matrix metalloproteases (MMPs) in the tenderisation of meat during storage has been
69 suggested, since they have the potential to degrade the connective tissue (Purslow, Archile-
70 Contreras, & Cha, 2012; Sylvestre, Balcerzak, Feidt, Baracos, & Bellut, 2002). The expression
71 of MMPs in muscle fibres are found to depend on the muscle fibre type (Cha & Purslow, 2010),
72 and the activity of the calpain system is also reported to vary between different muscles (Delgado,
73 Geesink, Marchello, Goll, & Koohmaraie, 2001). Thus, both these proteolytic systems can play
74 a role in explaining some of the variation in tenderness between different bovine muscles.
75 By comparing biochemical characteristics of six muscles of the beef round with the more studied
76 *longissimus dorsi* muscle, postmortem proteolysis was found to give a good indication of the
77 tenderization that takes place in these different muscles during ageing (Anderson et al., 2012).
78 However, Anderson et al. (2012) also concluded that postmortem proteolysis by itself was not
79 sufficient to explain the overall variation in tenderness between these muscles, and that factors
80 such as pH decline, sarcomere length, connective tissue content and muscle fibre type would
81 improve the explanation. This illustrates the need to apply a broad array of biochemical methods
82 in order to reveal what mechanisms govern the differences in meat tenderness between different
83 muscles.
84 Thus, the objective of this study was to investigate the relationship between postmortem
85 proteolysis, muscle pH decline, sarcomere length, intramuscular fat and meat tenderness
86 (Warner-Bratzler shear force) in four different bovine muscles.
87
88 **2. Materials and methods**
89 *2.1 Source of samples*

A total of 22 steers were used in this experiment, representing four different breed groups; Jersey (n = 5), Norwegian Red (NRF, n = 6), Angus × Jersey (A×J, n = 6), and Angus × NRF (A×N, n = 5). All animals were raised on the same farm in Rogaland County in Norway, and received the same treatment and feeding for the last 22 months of their lives, which consisted of grazed pasture during the summer, and indoor feeding during winter with roughage *ad libitum* and concentrate at 0.5 kg per day. Further details of animals and feeding are given in Rødbotten, Gundersen, Vermeer, and Kirkhus (2014). All animals were slaughtered in one day at approximately 23 months of age at a commercial abattoir. The carcasses were not electrically stimulated, and each carcass was split in half and placed in a cooler at 10°C for 18 h, followed by 4°C from then on. This conditioning approach is normal practice at slaughter facilities without electrical stimulation in Norway, in order to limit the risk of cold shortening. At 2 d postmortem, four muscles were excised from the left side of each carcass; *biceps femoris* (BF), *infraspinatus* (IS), *longissimus lumborum* (LL), and *psoas major* (PM). The muscles were vacuum-packed and stored at 4°C until 13 d postmortem.

2.2 Meat quality analyses

Measurements of Warner-Bratzler shear force (WBSF) were performed at 13 d postmortem as described by Veiseth-Kent et al. (2010). Briefly, a 3.5-cm thick slice of each muscle was vacuum-packed and heated in a water bath at 70.5°C for 50 min, before it was chilled in ice water for another 50 min. We have previously monitored the temperature increase inside numerous meat samples from various muscles during the cooking treatment. It takes approximately 40-45 minutes for the internal temperature to reach 70^0C, and since the water bath is set at 70.5^0C the temperature will never be higher. From each cooked slice, 10 rectangular pieces (1 × 1 × 3 cm) were cut along the muscle fibre direction, and these were sheared perpendicular to the fibre direction with a WBSF device with a V-shaped blade

attached to an Instron Materials Testing Machine (Model 4202, Instron Engineering Corporation, High Wycombe, UK). For data analysis, the average maximum force for the 10 parallels was used. Measurements of pH were performed at 1.5, 5, and 48 h postmortem by inserting a glass-stick pH probe (InLab427 Combination pH Puncture Electrode, Mettler Toledo Intl. Inc., Greifensee, Switzerland) and a temperature probe, both connected to a pH-meter (Portamess 911 pH, Knick Elektronische Messgeräte, Berlin, Germany), into the muscles. Sarcomere length (SL) was measured at 13 d postmortem as described by Rødbotten, Lea, and Hildrum (2001). For measurement of intramuscular fat, a slice of approximately 2.5 cm thickness was cut from each sample. All external fat was removed before homogenization (Retsch Grindomix GM200, Retsch GmbH, Haan, Germany), and fat content was measured with an NMR instrument (Maran Ultra LF-NMR, Resonance Instruments Inc., Witney, UK) equipped with a 23-Hz permanent magnet as described by Sorland, Larsen, Lundby, Rudi, and Guiheneuf (2004).

2.3 Microstructural analysis

Microstructural analysis included measurement of four different structural features; fractured muscle fibres, contracted muscle fibres, muscle fibre-fibre detachments (FF detachments) and muscle fibre-perimysium detachments (FP detachments). For detailed description of the analysis, see Veiseth-Kent et al. (2010). Due to resource limitations, fractured and contracted muscle fibres were measured in all four muscles from 11 animals, while FF and FP detachments were measured in two muscles (IS, LL) from all animals. Briefly, muscle samples (2 × 2 × 3 mm) were taken at 13 d postmortem and fixed with 2.5 % glutaraldehyde in cacodylate buffer before embedding in plastic resin. For analysis of fractured and contracted muscle fibres the samples were sectioned longitudinal to the fibres, while for FF and FP detachments samples were sectioned perpendicular to the fibres. After staining of the

sections with toluidine blue, optical microscopy was performed and images were captured. Fractured and contracted muscle fibres were counted for a minimum of 90 muscle fibres per sample. For quantification of FF detachments, the number of muscle fibres that were attached vs. partly or completely detached from their neighbouring muscle fibres was counted for a minimum of 600 muscle fibres per sample. Likewise, the quantification of FP detachments involved the counting of muscle fibres that were attached vs. partly or completely detached from the perimysium for a minimum of 90 muscle fibres per sample.

2.4 Western blotting

Samples for Western blotting were frozen in liquid nitrogen at 2 and 13 d postmortem. For extraction, 100 mg of frozen muscle was homogenized in 1 mL extraction buffer (50 mM tris, 10 mM EDTA, pH 8.3) using Precellys 24 (Bertin Technologies, France) at 6,000 rpm for 2 × 20 s. Following the homogenisation, 500 µL of the homogenate was mixed with an equal amount of protein denaturing buffer (125 mM tris, 4 % SDS, 20 % glycerol, pH 6.8) and heated in a water bath at 50°C for 25 min. The samples were then centrifuged (20 min, 7,800 × g, 4°C) to remove any insoluble components. Protein concentrations were determined using a commercial kit (*RC-DC* Protein Assay, Bio-Rad, Richmond, CA) with BSA as a standard. Protein extracts (12 and 20 µg protein for troponin T and MMP-generated degradation products of aggrecan, respectively) were then subjected to SDS-PAGE. All samples from one animal (i.e. 2 time points for 4 muscles) were run on the same gel, and each gel included two lanes with a 0-h reference sample from the LL of a young NRF bull to adjust for variation between blots. Following electrophoresis, the proteins were transferred onto nitrocellulose membranes using an iBlot™ gel transfer device (Invitrogen, Life Technologies Ltd., Paisley, UK). All blots were blocked with 2 % ECL Advance blocking agent (GE Healthcare Biosciences, Piscataway, NJ) in TPBS (1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 140 mM NaCl,

2.7 mM KCl, 0.1 % Tween-20, pH 7.4). The primary antibodies were a mouse anti-troponin T used at a 1:2,000 dilution (Clone JLT-12, Sigma Aldrich, Saint Louis, MO) and mouse anti-aggrecan used at a 1:50 dilution (BC-14, Novus Biologicals, Cambridge, UK), and the secondary antibody was a Cy3-conjugated goat anti-mouse used at a 1:2,500 dilution (GE Healthcare Biosciences). Both primary and secondary antibodies were diluted in TPBS and incubated at room temperature for 1.5 h with gentle shaking, and membranes were washed with TPBS 3 × 5 min after both incubations. Membranes were scanned using Ettan DIGE Imager (GE Healthcare Biosciences), and the resulting images were used for quantification of the 30-kDa fragment of troponin T and two fragments of aggrecan by the ImageQuant TL software (Version 7.0, GE Healthcare Biosciences). Within each blot, all band densities were calculated as a percentage of the average density of the full-length troponin T and the 43kDa fragment of aggrecan in the reference sample.

2.5 MMP-2 analysis

MMP-2 activity was measured using gelatine zymography. Samples for MMP-2 analysis were co-homogenised with the samples for Western blotting. Following the removal of 500 µL homogenate for Western blotting, the remaining homogenate was centrifuged (20 min, 7,800 × g, 4°C), and the supernatant was collected and stored at -80°C until further analysis. Prior to electrophoresis, the samples were diluted 1:2 with a sample buffer (62.5 mM tris, 10 % glycerol, 2 % SDS, 0.0025% Bromophenol blue, pH 6.8). The samples (20 µL) were applied onto Novex® 10% Zymogram Gelatin gels (Invitrogen), and run at 125 V for 100 min in a standard SDS-PAGE electrophoresis buffer (25 mM tris, 192 mM glycine, 0.1 % SDS, pH 8.3). Similar to the Western blotting, all samples from one animal were run on the same gel, and each gel included two lanes with the 0 h reference sample from the LL of a young NRF bull to adjust for variation between gels. Following electrophoresis, the gels were washed 2 ×

15 min at room temperature in a renaturing buffer (2.5 % Triton X-100), before incubation at 37°C for 20 h in an incubation buffer (50 mM tris, 5 mM $CaCl_2$, pH 8.0). Finally the gels were stained with Coomassie brilliant blue R-250 (0.1 % R-250, 50 % methanol, 7 % acetic acid) for 1 h and destained (20 % methanol, 7 % acetic acid) for 2 h. The gels were scanned using an Epson Expression 1680 Pro (Epson, Nagano, Japan), and the resulting images were used for quantification of the two MMP-2 bands (i.e. pro and active form) by the ImageQuant TL software. Within each gel, all band densities were calculated as a percentage of the average density of the pro-MMP-2 band in the reference sample.

2.6 Immunohistochemistry

Muscle samples (10 × 10 × 4 mm) were taken at 2 and 13 d postmortem, and quickly embedded in Tissue-Tek ™ OCT compound (Sakura Finetek USA Inc, Torrance, CA, USA) and frozen in liquid nitrogen before storage at -80°C. Five-µm-thick sections were cut using a cryostat (CM3050 S, Leica Biosystems, Nussloch, Germany) and collected on poly-L-lysine-coated glass slides. Sections were fixed for 5 min in acetone at -20°C and air-dried. After rinsing twice with DPBS (Gibco, ThermoFisher Scientific, MA, USA), the sections were permeabilized in 0.5% Triton X-100 in PBS for 15 min, and incubated with 5% milk in DPBS-t (0.01% tween) for 1 h before incubation with primary antibody (rabbit anti-Aggrecan, H-300, 1:100 dilution, sc-25674, Santa Cruz, CA, USA) and DAPI (Molecular probes, Invitrogen, Paisley, UK) at 4°C overnight. After three washes in DPBS-t the sections were incubated with secondary antibody (DyLight 549-conjugated mouse anti-rabbit, 1:400 dilution (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA)) for 2 h before using Dako fluorescent mounting medium (Glostrup, Denmark). The cells were examined by fluorescence microscopy analysis (apotome mode) (ZEISS Axio Observer Z1 microscope, Jena, Germany), and images were processed using Adobe Photoshop CS3. The objective used

with fluorescence microscopy was a LCI Plan-Neofluor 25x/ 0.8 1mm Korr M277 objective oil.

2.7 Statistical analysis

Statistical analysis of the data was performed using the GLM procedure in Minitab (Version 17.1.0). Initially, models that included the random effects of Breed and Animal (nested within Breed), and the fixed effects of Muscle and Sampling day (when applicable), and all first order interactions were used. These analyses revealed no significant breed-related effects. Based on that result, and since the main interest was to compare the different muscles, the 22 animals were treated as replicates and focus the attention on the potential muscle-related differences. After this adjustment, the statistical models included the fixed effects of Muscle and Sampling day (when applicable), and their interaction. The significant level was set to $P < 0.05$, and when the effect of a factor was significant, means were separated using the Tukey's method. In order to visualise the main variations in the data, principle component analysis (PCA) was performed using The Unscrambler® X, version 10.4.1 (CAMO A/S, Oslo, Norway). The variables included in the PCA were WBSF, sarcomere length, IMF, muscle pH at all time points, and the occurrence of the 30-kDa fragment of troponin T and the MMP-2 activity measured at 2 and 13 d postmortem. All variables were weighted (1/standard deviation), and full cross validation keeping out one single muscle at a time was performed. In addition, Pearson's product-moment correlation analysis (Minitab) was run to assess the relationship between WBSF and the other variables across the muscles.

3. Results

3.1 Warner-Bratzler shear force, sarcomere length, intramuscular fat and muscle pH

In the present study, effects of muscle type were found for WBSF, sarcomere length, intramuscular fat (IMF) and muscle pH (Table 1). The WBSF measured at 13 d postmortem storage was greater ($P < 0.001$) for LL compared to the other muscles. The sarcomeres were longest ($P < 0.001$) for PM, intermediate for IS, and shortest for BF and LL, while for intramuscular fat (IMF), the IS and PM muscles had greater IMF content ($P = 0.038$) than BF and LL. There was an effect of muscle type on muscle pH at all time points ($P < 0.001$). The PM showed a more rapid pH decline during the first 5 h postmortem compared to LL and BF, while at 48 h postmortem the muscle pH was highest in IS, intermediate for BF, and lowest for LL and PM.

3.2 Microstructural changes

The microstructural analysis revealed an effect of muscle on fractured ($P < 0.001$) and contracted ($P < 0.001$) muscle fibres (Figure 1, Table 2). The highest incidence of fractured muscle fibres was found in LL and BF, while IS and PM had the lowest degree of fractured fibres. Contracted muscle fibres were observed more frequently in IS compared to BF and PM, while LL showed an intermediate frequency. Only two muscles (i.e. IS and LL) were analysed for FF and FP detachments, and no differences were seen between these two muscles (Table 2).

3.3 Degradation of troponin T

To investigate the postmortem degradation of troponin T, the level of a 30-kDa fragment of troponin T in samples taken at 2 and 13 d postmortem was quantified (Figure 2A). The analysis showed an interaction effect ($P = 0.003$) between muscle and sampling day (Figure 2B). At 2 d postmortem, the occurrence of the troponin T fragment was similar in all muscles. However, at 13 d postmortem, the level was higher in LL compared to IS, while PM and BF

were intermediate and not different to any muscles. For LL and BF the level of the troponin T fragment increased during the storage period, while for IS and PM no change was observed from 2 to 13 d postmortem.

3.4 MMP-2 activity

The activity of MMP-2 was measured using gelatine zymography (Figure 3A) and was found to vary between muscles ($P = 0.022$) and sampling day ($P = 0.001$), while no interaction effect was observed. Among the muscles, IS had a reduced amount of MMP-2 activity compared to both LL and PM, while BF was similar to all muscles (Figure 3B). Moreover, the activity of MMP-2 was increased from 2 to 13 d postmortem, with more than a doubling over that time period (Figure 3C). The activity of the latent pro form of MMP-2 was also assessed in all muscles, and the level of activity for the pro form was inversed compared to the active form (data not shown).

3.5 Degradation of connective tissue

The postmortem degradation of connective tissue in relation to MMP-2 activity was measured by use of a monoclonal antibody recognising MMP-degradation epitopes in Aggrecan. The occurrence of two bands (i.e. 31- and 46-kDa fragments) on Western blots was quantified in samples taken at 2 and 13 d postmortem (Figure 4A). For the 46-kDa fragment, no muscle effect was seen (data not shown). However, for the 31-kDa fragment, an interaction effect ($P = 0.025$) between muscle and sampling day was observed (Figure 4B). For the IS muscle the occurrence of this fragment did not change from 2 to 13 d postmortem, but in the LL muscle an increase in this fragment was seen at 13 d postmortem. The degradation of aggrecan was also investigated using immunohistochemistry with antibody staining for Aggrecan (H-300)

288 in LL and IS muscles at 2 and 13 d postmortem, and showed a reduction in fluorescence
289 intensity for both muscles during the postmortem storage period (Figure 5).
290
291 *3.6 Simple correlation and principle component analysis*
292 The simple correlation analyses revealed three significant correlations with WBSF across the
293 muscles, namely sarcomere length (r = - 0.250, P = 0.019), IMF (r = - 0.263, P = 0.013) and
294 pH 48 h (r = - 0.289, P = 0.006). The other statistical approach, principle component analysis,
295 is a multivariate statistical method used to detect clusters and correlations in a data set (Næs,
296 Isaksson, Fearn, & Davies, 2002). During a PCA, principal components (PCs) are calculated
297 and used to construct a coordinate system, where PC-1 explains the most variation in the data,
298 PC-2 the second largest variation, and so on. The resulting score plot, where all samples are
299 plotted according to the new coordinates from the transformed data, allows for easy
300 interpretation of the main variation in the data set, and clusters of the samples are often visible
301 using the first few PCs. A corresponding plot of variables, the correlation loading plot,
302 visualises the correlations between the different variables. In the current study, the PC-1 and
303 PC-2 explained 29 and 20 % of the variation in the data set, respectively. The PM muscles
304 form a separate cluster on the PC-1 axis in the score plot (Figure 6A) compared to the other
305 muscles, which form slightly overlapping clusters mainly along the PC-2 axis. The IS muscle
306 is clustered in the left-hand corner and the LL muscle in the centre bottom of the score plot,
307 while the BF overlaps between these two clusters. The correlation loading plot (Figure 6B)
308 shows that the variation along PC-1 is strongly influenced by sarcomere length and muscle
309 pH decline and ultimate pH level, since these variables are localised between the inner and
310 outer circles in the plot. The variation along PC-2 is more related to degradation of troponin
311 T, MMP-2 activity and WBSF.
312

4. Discussion

4.1 Differences in Warner-Bratzler shear force

Traditionally, the loin muscles have been the most desirable and valuable muscles of a beef carcass. However, more attention has now been put on muscles in the chuck and round as a measure to assure optimal use of and better price for the different muscles in a beef carcass (Belew et al., 2003; Kukowski, Maddock, & Wulf, 2004; Rhee et al., 2004). Regarding tenderness, the WBSF values in the present study were relatively low, indicating that the tenderness level of all muscles were acceptable. Nevertheless, LL had higher WBSF values compared to the other three muscles. This is partly in agreement with previous studies (Belew et al., 2003; Hildrum et al., 2009; Kukowski et al., 2004; Von Seggern et al., 2005), however several of these also found significant differences between IS and PM (Belew et al., 2003; Rhee et al., 2004), and BF and LL (Hildrum et al., 2009; Rhee et al., 2004; Shackelford et al., 1995; Von Seggern et al., 2005). In all studies including this, the IS and PM are found to be very tender muscles, and thus the ability to separate these two muscles when it comes to tenderness may be limited. Most surprising in the present study, was the fact that BF obtained the same WBSF level as IS and PM, while other studies have found the WBSF values of BF to be comparable or even higher than LL (Hildrum et al., 2009; Shackelford et al., 1995). One potential reason for this discrepancy could be that the samples for WBSF were collected from the proximal end of the BF in the present study. When studying tenderness variation within beef muscles, Senaratne, Calkins, Mello, Pokharel and Hinkle (2010) also found significantly lower shear force values in the proximal end of the BF muscle. Thus, the results presented for the BF muscle in the current study may not be representative to the more distal parts of the muscle.

To shed light on the mechanisms governing variation in meat tenderness between these muscles, assessments of pH decline and ultimate pH level, sarcomere length and various

338 factors related to postmortem tenderisation, including calpain-mediated degradation of
339 troponin T, MMP-2 activity, and degradation of connective tissue components, was
340 performed.
341
342 *4.2 Sarcomere length and intramuscular fat*
343 In order to have some assessment of the influence of background toughness and the
344 toughening phase on WBSF variations in the present study, measurements of IMF and
345 sarcomere length were performed, respectively. When it comes to the intermuscular
346 differences in IMF, the results are in agreement with other groups, particularly showing that
347 IS has more IMF than LL and BF (Highfill et al., 2012; Kadim et al., 2013; McKeith, DeVol,
348 Miles, Bechtel, & Carr, 1985; Von Seggern et al., 2005). In addition, Highfill et al. (2012)
349 also showed that PM had more IMF than LL and BF. Conversely, other groups have reported
350 no differences in IMF between LL and IS or PM (Marino, della Malva, & Albenzio, 2015;
351 Purchas & Zou, 2008). The intermuscular differences in sarcomere length appear to be more
352 consistent between studies including this, with PM and IS having the longest, and LL and BF
353 having the shortest sarcomeres (McKeith et al., 1985; Purchas & Zou, 2008; Rhee et al.,
354 2004). Although the differences in IMF and sarcomere length in the present study may
355 explain the low WBSF values for IS and PM, they do not explain the variation in WBSF
356 between LL and BF, since BF and LL had similar IMF and sarcomere lengths but different
357 WBSF values.
358
359 *4.3 Postmortem pH decline and ultimate pH*
360 In accordance with previous studies on lamb, pork and beef, the PM had a more rapid pH
361 decline than LL and BF (Ilian et al., 2001; Koohmaraie, Seideman, Schollmeyer, Dutson, &
362 Babiker, 1988; Melody et al., 2004). Melody et al. (2004) suggested that the accelerated pH

363 decline in PM could be a result of its higher percentage of slow muscle fibres and faster rate
364 of temperature decline, leading to higher Ca^{2+} concentrations in the sarcoplasm of the muscle
365 fibres and thus stimulated metabolism. Unfortunately it was not possible to measure pH of the
366 IS during the early postmortem period due to its location in the carcass, so information
367 regarding its pH decline is missing. However, in a study of different dromedary camel
368 muscles, measurements of pH decline was performed on both LL, IS and BF muscles from 1
369 to 48 h postmortem (Kadim et al., 2013). The results showed that the LL had a slightly more
370 rapid pH decline postmortem, while the pH decline in IS and BF were similar. However, the
371 measurements were performed on hot-boned muscles, so the effect of natural variation in
372 temperature decline caused by the muscles' location within the carcass on pH decline was
373 eliminated (Kadim et al., 2013). Thus, it is difficult to know whether the pH decline in bovine
374 IS muscles attached to the carcass during chilling would be comparable.
375 The highest ultimate pH level in the present study was found for the IS muscle, which is in
376 accordance with earlier studies in beef (Hildrum et al., 2009; Purchas & Zou, 2008; Von
377 Seggern et al., 2005). For the remaining muscles, the BF had a higher ultimate pH than LL
378 and PM, which were similar. This finding is contradictory to previous studies in beef that
379 have reported either no differences in ultimate pH among these three muscles (Koohmaraie et
380 al., 1988), or higher ultimate pH in PM compared to LL muscles (Ilian et al., 2001).
381
382 *4.4 Calpain-mediated proteolysis and muscle fibre fractures*
383 Calpain-mediated proteolysis of key myofibrillar and associated proteins is known to play a
384 central role in the tenderisation process occurring in meat during cooler storage (Lonergan et
385 al., 2010; Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995). Firstly, it is know that
386 calpain activity varies between different muscles, where LL usually is reported to have an
387 increased level compared to BF and PM (Ilian et al., 2001; Koohmaraie et al., 1988). Little is

388 known about calpain activity in IS, but in a study by Delgado et al. (2001), where LL, BF and
389 IS were compared in normal and callipyge lambs, the activity seemed to be comparable to LL
390 and BF. Secondly, the rate of postmortem pH decline in muscle is known to affect the activity
391 of the calpain proteolytic system (Koohmaraie, 1992). Specifically, a rapid pH decline has
392 been associated with earlier Calpain-1 autolysis and loss of proteolytic activity, eventually
393 leading to reduced degradation of key myofibrillar and associated proteins (Bee, Anderson,
394 Lonergan, & Huff-Lonergan, 2007). Studies in pork showed that PM had a faster pH decline,
395 increased rate of Calpain-1 autolysis and reduced postmortem degradation of troponin T and
396 desmin compared to LL (Melody et al., 2004). In the present study, calpain-mediated
397 proteolysis was assessed through the detection of a 30-kDa fragment of troponin T known to
398 result from calpain activity. The accelerated pH decline observed in the PM did not result in
399 reduced postmortem proteolysis of troponin T compared to LL and BF, which had slower pH
400 declines. In fact, the level of troponin T degradation was similar in these three muscles after
401 both 2 and 13 days postmortem. After 13 days, the only difference in troponin T degradation
402 in the present study was seen between the IS and LL muscles, with more degradation in the
403 latter. This is in accordance with Rhee et al. (2004) who reported increased postmortem
404 degradation of desmin in LL compared to IS.
405 In the present study, microstructural quantification of fractured muscle fibres was performed,
406 which previously has been shown to be positively correlated to calpain activity and negatively
407 correlated to WBSF in LL muscles (Veiseth-Kent et al., 2010). The highest occurrence of
408 muscle fibre fractures was seen in the muscle with most troponin T degradation, namely the
409 LL. Moreover, the IS, which had the lowest amount of troponin T degradation, also showed
410 less muscle fibre fractures compared to the LL. Another method for assessing physical
411 fragmentation resulting from postmortem degradation is the myofibrillar fragmentation index
412 (MFI), and similarly to the present study, Purchas and Zou (2008) found MFI to be increased

in the LL compared to IS muscle. Interestingly, the BF muscle had similar levels of troponin T degradation and muscle fibre fractures as the LL muscle in the present study, while the PM, although similar levels of troponin T degradation, had very little muscle fibre fractures. Thus, the relationship between troponin T degradation and muscle fibre fractures appears to vary between muscles. Moreover, while varying degree of muscle fibre fractures have been reported to show a negative correlation with WBSF in the LL muscle (Veiseth-Kent et al., 2010), it cannot explain the variation in WBSF between the different muscles in the present study. The main reason for this could be that there are other factors, such as e.g. IMF and SL, that have an larger impact on the WBSF values for the different muscles in the current study, while the study by Veiseth-Kent et al. (2010) was focusing on the LL muscle only, and thus was less influenced by these other factors.

4.5 MMP-activity, connective tissue degradation and muscle fibre detachments

Matrix metalloproteinases (MMPs) are a family of more than 20 different zinc-dependent endopeptidases. The MMPs are involved in degradation of extracellular matrix, and play important roles in normal and pathological tissue remodelling processes such as embryonic development and tissue repair (Nagase & Woessner, 1999). In muscle, MMPs are known to play regulatory roles during growth and development (Carmeli, Moas, Reznick, & Coleman, 2004). More recently, a possible role of MMPs in the breakdown of connective tissue in meat has been suggested (Purslow et al., 2012), and the expression of MMPs is also found to vary between heart and skeletal muscles (Cha & Purslow, 2010). Balcerzak, Querengesser, Dixon, and Baracos (2001) characterized the MMPs, including their activators and inhibitors, present in bovine skeletal muscle and connective tissue, and detected activity of MMP-2 in both tissue types. Using gelatin zymography, the activity of the latent pro form and the active form of MMP-2 was detected in all four muscles, similar to what Sylvestre et al. (2002) found in

438 ovine LL and *semimembranosus* (SM) muscles. The activity of the latent pro form and the
439 active MMP-2 protease was inversed in the present study, showing that as the latent pro form
440 is being activated, the level of MMP-2 activity increases in the tissue during postmortem
441 storage.
442 The IS muscle had the lowest level of MMP-2 activity in this study, while LL and PM had the
443 highest. No reports exist on MMP-2 activity in different bovine muscles, however one report
444 has shown that fibroblasts isolated from three different muscles had different MMP-2
445 activities (Archile-Contreras, Mandell, & Purslow, 2010). Reports on rodents have shown that
446 MMP-2 activity is different in slow versus fast twitch muscles, especially during muscle
447 regeneration (Zimowska, Brzoska, Swierczynska, Streminska, & Moraczewski, 2008), and
448 fast twitch type II fibres have been found to have higher intracellular MMP-2 activity than
449 slow twitch type I fibres (Hadler-Olsen, Solli, Hafstad, Winberg, & Uhlin-Hansen, 2015).
450 This is in line with the current findings, where IS, known to contain about 80% slow twitch
451 type I fibres (Totland & Kryvi, 1991), had the lowest level of MMP-2 activity.
452 In addition to measuring MMP-2 activity, the degradation of a known target of MMP-2 was
453 measured to see if the actual degradation of connective tissue components reflected the
454 changes observed in MMP-2 activity. Aggrecan is a connective tissue component
455 predominantly found in cartilage, although also found in skeletal and ocular muscles
456 (Torgersen et al., 2014; Yamane, Matsuo, Hasebe, & Ohtsuki, 2003), and is a known substrate
457 of MMP-2 (Nguyen, Murphy, Hughes, Mort, & Roughley, 1993). The antibody used for
458 Western blotting in this study recognizes the N-terminal neoepitope sequence generated at the
459 MMP cleavage site in the interglobular domain of aggrecan, and this analysis was performed
460 on the most contrasting muscles for MMP-2 activity, namely the IS and LL muscles. Indeed,
461 the elevated MMP-2 activity in LL compared to IS was mirrored with increased levels of
462 aggrecan fragments, and thus provide further evidence to support a role of MMP-2 in

463 postmortem proteolysis in bovine muscle. The degradation of aggrecan during postmortem
464 storage was also confirmed by immunohistochemistry analysis, where a great reduction in
465 aggrecan level was seen from 2 to 13 days postmortem, however no apparent differences were
466 seen between IS and LL.
467 The assessment of FF and FP detachments performed in this study is related to the interaction
468 between muscle fibres and intramuscular connective tissue, and reflect the strength and
469 integrity of the endomysium and perimysium, respectively. Indeed, previous studies have
470 shown that the structural integrity and strength of the endomysium and perimysium are
471 weakened during postmortem storage of muscle (Hannesson, Pedersen, Ofstad, & Kolset,
472 2003; Nishimura, Hattori, & Takahashi, 1995). However, in the present study, no differences
473 in FF and FP detachments were seen between LL and IS, even though the degradation of
474 connective tissue components seems to vary between the same muscles. Overall, the results
475 indicate that MMP-2 is activated in muscles during postmortem storage, and that MMP-2
476 activity varies between muscle types and thus could contribute to the WBSF differences
477 observed between them.
478
479 *4.6 Main determinants of variation in tenderness between muscles*
480 Several groups have investigated multiple parameters in order to unravel which factors
481 determine meat tenderness variation between different muscles by correlation analyses. Rhee
482 et al. (2004) investigated the relationships between WBSF, sarcomere length, collagen
483 concentration and desmin degradation in eleven different bovine muscles, including the four
484 muscles of the current study. They found, when including all muscles in their study, a
485 negative correlation between WBSF and sarcomere length and a positive correlation between
486 WBSF and collagen concentration, while no correlation was seen for desmin degradation. For
487 ovine muscles, Starkey, Geesink, Collins, Oddy and Hopkins (2016) investigated the

relationships between sarcomere length, collagen content, muscle pH, intramuscular fat, desmin degradation and meat tenderness in LL, BF and SM muscles. When investigating the relationships across all muscles, they found WBSF to be negatively correlated to sarcomere length, soluble collagen and desmin degradation. Interpretation of the score and correlation loading plots (Figure 6A and 6B) revealed that the major factors contributing to the observed variations between the muscles are sarcomere length, muscle pH decline and ultimate pH level. Indeed, the PM muscles from all animals clustered separately from the other muscles, and had the longest sarcomere length, accelerated pH decline, and low ultimate pH. The importance of sarcomere length for the low WBSF values observed for the PM muscle is thus in agreement with the previous reports (Rhee et al., 2004; Starkey et al., 2016). The IS muscle clustered in the left-hand corner of the score plot, an area positively correlated to high ultimate pH levels and negatively correlated to MMP-2 activity and troponin T degradation, while the LL muscle is localised on the opposite side of the plot, correlated with increased postmortem proteolysis and lower ultimate pH levels. The BF muscle, forming an overlapping cluster between the IS and LL muscles, indeed has intermediate levels of both postmortem proteolysis and ultimate pH.

With respect to correlations between WBSF and the other variables across all muscles, we found a negative correlation between WBSF and sarcomere length as reported by both Starkey et al. (2016) and Rhee et al. (2004). In addition, similarly to Rhee et al. (2004), we found no significant correlation with postmortem proteolysis, even though increased levels of postmortem proteolysis have been associated with tenderisation of meat (Lonergan et al., 2010; Taylor et al., 1995). However, when comparing different muscles, this relationship between WBSF and postmortem proteolysis seems to be weakened (Rhee et al., 2004; Starkey et al., 2016), as also shown in the current study. The most likely reason for this is that other factors such as sarcomere length, intramuscular fat and connective tissue are of more

importance when it comes to determining variation in tenderness levels between muscles. Indeed, we observed negative relationships between WBSF and both sarcomere length and IMF across the muscles. Nevertheless, the results clearly show that there is a large variation in postmortem proteolysis between the muscles, which is important to consider when optimising postmortem handling of various beef muscles.

5. Conclusions

The current study provides further support to the muscle-dependent importance of pH decline, SL, IMF and postmortem proteolysis for meat tenderness. However, the more novel aspect of this work relates to the potential for connective tissue degradation in different muscles, measured as MMP-2 activity and degradation of aggrecan. Even though almost all the parameters assessed in this study varied between the muscles, neither of them could explain the muscle-related differences in meat tenderness. In conclusion, this study has illustrated the need to assess multiple factors that may influence ultimate meat tenderness in order to understand and explain intermuscular differences. In addition, the muscle-related variation in proteolytic activity described in this study illustrates that some muscles, e.g. LL and BF, may benefit greatly from a postmortem storage period before consumption with respect to tenderness due to their high proteolytic activity, while others, e.g. IS and PM, will have low or no benefit of such a storage period. This is useful knowledge for the meat industry for optimising processing and storage procedures for different beef muscles.

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FIGURE CAPTIONS

Figure 1. Images of bovine muscles stored for 13 d postmortem used for microstructural analyses, stained with 0.1 g/100 mL of toluidine blue dissolved in 0.1 M sodium acetate. Muscle fibre-fibre detachments and muscle fibre-perimysium detachments were quantified on cross-sections of the muscles (A), and are indicated with arrow heads and arrows, respectively. Fractured muscle fibres (B) and contracted muscle fibres (C) were quantified on longitudinal sections, and are indicated with arrows and stars, respectively. The scale bars indicate 50 μm.

Figure 2. A representative Western blot indicating the 30-kDa troponin T fragment (A), and the quantified levels of the 30-kDa troponin T fragment (Mean ± Standard Error of Mean) in the different muscles (B). Different letters within figure B indicate significant differences between means. Ref – 0 h reference sample from LL, BF – *biceps femoris*, IS – *infraspinatus*, LL – *longissimus lumborum*, PM – *psoas major*. $P_{\text{Muscle}\times\text{Sampling day}} = 0.003$.

Figure 3. A representative gelatine zymogram for measurements of pro-MMP-2 and MMP-2 activity in different muscles (A), and the quantified activity of MMP-2 (Mean ± Standard Error of Mean) in different muscles (B), and at different sampling days (C). Different letters within figures B and C indicate significant differences between means. Ref – 0 h reference sample from LL, BF – *biceps femoris*, IS – *infraspinatus*, LL – *longissimus lumborum*, PM – *psoas major*. $P_{\text{Muscle}} = 0.022$, $P_{\text{Sampling day}} = 0.001$.

Figure 4. A representative Western blot indicating the 31- and 46-kDa aggrecan fragments (A), and the quantified levels of the 31-kDa aggrecan fragment (Mean ± Standard Error of Mean) at two time points postmortem in two bovine muscles (B). Different letters within

706 figure B indicate significant differences between means. IS – *infraspinatus*, LL – *longissimus*
707 *lumborum*. $P_{\text{Muscle}\times\text{Sampling day}} = 0.025$.
708
709 Figure 5. The occurrence of aggrecan in the endomysium at 2 and 13 d postmortem in two
710 bovine muscles. Muscle sections from *longissimus lumborum* (LL) and *infraspinatus* (IS)
711 were stained with rabbit anti-aggrecan (red) and DAPI (stains nuclei blue) followed by
712 DyLight 546-conjugated mouse anti-rabbit before fluorescence microscopy analysis. The left
713 upper and lower panels show high fluorescence intensity (arrows), whereas the right upper
714 and lower panels have almost undetectable fluorescence. Scalebar 50 µm.
715
716 Figure 6. Principal component analysis (PCA) of Warner-Bratzler shear force (WBSF),
717 sarcomere length (SL), intramuscular fat (IMF), muscle pH at 1.5, 5 and 48 h, and the
718 occurrence of the 30-kDa fragment of troponin T (TnT) and MMP-2 activity (MMP-2)
719 measured at 2 and 13 d postmortem from all muscles in the experiment, showing the first two
720 principal components (PC-1 and PC-2). The score plot (A) of all muscles according to PC-1
721 and PC-2, and the corresponding correlation loading plot (B) of the measured variables where
722 the inner circle and the outer circle mark the limits for 50 % and 100 % explanation of the
723 variation in the data, respectively. Explained variance for each PC is indicated on the axes. BF
724 – *biceps femoris*, IS – *infraspinatus*, LL – *longissimus lumborum*, PM – *psoas major*.
725

Table 1. Warner-Bratzler shear force (WBSF), intramuscular fat (IMF) and sarcomere length measured at 13 days postmortem, and muscle pH during the first 48 hours postmortem in four bovine muscles (Mean ± Standard Error of Mean)

	WBSF (N/cm^2)	Sarcomere (µm)	IMF (%)	pH 1.5 h	pH 5 h	pH 48 h
BF	37.3 ± 1.53^{b}	1.84 ± 0.018^{c}	5.04 ± 0.373^{b}	7.04 ± 0.05^{a}	6.46 ± 0.07^{a}	5.55 ± 0.01^{b}
IS	33.7 ± 1.11^{b}	2.26 ± 0.053^{b}	6.06 ± 0.394^{a}	n.m.[1]	n.m.	5.62 ± 0.02^{a}
LL	50.0 ± 2.32^{a}	1.85 ± 0.026^{c}	5.06 ± 0.380^{b}	6.90 ± 0.04^{a}	6.25 ± 0.07^{a}	5.46 ± 0.01^{c}
PM	36.7 ± 1.39^{b}	3.64 ± 0.034^{a}	6.30 ± 0.397^{a}	6.08 ± 0.07^{b}	5.72 ± 0.06^{b}	5.44 ± 0.01^{c}
P-values	$P < 0.001$	$P < 0.001$	$P = 0.038$	$P < 0.001$	$P < 0.001$	$P < 0.001$

BF – *biceps femoris*, IS – *infraspinatus*, LL – *longissimus lumborum*, PM – *psoas major*.

[abc] Different letters within the same column indicate significant differences between muscles.

[1] Not measured due to access difficulties while muscle was still attached to the carcass.

732 **Table 2.** Microstructural features measured at 13 days postmortem in four bovine muscles (Mean ± Standard Error of Mean)

	Fractured fibres	Contracted fibres	FF detachments	FP detachments
BF	27.4 ± 5.85[a]	2.3 ± 0.76[b]	n.m.[1]	n.m.
IS	11.1 ± 3.44[b]	7.1 ± 1.65[a]	31.9 ± 1.58	14.2 ± 1.27
LL	31.1 ± 5.06[a]	3.9 ± 0.97[ab]	34.3 ± 1.88	14.1 ± 1.10
PM	2.2 ± 0.50[b]	0.1 ± 0.08[b]	n.m.	n.m.
P-values	$P < 0.001$	$P < 0.001$	$P = 0.328$	$P = 0.949$

733 BF – *biceps femoris*, IS – *infraspinatus*, LL – *longissimus lumborum*, PM – *psoas major*, FF detachments – Fibre-fibre detachments, FP

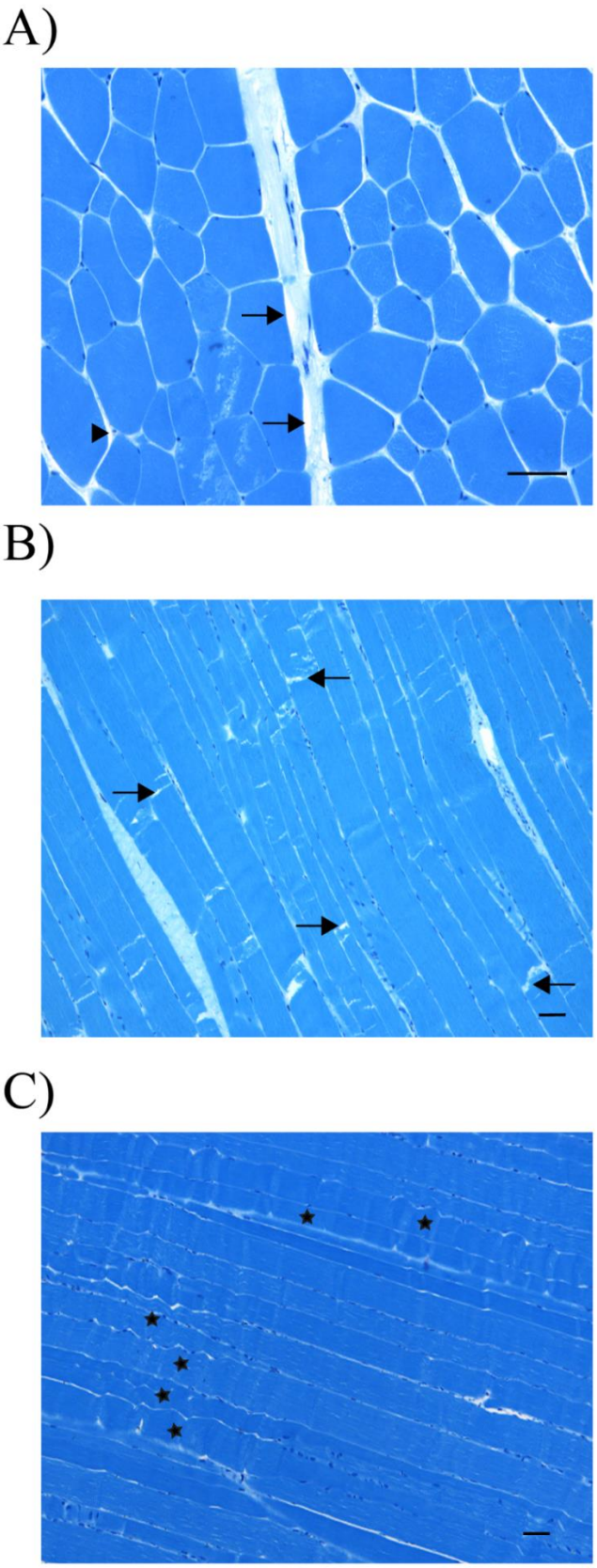
734 detachments – Fibre-perimysium detachments.

735 [abc] Different letters within the same column indicate significant differences between muscles.

736 [1] Not measured due to resource limitations.

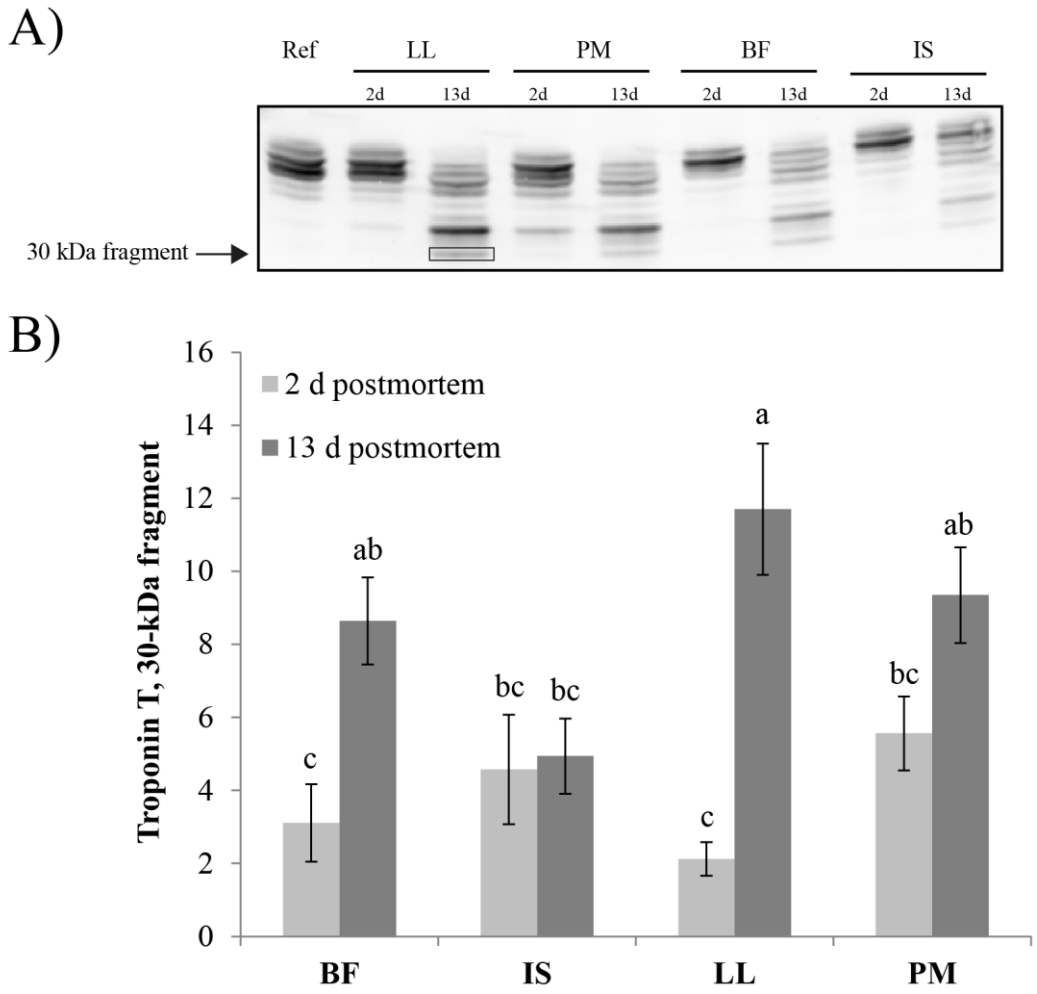
737

738 **Figure 1.**



739

740 **Figure 2.**



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742

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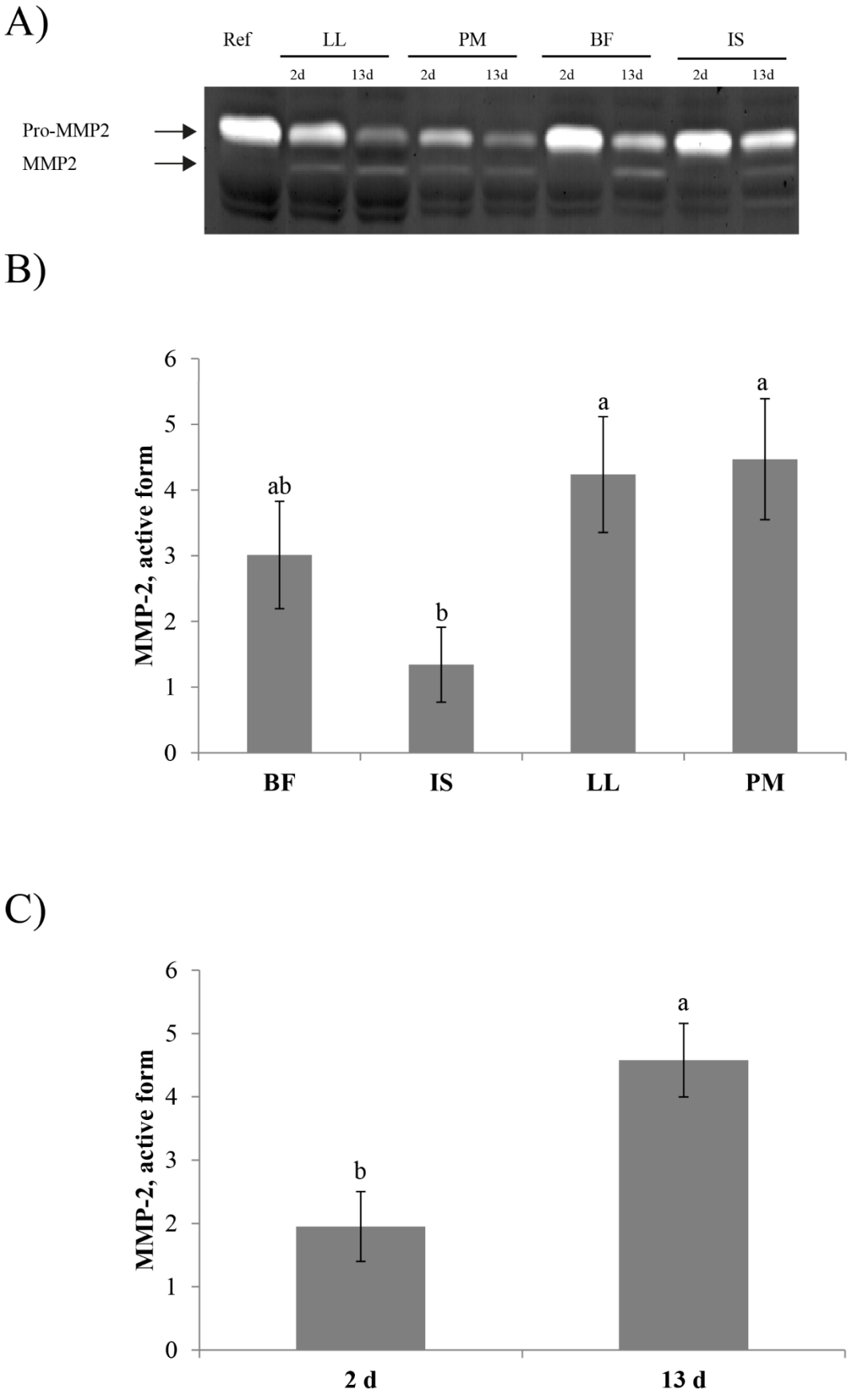
747

748 **Figure 3.**



749

750 **Figure 4.**

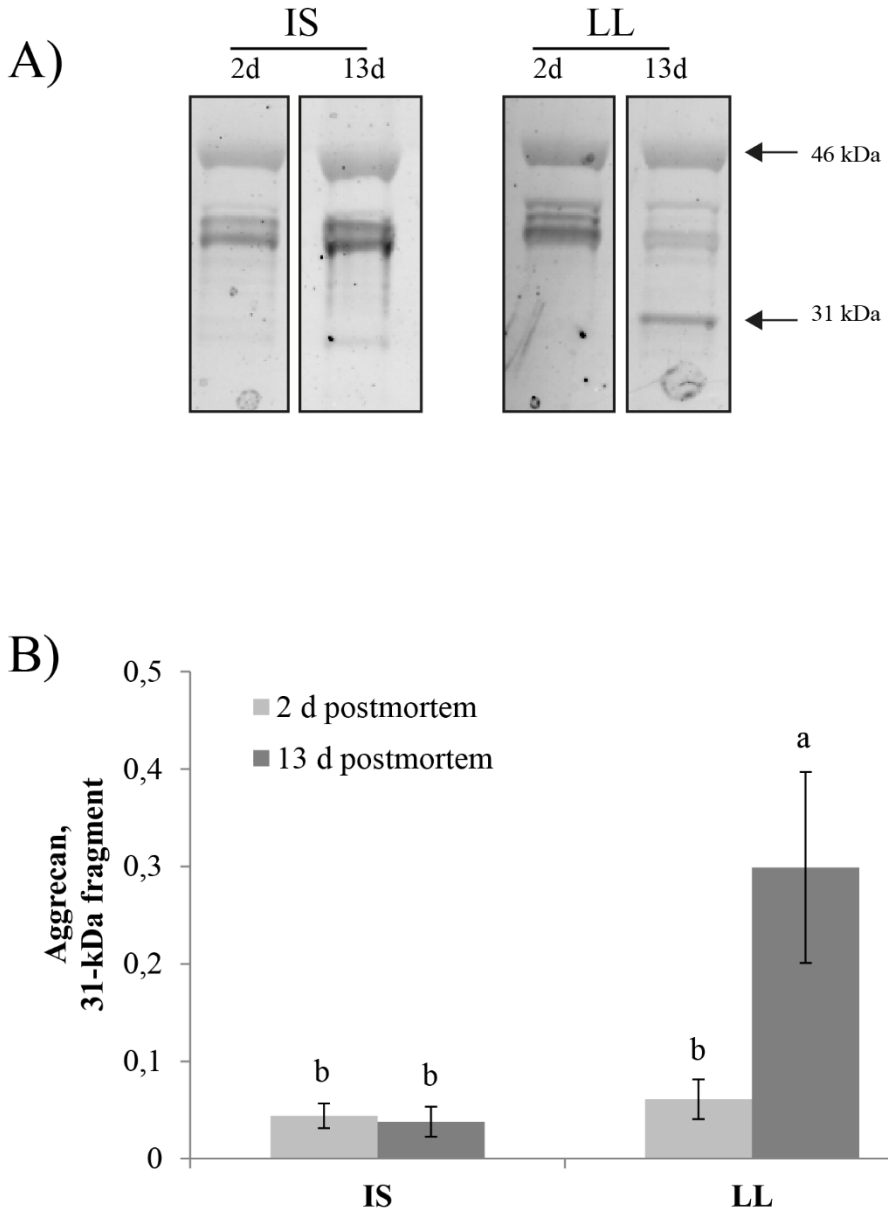


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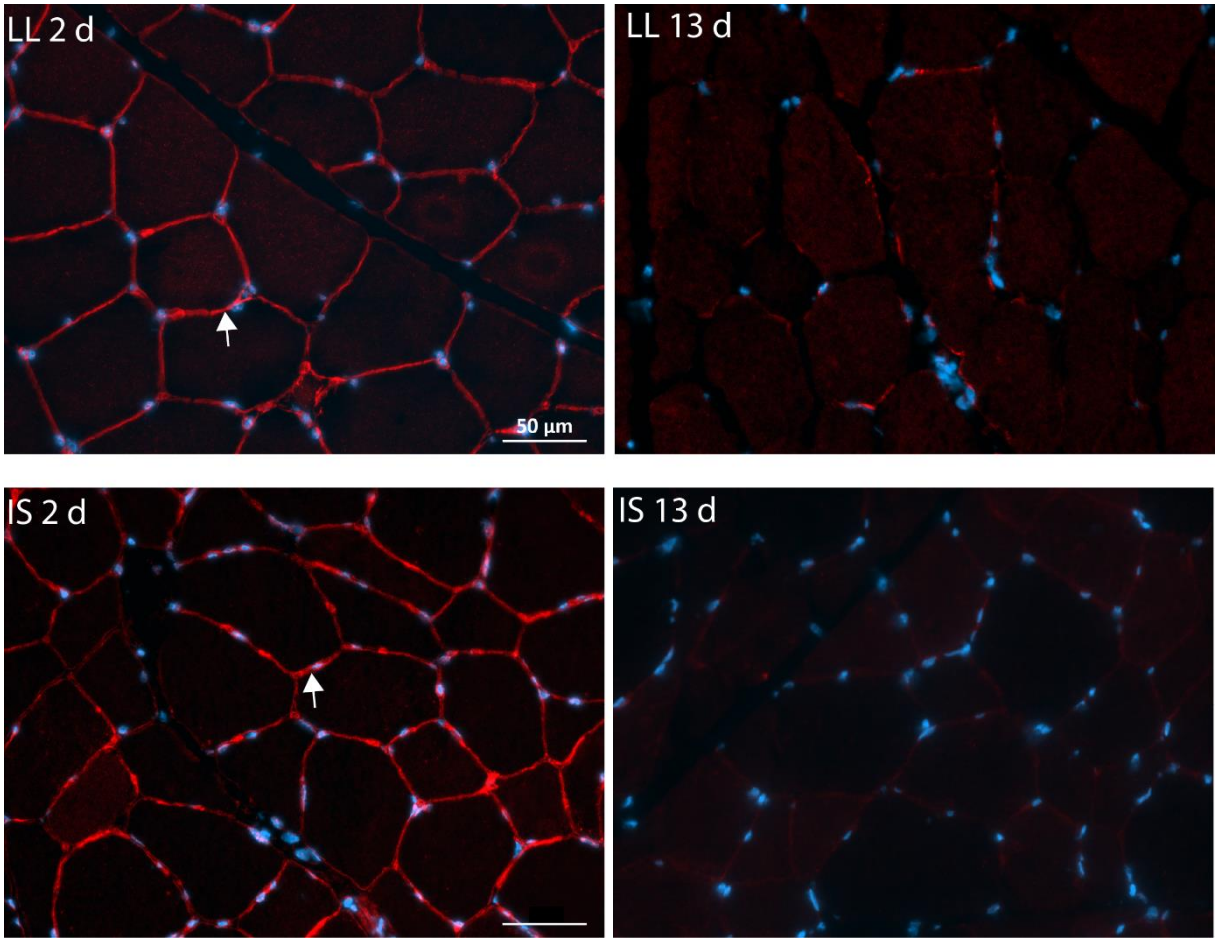
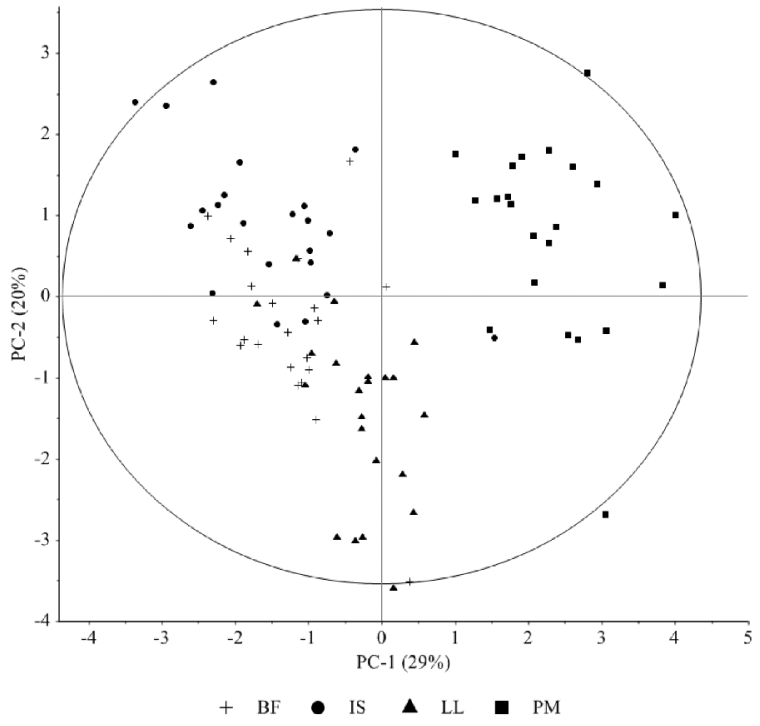
Figure 5.

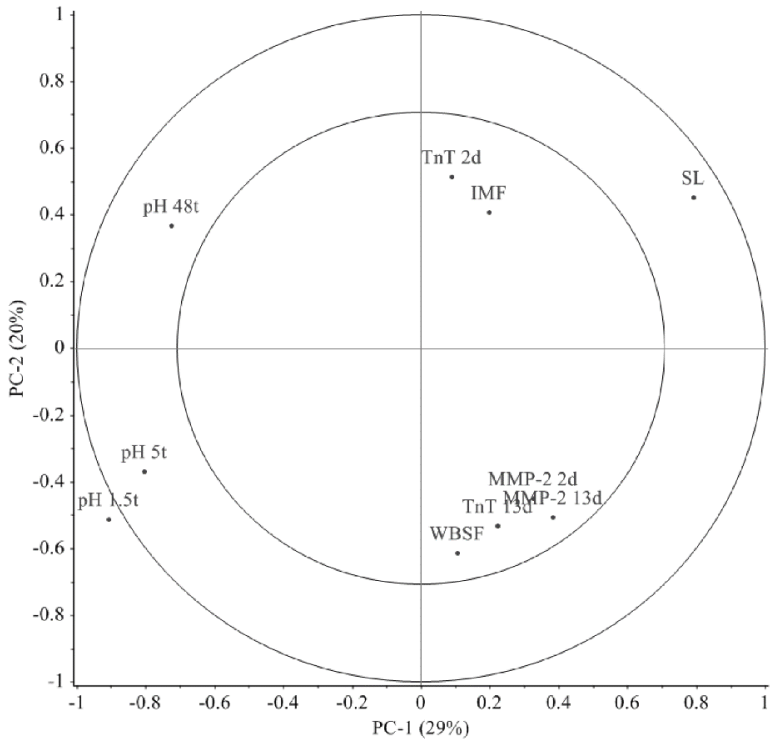


759 **Figure 6.**

A)



B)



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