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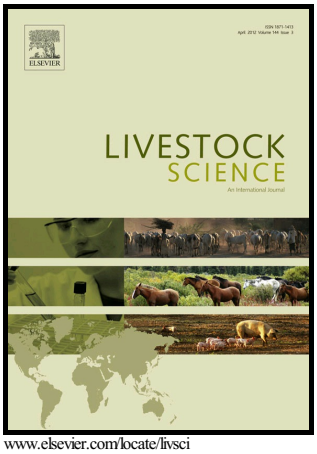

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The effects of gender and muscle type on the mRNA levels of the calpain proteolytic system and beef tenderness during *post-mortem* aging

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Abstract

The objective of this study was to determine the effects of gender and muscle type on the mRNA levels of the calpain system and the tenderization of beef meat. The *Longissimus thoracis* (LT) and the *Semimembranosus* (SM) were sampled from each bull, steer and heifer after routine slaughter (Six animals per group). The mRNA levels of µ-calpain, m-calpain, calpain-3 and calpastatin were quantified using real-time PCR. Concurrently, tenderness was determined following the Warner-Bratzler Shearforce (WBSF) procedure and rate of tenderization during *post-mortem* storage was calculated from the WBSF values of 7d and 35d aged steaks. The results show that bulls had significantly lower ($P < 0.01$) WBSF values than heifers which were accompanied by higher ($P < 0.01$) levels of µ-calpain and calpain-3 mRNA but similar levels of calpastatin as compared to heifers. There was a significantly higher ($P < 0.05$) calpastatin expression in steers, as compared to heifers. However, µ-calpain expression was lower ($P < 0.05$) in heifers whose meat was significantly tougher ($P < 0.05$) than that of steers. Steer meat was slightly tougher than that of bulls, while steers had had a tendency to express higher levels of calpastatin but similar µ-calpain and calpain-3 mRNA. The LT had lower ($P < 0.05$) WBSF values than the SM but these muscles tenderised at the same rate, and

had similar mRNA levels for all investigated genes. M-calpain mRNA levels were not significantly affected by muscle and gender ($P > 0.05$). Moreover, calpain 3 was negatively correlated to 7d WBSF values ($P < 0.05$). Despite the small sample size, these results suggest that variations in beef tenderness could be modulated through the differential expression of the members of the calpain system, specifically, μ-calpain, calpain 3 and calpastatin.

Keywords

Calpain, gene expression, gender, meat quality, beef cattle, calpastatin

Introduction

The calpain system consists of a large family of proteolytic enzymes whose *post-mortem* activities have been associated with enhanced meat quality characteristics. Specifically, the roles of the micromolar Ca^{2+} requiring μ-calpain, the millimolar Ca^{2+} requiring m-calpain (Dayton, 1982), the skeletal muscle-specific calpain-3 or p94 (Sorimachi et al., 1989) and calpastatin (Goll et al., 2003) have been extensively investigated in the degradation of several myofibrillar proteins.

Research has shown that Calpastatin regulates the activities of μ-calpain and m-calpain (Goll et al., 2003), where higher calpastatin activity leads to less tender meat (Morgan et al., 1993a). Therefore, as calpastatin losses its inhibitory effects during *post-mortem* aging, the ubiquitous calpains becomes increasingly active and cleaves myofibrillar proteins such as titin, desmin and vinculin which leads to increased tenderness (Taylor et al., 1995; Kemp et al., 2010).

Despite this, evidence about the activities of the individual members of the calpain system and their specific roles in the degradation of skeletal muscle proteins remain contentious. Huff-Lonergan et al. (1996) demonstrated that the *in vitro* degradation of desmin, nebulin, titin, vinculin, troponin-T, and other cytoskeletal proteins by μ-and m-calpain were similar to those observed in *post-mortem* muscle. However, Boehm et al. (1998) argued that m-calpain would not autolyse under the Ca^{2+} levels observed in *post-mortem* muscle. In addition, the degradation of myofibrillar proteins were significantly reduced in μ-calpain knock-out mouse, suggesting that m-calpain may be inactive in *post-mortem* muscle (Geesink et al., 2006). However, others

argue that the levels of calcium *in vivo* may not be accurately represented through *in vitro* assays and there are possibly other unknown cofactors that may be involved and may allow m-calpain to be active (Goll et al., 2003).

Similarly, no differences in *post-mortem* protein degradation were found between calpain-3 knockout and wild type mice (Geesink et al., 2005) despite the strategic location of the protease within the sarcomere (Sorimachi et al., 1996). Besides, calpastatin does not regulate calpain-3 activities (Ono et al., 2004), suggesting no significant role for this protease in meat tenderization. In contrast, significant correlations between the appearance of the autolysed calpain-3 and the degradation of nebulin in the *Longissimus* muscle of goats were reported (Ilian et al., 2004). Results from the *Longissimus* muscles of pigs showed a positive relation between calpain-3 mRNA and tenderness (Yang et al., 2012), conflicting those of Gandolfi et al. (2011) who found high calpain 3 expression in relation to higher shear force values.

This suggests that all three calpains may have proteolytic properties but the extent of their involvement during *post-mortem* aging may sometimes be difficult to ascertain. In other words, direct evidence at the protein levels are challenging in terms of quantification and the emulation of *in vivo* activity levels. In that case, quantification of the mRNA levels of candidate genes under varying experimental and physiological conditions may offer insights into the variable cellular demand of a specific protease.

It was thus presumed that these genes would be differentially regulated in bulls, steers and heifers and the variation would be indicative of differences in meat quality characteristics. Taking into consideration post-transcription modifications, the variations observed in mRNA concentrations of a particular gene may correlate to a great extent with the quantity and activity of the translated protein, as demonstrated by others for m-calpain and calpastatin (Parr et al., 1992; Ilian et al., 1999) and other genes (Tang et al., 2010).

Such information may be useful particularly to the meat industry as it would enhance the understanding of the molecular basis of meat tenderization, and may allow

amendment of pre-slaughter handling practices and *post-mortem* treatments that improves meat quality.

Therefore, the objective of this study was to quantify the mRNA levels of the large subunits of μ-calpain, m-calpain, calpain-3 and calpastatin in bovine skeletal muscles and to test the hypothesis that gender and muscle type have an effect on the expression of these genes in association with meat tenderness.

Materials and Methods

Sample acquisition and storage

Eighteen Hereford-cross cattle were obtained from a commercial farm in Yorkshire, North-East England and slaughtered at equivalent liveweight of approximately 550 kg following the regulations in an EU approved abattoir and in accordance with EU Directive 2010/63/EU. The carcases were weighed and then graded by expert meat graders following the EUROP carcass classification.

Six carcasses from each gender group, namely young bulls, steers and heifers, were selected for use in this study. Muscle tissue sub-samples for RNA extraction were collected from each carcass by making a deep incision approximately 10 cm into the forerib and topside section to access the *Longissimus thoracis* (LT) and the *Semimembranosus* (SM), respectively. These sub-samples were then immediately submerged in tubes containing RNA*later*™ stabilization reagent (Sigma-Aldrich, St. Louis, USA). This was carried out within 10 minutes after exsanguination. After 24 hours, the sub-samples were subsequently frozen at -20 ˚C in accordance with manufacturer recommendations (Sigma-Aldrich, St. Louis, USA). The rest of the carcass was stored in a 4 ˚C chiller to age for 7 days, for subsequent use in meat tenderness analysis.

Measurement of pH

Specifically, Carcass pH was taken at the centre of Longissimus muscle, at approximately 15 min and 24 hours after slaughter for pH0 and pH2d, respectively. This was then repeated after 1 week to obtain the pH7d. The pH0 and pH2d readings for bull carcasses were not available and thus only the pH7d could be reported for this group.

RNA extraction

Total RNA were extracted from 100 mg bovine skeletal muscle subsamples using the RNeasy fibrous tissue extraction kit (Qiagen, Germany), following manufacturer's instructions. To ensure that any carry-over genomic DNA was eliminated, the eluates were treated with DNase-I (Sigma-Aldrich, St. Louis, USA). Total isolated RNA was quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA), were the A260/A280 ratios between 1.40 and 2.16 indicated samples with optimal purity. Quality was assessed by performing electrophoresis on a 2 % agarose gel and by exposure to UV light using the SYNGENE system (SynGene Ltd., Cambridge, UK). The RNA was then aliquoted into microtubes and stored at -80 ˚C until required in downstream processes.

Quantitative PCR

The mRNA levels of μ-Calpain, m-Calpain, Calpain-3 and Calpastatin in the two skeletal muscles were determined by real-time quantitative PCR (qPCR). Firstly, the total RNA was reverse-transcribed to cDNA using the Verso^{TM} reverse transcriptase and oligo-dT primers from the Verso® cDNA synthesis kit (Abgene Ltd., Epsom, UK). Then an aliquot was taken from each tube to create a pooled cDNA mixture which was subsequently serially diluted 5-fold to generate a standard curve. Real-time PCR was performed in a 25 μl amplification reaction mixture containing 5 μl of cDNA, 12.5 μl ABsolute^{TM} Blue QPCR SYBR® Green master mix (ABgene Ltd., Epsom, UK), 300 nM of gene specific primers (Primerdesign Ltd., Southampton, UK) and PCR-grade water. Duplicate reactions for each study sample and triplicate reactions for standard curve subsamples were performed as a means of controlling between wells variation. The sequences of the primers used in the amplification of target genes are shown in Table 1. On the other hand, commercially available primers sets for the *Bos taurus* housekeeping genes (HKGs), namely Glyceraldehyde-3-phosphate dehydrogenase (GAPD), Peptidylprolyl isomerase-A (PPIA) and Eukaryotic initiation factor-2B Subunit 2 (EIF2B2), were used to amplify the reference genes (Primer Design, Southampton, UK). The GeneBank Accession Numbers for these HKGs genes are NM_001034034, NM_178320 and NM_001015593, respectively.

The following PCR cycling conditions were used in the Mastercycler® ep realplex thermal cycler (Eppendorf AG, Hamburg, Germany): One 15 min cycle of enzyme activation at 95 ˚C, 40 cycles at 95 ˚C for 15s, 30s annealing at 60 ˚C and 30s elongation at 72 ˚C. The threshold cycle value (Ct value) of each reaction tube was automatically determined using the realplex 2.2 software (Eppendorf AG, Hamburg, Germany).

Quantification of Gene expression

For each primer, the PCR efficiency and R^2 were calculated from the slope of the curve by plotting the Ct values against the cDNA concentration and using the formula: $E = (10^{(-1/slope)} -1)*100$.

For each sample, arithmetic mean of its technical replicates' Ct values were then converted into relative quantification values (Q) following the delta-Ct formula Q = E(ΔCt), where *E* is the efficiency of a specific gene and ΔCt is the difference between the lowest intra-gene Ct value and the mean Ct of the sample (min Ct – sample Ct). These Q values were further divided by the normalization factor (NF) value to obtain the normalized mRNA level of a specific target gene. The NF value was derived from the geometric mean of the Q values of selected reference genes. Specifically, the NF value was calculated from the geometric means of GAPD (NM_001034034), PPIA (NM_178320) and EIF2B2 (NM_001015593) using the geNorm algorithm (Vandesompele et al., 2002).

Determination of Meat Tenderness

i. Muscle preparations and aging treatments

Due to abattoir restrictions, meat samples for *post-mortem* aging could only be obtained one week after slaughter. Therefore, at 7d *post-mortem*, the *Longissimus thoracis* and the *Semimembranosus* muscles were removed from the right side forerib and the topside, respectively. Five steaks were cut from each muscle, vacuum sealed individually, and then randomly assigned to 7, 14, 21, 28 and 35d *post-mortem* aging treatments. Each steak was 2.54 cm thick and was cut perpendicular to muscle fibre orientation. The steaks for the 7d *post-mortem* aging were frozen immediately while the remaining steaks were aged at 4 °C for the designated aging period before freezing at -20 °C.

ii. Warner-Bratzler Shear Force analysis and calculations of Aging Response

Subsamples of approximately 100 x 100 mm were cut from each steak then suspended in individual plastic net bags at 4 °C to allow them to thaw and drip freely for 24 h. The steaks were then placed in individual self-sealing polypropylene bags and cooked for 20 min in a waterbath (Grant Optima^{TM} GD100, Grant Instruments Ltd., Cambridge, UK) to allow the sample's internal temperature to reach 71 °C. Cooking was made in a waterbath preheated to 80 °C.

The Warner-Bratzler shear force (WBSF) procedure (Bratzler, 1949) was then performed as an instrumental measure for tenderness. Specifically, using a sharp hand-held coring device, 6 cylindrical cores measuring 1.27 cm in diameter, were cut out from the middle of each cooked sample by drilling parallel to the longitudinal orientation of the muscle fibres. WBSF values were obtained with an automated standard texture analyser (1000S apparatus, Lloyd Instruments, Southampton, UK) fitted with a 60˚ V-notch blade and a 500 N load cell, preset to shear at a crosshead speed of 250 mm/min and to display maximum deformation in Newton (N). The mean value of the 6 cores was reported as the WBSF value (N) of that specific sample. In addition, the difference between 7d and the 35d shearforce values defined the aging response of the steak sample.

Statistical Analysis

The data were analysed using the SPSS statistical package (SPSS version 17, Inc, Chicago, IL). The normalized mRNA levels of each sample were first subjected to arcsine square root transformation (Ferguson and Takane, 1989), and then a two-way ANOVA was performed for each gene with gender and muscle as factors. The differences between the three gender groups for age at slaughter, liveweight, carcass parameters and each measure of pH were analysed using a one-way ANOVA in SPSS. On the other hand, the effects of gender, muscle type and *post-mortem* aging periods on the WBSF values and the aging response were analysed using a mixed-design ANOVA, with muscle type as a repeated measures variable and days *post-mortem* nested in each muscle type. Hot carcass weight was used as a covariate and the Bonferroni corrections were applied in all subsequent *Post-hoc*

analyses. The relationships between meat tenderness and gene expression were analysed by Pearson's correlation coefficients.

Results

The effects of gender and muscle type on the expression of the calpain system

Table 2 shows the mRNA levels of calpain system genes in the *Semimembranosus* and *Longissimus thoracis* of bulls, steers and heifers. Hot carcass weight was not a significant covariate for the relative mRNA levels of all the genes (P >0.05).
There were no significant interactions between gender and muscle type on the expression of μ-calpain. The main effects of muscle type was also insignificant (P = 0.85). However, the analysis revealed that gender had a highly significant effect (P = 0.001) with heifers showing lower μ-calpain mRNA levels than both steers and bulls. The mean mRNA levels for bulls were similar (P > 0.05) to those of steers, suggesting that castration did not regulate μ-calpain expression. The results also showed that gender and muscle type had no effects (P > 0.05) on the expression of m-calpain (Table 2).

Furthermore, there were no significant interactions (P > 0.05) between gender and muscle type on calpain-3 expression, mainly due to similar mRNA levels in both muscles. The effects of gender was however significant, where the mRNA levels of bulls appeared to be up-regulated as compared to those of heifers (P < 0.01). No other statistically significant differences were observed on the calpain-3 mRNA levels.

There were however significant interactions (P < 0.05) between gender and muscle on the expression of calpastatin. These were mainly due to higher mRNA levels in steers LT as compared to other samples. Therefore, the significant main effect of gender revealed that steers had higher levels of calpastatin mRNA than heifers (P = 0.01). There was also a tendency for steers to have higher levels of calpastatin mRNA than bulls (P = 0.09). The main effect of muscle type was also marginally significant (P = 0.06) due to higher calpastatin expression in steer LT.

Carcass measurements

The means ± SEM for age at slaughter, liveweight, carcass grading and of pH measurements in bulls, heifers and steers are reported in Table 3. The three gender groups differed significantly (P < 0.001) with regards to age at slaughter with heifers being the oldest, followed by steers and young bulls. In contrast no differences were found in liveweights and carcass weights although steers had a lower dressing percentage than bulls. The EUROP fatness score was highest in heifers, which were significantly different from bulls and steers ($P < 0.05$). In contrast, the carcasses of bulls had similar fatness scores as those of steers. There were also no differences in terms of carcass conformation between the gender groups. Steer carcasses had significantly higher pH values than heifers for both pH_0 and pH_{2d} readings ($P < 0.01$). The pH readings at early *post-mortem* (pH_0 and pH_{2d}) were not available for bull carcasses and therefore not reported. However, the 7d *post-mortem* pH values of carcasses from the three gender groups were obtained and no differences were found ($P > 0.05$). The carcasses of steers initially had a high pH value close to neutral which declined during *post-mortem* aging. Those of heifers, however, increased from 5.24 at day 2 to 5.85 on day 7 (Table 3).

WBSF Values and Aging Response of the LT and SM muscles of bulls, steers and heifers

Means ± SEM for WBSF are presented in Table 4. Hot carcass weight was not a significant covariate for shearforce values ($P > 0.05$). A significant ($P < 0.05$) gender X muscle interaction was observed for WBSF values. In both steers and bulls, the SM had higher shearforce values than the LT but no differences were found between heifer muscles. There was also a significant interaction ($P < 0.05$) between *post-mortem* aging period and muscle type where the LT tenderised earlier than the SM (Table 4). This means that the SM muscle benefited from prolonged aging beyond 21 d. The results further indicated a highly significant main effect of the *post-mortem* aging period ($P < 0.001$). It shows that, in general, the WBSF values of steaks aged for 21 d, 28 d and 35 d were significantly lower than those aged for 7 d. Prolonged aging tenderised the meat further as indicated by lower shearforce values between 28d and 35d. The main effects of gender were highly significant ($P < 0.001$) with heifers showing higher shearforce values than bulls and steers ($P < 0.05$). The differences between steers and bulls were marginally significant ($P = 0.06$) as steer

meat was slightly tougher. However, gender, muscle type and the covariate had no significant effect on aging response (Table 5).

Correlations between gene expression and WBSF values

Pearson's correlation coefficient was used to determine the relationship between the mRNA levels of the calpains system genes and 7d WBSF values in the LT and SM muscles. The mRNA levels of Calpain-3 in the LT ($r = - 0.51$; $P < 0.05$), showed a negative correlation with 7d WBSF values of the same muscle. This is consistent with its expected role as proteolytic enzyme were higher levels of the calpains would lead to lower shearforce values and more tender meat. No further correlations within a muscle were significant.

Discussion

The present study was carried out to determine the effects of gender and muscle type on the mRNA levels of μ-calpain (CAPN1), m-calpain (CAPN2), calpain-3 and calpastatin, as well as on the tenderness and aging response of beef. Factors that affect meat quality differences between gender groups and individual muscles are not clearly understood. Although the calpain enzymes are known to degrade muscle proteins, the specific roles of each enzyme in the tenderization process remain contentious. Taking into consideration various post-transcription regulatory mechanisms and modifications, an investigation at the mRNA level was carried out to give insight into the cellular demand for individual enzymes of the calpain system. Such gene expression information may suggest circumstances were certain calpains are required. Therefore, the purpose was to determine whether the variations at the mRNA level are associated with tenderness differences among the gender groups and muscle types.

The expression of the ubiquitous calpains and beef tenderness

The current results revealed a down-regulation of μ-calpain in heifers. This would lead to reduced proteolysis of myofibrillar proteins and poor tenderization, which was supported by higher shear force values in heifer meat in our study. Conversely, the higher μ-calpain mRNA levels found in intact males and steers denoted that higher proteolytic activities were eminent in the muscles of this group than there would be in

the muscles of heifers. This was emulated by the lower shearforce values in bulls and steers meat.

On the other hand, μ-calpain mRNA levels were similar between steers and bulls in the present study, which agrees with the results of Morgan et al. (1993b) who showed that 24 h calpain activities in steer and bull *longissimus* muscle were not different. However, Morgan et al. (1993b) found higher calpastatin activities in bull compared to steer muscles and proposed that this may have explained the higher shear-force values for bull muscle.

In the present study, we found that steer meat was slightly tougher than that of bulls, which was contrary to previous findings (Morgan et al., 1993b; Purchas et al., 2002; Zhang et al., 2010). On that basis, steers were expected to have lower shearforce values than bulls, and it was thus hypothesised that castration would lead to increased mRNA levels of calpains and reduce those of calpastatin. However, other studies found no significant differences in tenderness between bulls and steers (Boccard et al., 1979; Keane and Allen, 1998; Knight et al., 1999; Destefanis et al., 2003; Choi et al., 2010). It has been argued that these inconsistent effects of castration and gender on meat tenderness are due to various intrinsic factors, as well as the production systems and the age at slaughter (Field, 1971; Boccard et al., 1979; Purchas et al., 2002).

Accordingly, it has been suggested that older animals could produce much tougher meat (Cross et al., 1984; Gheisari et al., 2007) which may be the case with the animals in the present study where bulls were approximately 7 months younger than the steers and more than 11 months younger than the heifers (Table 3). However, it is important to note that commercial abattoirs prefer to slaughter at equivalent weight, which usually means that the slow growing steers and heifers are usually older. In Choi et al. (2010), commercial steers were on average 2 months older than bulls. This, however, did not affect meat tenderness possibly due to negligible collagen cross-linking at that age (Cross et al., 1984). Since the calpains do not degrade collagen, this might have provided background toughness in the present study. Therefore, as indicated by similar *post-mortem* aging responses, the meat of steers in the present study tenderised at the same rate as those of bulls. This

suggests similar rates of myofibrillar protein degradation and concurred with the similar levels of μ-calpain mRNA between bulls and steers in the present study.

Furthermore, although bulls were younger, no differences were observed with regards to liveweights and carcass weights, due to the expected rapid growth in bulls. This comparably faster rate of growth in bulls signifies higher muscle protein accumulation compared to castrates which is thought to be a reflection of the differences in the concentrations of anabolic androgens in these gender groups. Steers being castrates are expected to produce less testosterone (Hartwig et al., 1997; Fritsche and Steinhart, 1998), grow slower (Field, 1971; Seideman et al., 1982) and thus produce much tender meats. However, Fritsche and Steinhart (1998) reported that 25% of the bull samples analyzed showed lower testosterone levels than steers, a notable variability which may partly explain lower shearforce values in some meat from bull carcasses. It can therefore be speculated that the differences in concentrations of natural testicular and skeletal muscle androgens between intact and castrated males may sometimes not be large enough to result in transcriptional differences in the calpains.

As with the differences among the gender groups, muscles that are inherently tender are expected to express higher levels of proteolytic enzymes than those that are tough. The LT muscle and other skeletal muscles involved in support are generally more tender than locomotive muscles, such as the SM (Sullivan and Calkins, 2011). However, ovine LT tenderised at the same rate as the SM, and their mRNA levels for μ-calpain and m-calpain did not differ (Ilian et al., 2001), which is in agreement with our results in beef cattle. Both muscles in the present study also tenderised at the same rate suggesting equal rates of proteolysis. Therefore, these proteases play minor roles in determining meat quality differences between the LT and SM muscles. In addition, m-calpain expression was not affected by gender or muscle type. This supports exiting evidence that this isoform has little or limited involvement in *post-mortem* tenderization (Kemp et al., 2010). Others have suggested that both the LT and SM have oxidative-glycolytic (Type IIA) muscle fibre characteristics, which may have similar Ca^{2+} concentrations and proteolytic activities. However, the proportion of the different fibre types may differ among muscles and may influence the expression level of proteolytic enzymes (Kirchofer et al., 2002). Further studies are

thus warranted to investigate gene expression, tenderization and *post-mortem* proteolysis in muscles with distinct muscle fibre characteristics.

The influence of calpastatin can be countered by higher calpain levels

Since calpastatin expression may be responsive to the effects of castration (Zhang et al., 2010), bulls are thus expected to produce higher calpastatin levels and relatively tougher meats than steers. This is in contrast with the present results because steers had a tendency to express higher levels of calpastatin mRNA than young bulls. As a result of this elevated calpastatin content, there was a tendency for the WBSF values of steers to be higher than those of bulls.

Calpastatin mRNA levels in steers were also higher than those of heifers. Despite this, it was speculated that heifer steaks did not tenderise well since μ-calpain mRNA levels, which is necessary in the degradation of myofibrillar proteins, were significantly lower. The carcass pH_u values in heifer longissimus were also low while that of steers were high. High pH_u is associated with rapid autolysis of μ-calpain, faster degradation of myofibrillar proteins (Lomiwes et al., 2014) and may have resulted in much tender meat in steers as compared to heifers.

Furthermore, the regulation of calpastatin transcription in the LT, especially those of castrates, may not be in line with its expected activity in *post-mortem* muscle. The expression of calpastatin in the LT of steers was greater than that of the SM muscle, while the latter was found to have higher shear force values.

According to Walker et al. (2010), steers, when compared to heifers, had a tendency to have higher levels of β2-adrenergic receptor mRNA in the LT compared to the *Biceps femoris* (BF). Sequence analysis had identified several transcriptional motifs in the promoter region of the bovine CAST gene (Raynaud et al., 2005), while experiments show that that they are differentially regulated in response to β-adrenergic agonists (Cong et al., 1998a; Cong et al., 1998b; Sensky et al., 2006). Although androgen responsive elements have not been reported in the promoter of bovine Calpastatin, the Androgen Responsive Gene Database (ARGDB) has indicated this gene is generally up-regulated by androgens (Jiang et al., 2009). On the contrary, Labrie (2011) reported an increase in the levels of androgen receptors

in response to low levels of androgens due to castration. It can be deduced that castration may also result in an increase in the levels of β2-adrenergic receptor mRNA, that may lead to differential calpastatin transcription in a tissue specific manner.

It has also been suggested that calpain may cleave its inhibitor and allow proteolysis to occur more efficiently (Doumit and Koohmaraie, 1999). Hence, the elevated transcription of calpastatin observed in steer LT in the present study, could have been counter-acted by the elevated production of μ-calpain, as suggested by the increased gene transcription. This further suggests that the regulation of calpain activity and their involvement in tenderization is not mainly through the variation in calpastatin, but also through increased expression of the calpain proteases, and sometimes without altering the levels of calpastatin.

The expression of the "muscle-specific" calpain-3 and beef tenderness

In this study, the calpain-3 mRNA levels were negatively correlated with 7d WBSF values in the LT. This is in agreement with Yang et al. (2012), who reported a positive correlation between tenderness and high expression of calpain 3 in Sutai pigs.

The revelations that calpastatin does not inhibit the activities of calpain-3 (Ono et al., 2004), whereas variations in calpastatin content and activities has been associated with meat quality, has contributed to doubts about the involvement of this muscle-specific calpain in *post-mortem* proteolysis. In addition, Parr et al. (1999) reported no relationship between the abundance of calpain-3 protein and shearforce variations in porcine LD at 8 d *post-mortem*.

On the other hand, Ono et al. (2007) claimed that calpain-3 have common substrates with the ubiquitous calpains. These substrates include filamin-C, annexin, translation factors (Ono et al., 2007), cardiac ankyrin repeat protein (Laure et al., 2010) as well as calpastatin (Ono et al., 2004). Therefore, if a cleaved calpastatin have lower inhibitory potency than the intact one, then the involvement of calpain 3 in proteolysis would be supportive of μ-calpain activities and would lead to increased tenderness.

In the present study, calpain-3 mRNA levels did not differ between bulls and steers, but significant differences were observed between bulls and heifers. As with μ-calpain, a greater calpain-3 expression was found in bulls as compared to heifers. Therefore, lower shearforce values in bull steaks could in part be due to the increased expression of calpain-3 in its suggested supportive role to the μ-calpain activities as well as direct proteolysis of some muscle proteins. Similarly, Illian et al., (2004) hypothesized a synergetic catalysis of myofibril proteins by μ-calpain and calpain-3, due to the association between their autolysis and tenderization in the *Longissimus thoracis et lumborum* of lambs.

In addition, increased calpain-3 expression in bulls is possible since they exhibit faster growth rates and the proteolytic roles of the calpains in muscle development are to support myoblast fusion by cutting cytoskeletal membrane attachments (Goll et al., 2003). Indeed, the up-regulation of calpain-3 during fusion and differentiation in porcine cells further suggest its role in these growth processes (Theil et al., 2006).

It was also suggested that, by binding at N1 and M2 lines of myofibrils, calpain-3 may function as a protector of titin against unnecessary degradation (Sorimachi et al., 1996). Thus, higher levels of calpain-3 could also mean reduced degradation of myofibrillar proteins *in vivo*, which may support increased muscle growth. This, coupled with the fact that calpastatin does not inhibit calpain-3, may allow this protease to cleave titin when activators such as the concentration of Ca^{2+} (García Díaz et al., 2006) are in sufficient quantities, as in *post-mortem* muscle. Apart from this eminent suicide relationship between calpain-3 and titin, calpain-3 may degrade calpastatin (Ono et al., 2004) which would further enhance *post-mortem* proteolysis and tenderization.

Moreover, membrane associated calpain-3 was found to be essential for the recruitment of the glycolytic enzyme AldoA (Aldolase A) and the calcium release channel RyR (Ryanodine receptors) to the triads where it serves a structural support role (Kramerova et al., 2008). In that study, the reduced concentration of AldoA and RyR in Calpain-3-knockout mice also resulted in reduced calcium release, which is likely the cause of muscle weakness in muscular dystrophic patients (Kramerova et al., 2008). As a consequence of this reduced calcium release, the proteolytic

activities of the calcium-dependent calpains could be decreased in *post-mortem* muscles. Thus under this scenario a decreased expression of calpain-3 may indirectly result in less tender meat, as observed in heifer meats in the present study.

On the other hand, the present results shows that muscle type did not have an effect on the expression of calpain-3. Similarly, Ilian et al. (2001) reported that the levels of calpain-3 and the rates of tenderization between ovine LT and SM muscles did not differ significantly. In contrast, the levels of calpain-3 mRNA were significantly higher in the LT compared to the *Psoas major* (PM) in steers, and higher in the LT than the ST in male lambs (Ilian et al., 2001). Thus calpain-3 expression could not be implicated in meat quality differences between the LT and the SM in beef cattle. There is thus a need to carry out further research in more distinct muscles in beef cattle such as the *Psoas major, Semitendinosus, Biceps femoris,* to state a few.

Conclusions

Although the number of animals used in this study was small, the results indicate that tenderness differences among gender groups in cattle could in part be explained by variations in the expression of the calpain system at the mRNA level. The results suggest that calpain 3 may play a role in determining meat tenderness, possibly through a supportive role to μ-calpain, because gender groups that had an elevated transcription of both enzymes had lower WBSF values. There was, however, a lack of variation in the muscles at the mRNA level, and thus it appears that differences in tenderness between the LT and SM may not be modulated by calpains.

Further research that may involve investigating more muscles with distinct characteristics and to compare the mRNA levels with the contents and activities of the calpain system proteins is therefore recommended. It would also be ideal to perform further tests on a larger number of animals and to incorporate different ages at slaughter in order to further our understanding of the mechanisms of the calpain system in determining meat tenderness.

Conflict of Interests statement

None.

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TABLES

Table 1
The oligonucleotide sequences of the primers used to determine the expression of target genes

Target Gene	Gene Symbol	Accession number[1]	Primer	Tm[2] (˚C)	Nucleotide sequence 5′-3′
μ-Calpain	CAPN1	NM_174259	Sense	56.5	CGCCTCCCTTACCCTCAAT
			Anti-sense	56.8	CATCCACCCACTCACCAAAC
m-Calpain	CAPN2	NM_001103086	Sense	56.7	AACGACTTCCTGAGACACTATTC
			Anti-sense	56.7	GCCTCCAGTTCCCATCCAT
Calpain-3	CAPN3	NM_174260	Sense	57.2	AAAGACAACACAAGCCCTGATAA
			Anti-sense	57.3	ATTGCCGCTGTTCCTCACT
Calpastatin	CAST	AF159246	Sense	55.9	CAGCGTGACAACAAAGAACTT
			Anti-sense	56.2	GACTTTATCCTCTACGGGTTTATTC

[1]The primers were designed by Primerdesign Ltd. (Southampton, UK).
[2]The annealing temperature of 60 ˚C was used for all PCR reactions.

Table 2

The effects of gender and muscle type on the mRNA levels of the calpain system genes

	Bulls (n=6)		Heifers (n=6)		Steers (n=6)		*P*-Value		
Target Gene	LT	SM	LT	SM	LT	SM	Gender	Muscle	Interaction
μ-Calpain	0.10a±0.003	0.10a±0.003	0.09b±0.003	0.09b±0.003	0.11a±0.003	0.10a±0.003	***	NS	NS
m-Calpain	0.09±0.002	0.09±0.003	0.09±0.003	0.09±0.003	0.10±0.002	0.09±0.003	NS	NS	NS
Calpain-3	0.11a±0.003	0.11a±0.005	0.09b±0.003	0.10b±0.004	0.11ab±0.003	0.10ab±0.004	**	NS	NS
Calpastatin	0.09b±0.003	0.09b±0.004	0.09b±0.003	0.09b±0.004	0.11a±0.003	0.09b±0.004	*	0.06	0.04

[a,b,] Means within the same row that do not have a common superscript letter were significantly different ($P < 0.05$).

LT: *Longissimus thoracis* muscle

SM: *Semimembranosus* muscle

The values are Means ± SEM of relative gene expression levels

Table 3
The means and SEM for the age at slaughter, liveweight and carcass data of bulls, heifers and steers

Parameter	Gender			
	Bulls (n = 6)	Heifers (n = 6)	Steers (n = 6)	*p*
Age at slaughter, d	547.17^{c} ± 2.09	889.00^{a} ± 0.63	764.33^{b} ± 2.04	0.001
Liveweight, kg	571.58 ± 20.11	551.75 ± 6.08	546.25 ± 17.51	0.506
Carcass weight, kg	319.58 ± 10.75	301.27 ± 5.18	296.80 ± 11.51	0.236
Dressing percentage, %	55.93^{a} ± 0.29	54.59^{b} ± 0.51	54.27^{b} ± 0.41	0.030
EUROP fatness[1]	3.42^{b} ± 0.15	4.80^{a} ± 0.23	3.92^{b} ± 0.08	0.001
EUROP conformation[1]	2.17 ± 0.17	2.00 ± 0.01	2.00 ± 0.01	0.391
pH_0	-x	6.30^{b} ± 0.12	7.00^{a} ± 0.06	0.001
pH_{2d}	-x	5.24^{b} ± 0.24	6.48^{a} ± 0.10	0.002
pH_{7d}	5.86 ± 0.04	5.85 ± 0.09	5.72 ± 0.03	0.190

^{a,b,c} Means within the same row that do not have a common superscript letter were significantly different ($P < 0.05$).

[1]The fatness and conformation scores of EUROP classification system were converted to numerical scores. A higher number indicates a better conformation or higher carcass fatness level.

^{x} pH readings for bull carcasses at time of slaughter and the day after were not available.

Table 4

The means and SEM for the WBSF values (N) of the *Longissimus thoracis* and *Semimembranosus* among bulls, heifers and steers at various *post mortem*-aging periods

	Gender		
	Bulls	Heifers	Steers
Variables	(n = 6)	(n = 6)	(n = 6)
Longissimus thoracis			
Aging period			
7d	30.03aXY ± 2.54	39.30bcXY ± 2.40	31.88abXY ± 2.45
14d	25.78aYZ ± 2.17	32.82bcYZ ±2.05	31.87abYZ ± 2.09
21d	23.15aZ ± 2.03	32.40bcZ ± 1.92	27.40abZ ± 1.96
28d	23.80aZ ± 1.88	31.64bcZ ± 1.78	25.29abZ ± 1.82
35d	23.82aZ ± 1.61	30.94bcZ ± 1.52	25.03abZ ± 1.55
Semimembranosus			
Aging period			
7d	36.93bW ± 1.89	38.87cW ± 1.78	39.47cW ± 1.82
14d	36.61bWX ± 1.30	38.51cWX ± 1.23	39.34cWX ± 1.26
21d	34.74bWX ± 1.76	36.06cWX ± 1.66	36.20cWX ± 1.70
28d	30.07bWY ± 1.59	34.87cWY ± 1.50	37.56cWY ± 1.53
35d	27.80bYZ ± 0.96	34.03cYZ ± 0.91	29.65cYZ ± 0.93

a,b,c,d – Means without a common superscript indicate significant differences between each gender and muscle type ($P < 0.05$).

W,X,Y,Z – Means without a common superscript indicate significant differences between each postmortem aging period and muscle type ($P < 0.05$).

P-values for:

Effects of Gender:	0.001
Effects of Muscle:	<0.001
Effects of *post mortem* Aging:	<0.001
Gender x Muscle:	0.04
Gender x *Post mortem* Aging:	0.63
Muscle x *Post mortem* Aging:	0.04
Gender x Muscle x *Post mortem* Aging:	0.28

Table 5

The means and SEM for the aging responses (N) of *Longissimus thoracis* and *Semimembranosus* among bulls, heifers and steers

	Muscle	
Gender[1]	*Longissimus thoracis*	*Semimembranosus*
Bull	8.42 ± 2.14	8.59 ± 1.34
Heifer	7.62 ± 4.44	5.03 ± 2.26
Steer	5.39 ± 2.43	10.18 ± 2.56

P-values for:

Effects of Gender: 0.755

Effects of Muscle: 0.698

Gender x Muscle: 0.336

Aging response was obtained by subtracting 35d WBSF from 7d WBSF values (N).

[1]n = 6 for each gender group.

Highlights

- Muscle type had no effect on gene expression and rate of tenderization.
- Sex affected mRNA levels and shearforce values.
- Calpain 3 was negatively correlated to 7d shearforce values.
- The combined elevated mRNA levels of calpain 1 and 3 may enhance meat Tenderness.