A post-transcriptional mechanism regulates calpastatin expression in bovine skeletal muscle[1]

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ABSTRACT: The objective of this study was to investigate whether single nucleotide polymorphisms (SNP) in the calpain 1 (*CAPN1*), calpain 3 (*CAPN3*) and calpastatin (*CAST*) genes, which have been shown to be associated with shear force and tenderness differences in the skeletal muscle of cattle, contribute to phenotypic variation in muscle tenderness by modulating the transcriptional activity of their respective gene. The mRNA expression of the calpain and *CAST* genes was assessed in the longissimus lumborum muscle (LLM) of cattle from two herds located in distinct production zones on the east (New South Wales, NSW) and west (Western Australia, WA) of Australia. The cattle in the herds were mainly Brahman cattle (*Bos indicus*) with smaller populations of Angus cattle (*Bos taurus*). There were 191 steers in the WA herd and 107 steers and 106 heifers in the NSW herd. These herds were established by choosing cattle from the diverse population which had different single nucleotide polymorphism (SNP) genotypes at the *CAPN1*, *CAPN3* and *CAST* loci. Using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), the transcriptional activities of the *CAPN1* and the *CAST* genes, but not the *CAPN3* gene, were found to differ between favorable, positively associated with tenderness, and unfavorable, negatively associated with tenderness, allelic variants of these genes. These findings suggest that the muscle shear force and consumer taste panel differences in tenderness explained by the *CAPN1* and *CAST* gene markers are a consequence of alterations in their mRNA levels, which may ultimately influence the protein activity of these genes, thereby altering the rate and(or) the extent of postmortem proteolysis in skeletal muscle. Of particular importance were the significantly lower type II and type III *CAST* 5′ splice variant mRNA levels that were detected in the LLM muscle of Brahman and Angus cattle with 2 favourable alleles of the *CAST:c.2832A > G* polymorphism. Moreover, a reduction in the abundance of an alternative polyadenylated variant of the *CAST* transcript, terminated at the proximal polyadenylation site, provides a unique insight into the potential involvement of a post-transcriptional regulatory mechanism which may influence protein expression levels in bovine skeletal muscle.

Key words: alternative polyadenylation, calpastatin, cattle, gene expression, single nucleotide polymorphism, tenderness

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INTRODUCTION

Muscle tenderness is controlled to a large extent by the postmortem proteolysis of myofibrillar proteins in skeletal muscle. Members of the calpain family which are calcium activated cysteine proteinases, play an integral role in this process (Koohmaraie, 1992). The ubiquitously expressed μ-calpain (**CAPN1**) and m-calpain (**CAPN2**) are the most extensively studied isoforms, while the muscle-specific p94 isoform (**CAPN3**), has also been implicated in muscle tenderness (Barendse et al., 2008), although its biological role in this context remains unclear. In contrast, the interplay between the activity of CAPN1 and CAPN2, and their endogenous inhibitor, calpastatin (**CAST**), in modulating the rate and extent of postmortem proteolysis in skeletal muscle has been well documented (Koohmaraie and Geesink, 2006). In cattle, several studies have demonstrated genetic associations between single nucleotide polymorphisms (**SNP**) in the *CAPN*1 and *CAST* genes and differences in meat tenderness (Van Eenennaam et al., 2007; Johnston and Graser, 2010; Allais et al., 2011). Recent studies by Cafe et al. (2010a,b) and Robinson et al. (2012) demonstrated the presence of additive effects for improvements in beef tenderness for Brahman cattle with favorable allele combinations in the *CAPN*3, *CAST* and *CAPN*1 genes.

The association between these gene markers and measurable phenotypic consequences on meat tenderness supports the notion that the transcriptional activity of the calpain and calpastatin genes may be influenced by the SNP residing in these genes. With this in mind, LLM samples from the studies of Cafe et al. (2010a,b) were used to explore whether SNP for the *CAPN*1, *CAPN*3 and *CAST* genes were associated with transcriptional changes in the activity of these genes in line with their known biological functions, and whether such modulation could account for the phenotypic variation in tenderness previously reported in the LLM muscle of these cattle.

MATERIALS AND METHODS

Use of animals and the procedures performed in this study were approved by the New South Wales (**NSW**) Department of Primary Industries Orange Agricultural Institute Animal Ethics Committee (Approval Numbers ORA/06/001 and ORA 06/004), Commonwealth Scientific and Industrial Research Organisation (**CSIRO**) Rockhampton Animal Experimentation Ethics Committee (Approval Number RH216–06), and the Department of Agriculture and Food, Western Australia (**WA**) Animal Ethics Committee (Approval Number 2–06–11).

Tenderness Markers and Genotyping

Tests for 4 commercially available gene markers for beef tenderness were performed at the laboratories of CSIRO Livestock Industries, Brisbane, Queensland, Australia. The 4 markers were SNP within genes controlling the calpain proteolytic system, specifically *CAST* (*CAST:c.2832A > G;* (Barendse, 2002), *CAPN3* (*CAPN3:c.1538+225G > T*; (Barendse et al., 2008), and 2 in *CAPN1: CAPN1–4751* (*CAPN1:g.6545C > T;* (White et al., 2005) and *CAPN1–316* (*CAPN1:c.947C > G;* (Page et al., 2002). For each marker, the alleles differ in their effects on meat tenderness, and the favorable allele is associated with more tender meat, although it is not known whether any of these SNP are causal. The favorable alleles are 'A' at *CAST*, 'G' at *CAPN3*, 'C' at *CAPN1–4751* and the forward strand 'C' at *CAPN1–316*. The number of favorable alleles (0, 1 or 2) for each marker will be used in this paper to designate the genotype of cattle for each marker.

Blood samples were collected from weaner cattle into 6 mL vacutainers containing 10.8 mg K_2EDTA (Becton, Dickinson and Company, Sydney, Australia), chilled and transported to the laboratory. DNA was extracted from 200 μL of whole blood using Qiagen columns following the manufacturer's instructions (Qiagen, Hilden, Germany).

The SNP were genotyped using the Taqman MGB allele discrimination method (Applied Biosystems, Foster City, CA) using the previously published Taqman probes and primers (Barendse et al., 2007, 2008).

Sources of Cattle, Experimental Designs and Procedures

Two concurrent experiments were conducted, 1 at NSW Department of Primary Industries Agricultural Research and Advisory Station, Glen Innes, NSW (29°44′S, 151°42′E, altitude 1,057 m) and 1 at the WA Department of Agriculture and Food Vasse Research Station near Busselton, WA (33°45′ S,115°21′ E, altitude 25 m). The experiments were primarily designed to assess the effects of calpain-system gene markers (*CAST*, *CAPN3*, *CAPN1–4751*, and *CAPN1–316*) and molecular value predictions (**MVP**) on production and beef quality characteristics, as well as identify interactions of the gene markers with production (sex and hormonal growth promotant [**HGP**] implantation) and processing (carcass suspension, muscle, and duration of the postmortem aging period) effects in Brahman cattle (Cafe et al., 2010a; Robinson et al., 2012; Greenwood et al., 2013). Information on marker status was used to select Brahman cattle to achieve divergence in the number of favorable alleles for the *CAST* and *CAPN3* markers, with the groups being as balanced as possible for the *CAPN1–4751* marker. A small group of Angus cattle with favorable alleles for the *CAST* and *CAPN3* markers, as balanced as possible

for the *CAPN1–316* marker, were also selected as positive controls for biological studies on the calpain system.

New South Wales (NSW) study

The experiment for the Brahman cattle in NSW was designed to compare favorable alleles for *CAST* (0 or 2) × favorable alleles for *CAPN3* (0 or 2) × hormonal growth promotant (**HGP**) treatment (with or without HGP containing 200 mg trenbolone acetate and 20 mg 17β-estradiol (Revalor-H [registered for both steers and heifers], Virbac, Milperra, Australia) implanted in the ear 2 wk after arrival at the feedlot) × gender (heifer or castrate male) in Brahman cattle. The design for the Angus controls in NSW included contrasts of HGP status and gender, and was also chosen to maximize the accuracy of estimating the effect of *CAPN1–316* marker status (Robinson et al., 2007). The number of Brahman and Angus animals within each allelic status for the 4 calpain system gene markers and within gender and HGP category in the NSW experiment are also presented in Table 1.

Brahman cattle were sourced at weaning (6 to 8 mo of age) from 4 commercial and 3 research herds as described by Cafe et al. (2010a). The research herds supplied only heifers, and the commercial herds supplied both steers and heifers, which were born during the same season as the research herds and weaned at 7 to 8 mo of age. The Angus cattle were sourced from 2 research herds in northern NSW.

A total of 1,090 weaned calves were DNA tested (gene marker frequencies for the Brahman cattle are shown in Cafe et al., 2010a), and the results used to select 164 steers and heifers in groups that were homozygous for favorable and unfavorable *CAST* and *CAPN3* gene markers, and as balanced as possible across groups for *CAPN1* gene marker allelic status.

The cattle were managed as described by Cafe et al. (2010a). The Brahman cattle were transported to the Queensland Department of Primary Industries Brigalow Research Station (Theodore, Queensland, Australia), where they were held up to 4 wk while undergoing a cattle tick treatment program required for transport to Glen Innes, NSW for backgrounding. Selected Angus cattle were also transported to Glen Innes at this time. After 4 mo of grazing, calves were allocated to 4 backgrounding groups balanced for gender, gene marker status, origin, and previous management. At the end of the 6-mo backgrounding period, all calves were transported to the Australian Cooperative Research Centre for Beef Genetic Technologies "Tullimba" Research Feedlot near Kingstown, NSW. Upon arrival calves were segregated by gender, and individual animals were allocated to treatment (implant/none) and management groups aiming for the greatest possible balance of genotypes, gender, BW, and treatments across property of origin and other management groups, and to minimize the error variance of comparisons of marker and treatment effects.

Table 1. Number of Brahman and Angus cattle by gender, HGP-status, and genotype (number of favorable alleles) for calpain-system gene markers in the New South Wales (NSW) and Western Australian (WA) experiments

	Experimental site			
Variable	NSW	NSW	WA	WA
Breed	Brahman	Angus	Brahman	Angus
Gender				
Heifers	82	24	–	–
Steers	82	25	173	20
HGP				
No HGP	83	24	86	10
HGP	81	25	87	10
CAST[1]				
0	66	–	51	–
1	10	–	61	–
2	88	49	61	20
CAPN3[1]				
0	88	–	43	–
1	8	1	74	–
2	71	48	56	20
CAPN1–4751[1]				
0	89	1	83	–
1	67	18	75	4
2	8	30	15	16
CAPN1–316[1]				
0	149	17	145	4
1	15	21	27	12
2	0	11	1	4

[1]Gene markers for *CAST* = calpastatin; *CAPN3* = calpain 3; and *CAPN1–4751* and *CAPN1–316* = calpain 1.

After 2 wk in the feedlot during a 4-wk adaption period to grain-based diets, half of the cattle were implanted with an HGP containing 200 mg trenbolone acetate and 20 mg estradiol-17β (Revalor-H; Virbac). All cattle were fed a grain-based diet formulated to provide 12.0 MJ ME/kg, 16.0% CP, and 10.5% ADF (DM basis). Cattle were 17 to 19 mo of age at the end of the 117-d finishing period.

The NSW cattle were transported 370 km (approximately 7 h) from the feedlot to John Dee abattoir, Warwick, QLD for slaughter. Four replicates were transported at the same time and the remaining 4 replicates were transported 2 d later. Both groups left the feedlot midmorning. They were provided with water during lairage and there was no mixing of replicates at any stage. Cattle were slaughtered by captive bolt and exsanguination around noon the day after leaving the feedlot.

Western Australia (WA) study

Only steers were used in the WA experiment, which was designed to compare favorable alleles for *CAST* (0,

1 or 2) × favorable alleles for *CAPN3* (0, 1 or 2) × HGP treatment (with and without Revalor-H during feedlotting) in Brahman cattle. The design for Angus cattle included a contrast of HGP status and was also chosen to maximize the accuracy of estimating *CAPN1–316* marker effect. The number of Brahman and Angus animals within each allelic status for the 4 calpain system gene markers and within HGP category in the WA experiment are also shown in Table 1.

Brahman cattle were sourced at weaning (6 to 8 mo of age) from 4 producers in the Northern Agricultural region of WA, and the Angus cattle from a commercial herd in the south-west of WA. A total of 574 calves were tested for calpain-system gene marker status (gene marker frequencies for the Brahman cattle are shown in Cafe et al., 2010a) and 173 steers were then selected based on their initial DNA tests for the calpain system gene markers to create similarly-sized groups of cattle that were homozygous or heterozygous for favorable and unfavorable *CAST* and *CAPN3* gene markers, as balanced as possible for *CAPN1* gene marker allelic status.

The cattle were managed as described by Cafe et al. (2010a). The selected cattle were transported to Vasse Research Station for backgrounding and finishing. Calves were grazed for 6 mo on pasture in groups based solely on BW before allocation into replicates (n = 4), feedlot pens (n = 12), and HGP implant treatments, balanced for gene-marker status, property of origin, and previous management groups. After an additional 2 mo grazing pasture, steers were transferred to the pens in the feedlot facility and, following a 2-wk adaption period, fed a high-grain diet containing 10.8 MJ ME/kg DM and 13.4% CP. As in the NSW experiment, half of the steers received a combination trenbolone acetate-estradiol-17β implant (Revalor-H; Virbac) 2 wk after arrival at the feedlot. At the end of the 80-d feedlot phase, steers were 21 to 24 mo of age.

Cattle from 6 feedlot pens were transported approximately 100 km to Harvey Beef, Harvey, WA, for slaughter the following day. The remaining one-half of the cattle were transported 2 d later and slaughtered the next day. There was no mixing of cattle from different feedlot pens during transport or in lairage before slaughter by captive bolt and exsanguination.

Sample Collection and Total RNA Purification

Samples of LLM were collected from the carcass of each animal within 30 min of slaughter and snap frozen in liquid nitrogen. Each frozen muscle sample was wrapped in aluminum foil and pulverized with a hammer. Approximately 100–200 mg of muscle tissue powder was mixed with Trizol reagent (Invitrogen, Carlsbad CA) and homogenized with a rotor-stator (IKA, Staufen, Germany). The upper aqueous phase was obtained from each homogenized sample as per the manufacturer's instructions. The aqueous phase from each sample was mixed with ethanol (Sigma, St Louis, MO) and RLT buffer (Qiagen), and applied to a RNeasy column (Qiagen). The columns were processed according to the manufacturer's instructions, including an on-column RNase-free DNase treatment (Qiagen). Total RNA was eluted in 50ul of EB buffer (Qiagen). The concentration and purity of total RNA was determined using UV spectrophotometry (Nanodrop 1000, Thermo Fisher Scientific, Waltham, MA). The integrity of the 28S and 18S ribosomal RNAs was confirmed with agarose gel electrophoresis.

Table 2. Oligonucleotide sequences used to synthesize complementary DNA

Primer name[1]	Oligonucleotide sequence (5′-3′)
*CALP1*cdna	CATCCGCATTTCATAG
*CALP3*cdna	TGCTATCTGCCTGAAA
*CAST*cdna	TCAGCTTTTGGCTTGG
*RPLP0*cdna	ATGGATCAGCCAAGAA
*CASTIII*cdna	GCGGAGGGGGCGATG
*CASTII*cdna	TCTTGTACCACCAGCTT
*CASTIII ex5–6*cdna	TGGCTCTGTGTGTTCCTTTG
OligodT-VN	TTTTTTTTTTTTTTTVN
Adapter-oligodT[2]	GACATCGTACCTGACTCATCGCACTT-TTTTTTTTTTTTTTTT

[1] Abbreviated mRNA transcript identifiers for *CALP1* = calpain 1; *CALP3* = calpain 3; *CAST* = 'total' calpastatin; *RPLP0* = Ribosomal Protein, Large, P0; *CASTIII*= type III calpastatin splice variant; *CASTII* = type II calpastatin splice variant; *CAST ex5-6* = calpastatin exon5-exon6; OligodT-VN= thymidine homopolymer with 3' degenerate nucleotides (V = A,C,G and N= A,C,G,T).

[2]The underlined portion of the Adapter-oligodT sequence highlights the antisense primer sequence which was used to quantitatively measure the calpastatin mRNA polyadenylation variants.

Reverse Transcription

The concentration of total RNA for all samples was normalized to 100 ng/μL with the aid of a liquid handling robotics system (EpMotion 5075; Eppendorf, Hamburg, Germany). Complementary DNA (cDNA) was synthesised from 500 ng of total RNA using the High Capacity cDNA Synthesis Kit (Applied Biosystems). The cDNA synthesis reactions contained 25 units of Multiscribe reverse transcriptase, 4 units RNaseOUT ribonuclease inhibitor (Invitrogen), 5 μM oligodT-VN primer (Table 2) and 200nM gene-specific primers (Table 2). The synthesis of cDNA was performed in 96 well plates at 39°C for 2 h and the reverse transcriptase was subsequently inactivated at 65°C for 20 min. The cDNA stocks were diluted 1:4 with 10 mM Tris pH 8.0 (Ambion, Austin, TX) and stored at −80°C until required. For the quantitative assessment of alternatively polyadenylated *CAST* mRNA

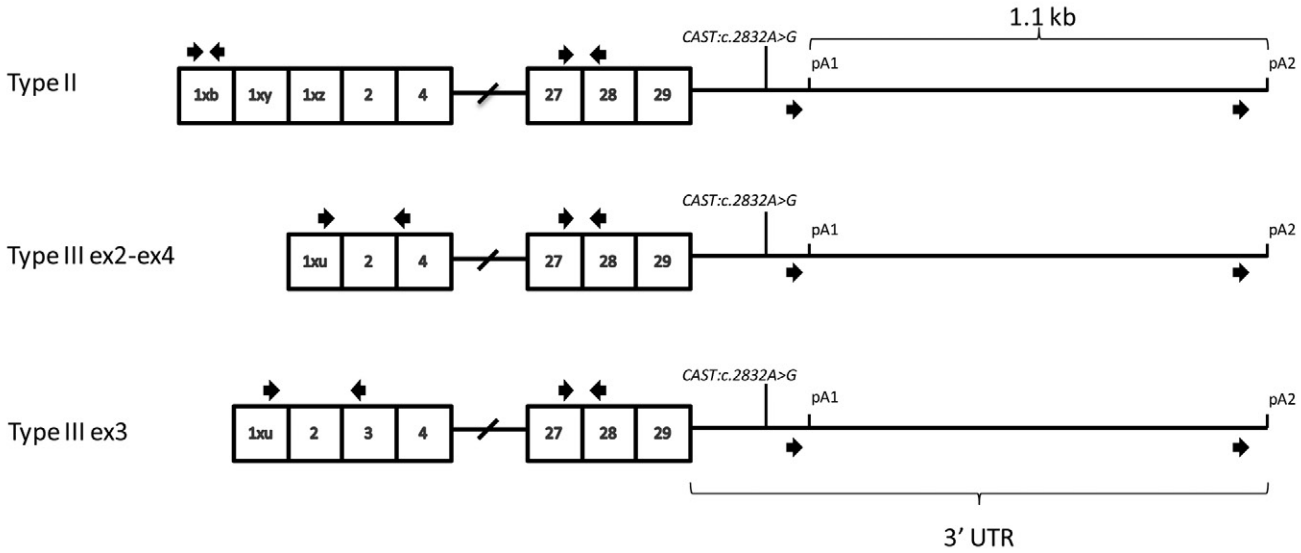

Figure 1. Schematic diagram of the calpastatin (*CAST*) *type II*, *type III ex2-ex4*, and *type III ex3* mRNA splice variants. Truncated mRNA transcripts are depicted. Exon numbers are marked within the boxes, including the exon 1 splice variants (1xb, 1xu, 1xy, 1xz). The arrows at the 5′ end of each variant indicate the location of the transcript-specific PCR primers. The arrows above exons 27 and 28 mark the location of the primers that were used to measure "total" calpastatin mRNA levels. The positioning of the pA1 and pA2 polyadenylation signals and the distance between them in kb are marked on the 3′ untranslated region (3'UTR). The location of the CAST:c.2832A > G SNP is shown and the arrows upstream of the pA1 and pA2 sites mark the locations of the *CASTpA1* and *CASTpA2* primers, respectively.

transcripts, the same reverse transcription reagents and procedure was applied but the reactions were primed with an oligodT primer that contained a 24 nucleotide adaptor sequence at the 5′ end (Table 2).

Real-Time Quantitative PCR

A liquid handling robotics system (EpMotion 5075; Eppendorf) was used to dilute the stock cDNA and to set up the real-time PCR assays. The stock cDNA (1:4) was diluted a further fivefold with 10 mM Tris-HCl pH 8.0 (Ambion) immediately before use in real-time PCR. For each sample, 5μl of 1:20 diluted cDNA was combined with 30μl of 2× PowerSYBR reagent (Applied Biosystems) and either 200nM or 400nM of forward and reverse oligonucleotides (Sigma-Genosys, Sydney, Australia) and nuclease-free water (Ambion), thus producing a cDNA/SYBR green master mix. Ten microliters of the cDNA/SYBR green master mix for each sample was transferred in triplicate to a 384 well MicroAmp plate, which was subsequently covered with a clear plastic seal (Applied Biosystem). For each gene, three and a half 384 well plates were required to screen the cDNA samples from the NSW and WA herds (n = 404 cattle). To account for inter-run variation for each gene assay, a common set of 4 pooled cDNA samples was included on each 384 well plate and these were used as inter-run calibrators. For each gene, the first 384 well plate run included a no template control and a 7 point standard curve. The first point in the standard curve contained 1:8 diluted cDNA prepared from pooled skeletal muscle cDNA that had been reverse transcribed with the same reagents as the samples from the NSW and WA herds. The remaining points in the standard curve were prepared with 6 consecutive twofold serial dilutions of the same pooled cDNA. Quantitative PCR was performed on 384 well real-time PCR machines (7900; Applied Biosystems) using the following cycling parameters: 95°C/10 min for 1 cycle, and 95°C/15 s and 60°C/1 min for 40 cycles, with data acquisition occurring at the 60°C step. The primers used to quantify the *CAPN*1, *CAPN*3 and *CAST* genes are described in Table 2.

Quantitative assessment of CAST 5′ *splice variants*

Multiple promoters upstream of the *CAST* gene initiate the synthesis of mRNA transcripts that have unique 5′UTR and transcript-specific exons preceding exon 2 (Fig. 1). The level of *CAST* mRNA transcripts synthesized from the type II and type III promoters was assessed using qRT-PCR assays which targeted these unique segments of DNA. Antisense orientated transcript-specific primers were also designed against the type II and type III *CAST* splice variants to facilitate cDNA synthesis. Two isoforms of the *CAST* type III 5′ splice variant were assessed: a transcript that contained exon 3 (termed *CASTIII-ex3*) and a transcript that lacked exon 3 (termed *CASTIII-ex2ex4*). The same cDNA synthesis and real-time PCR procedures as described previously were used to quantitatively measure the mRNA transcript levels of the *CAST* 5′ splice variants.

Table 3. Oligonucleotide sequences used for quantitative reverse transcriptase PCR assays (qRT-PCR)

Assay name[1]	Accession number	Oligonucleotide sequence (5′-3′)
CALP1	NM_174259	Sense: GGACATGGAGATCAGGCGTCAA Antisense: AACTCCACCAGGCCCAGTTT
CALP3	NM_174260	Sense: TCCCACCTAAATACCAACATCT Antisense: CCTGAGAGGGCATCAATGG
CAST	NM_001030319	Sense: GAAGCCCAAGGCATCAGAGA Antisense: AGCAGGCTTCTTGTCTTTGTC
RPLP0	NM_001012682	Sense: CAACCCTGAAGTGCTTGACAT Antisense: GCAAGTGGGAAGGTGTAATCA
CASTII	NM_174003	Sense: TTAGTCCCAGTCAGGTCTGC Antisense: ACGATGGATGTGTTCCGAGA
CASTIII (ex3)	AH014526	Sense: AAGCCGGGCTGAAGTCTC Antisense: TGTTAGGAGAATGCGGTCCT
CASTIII (ex2–ex4)	AH014526	Sense: AAGCCGGGCTGAAGTCTC Antisense: TCAGGTTCTGTTTTTACAGCCTT
CASTpA1[2]	AH014526	Sense: CAGCATGTGGCTTAATGGAA Antisense: CGTACCTGACTCATCGCACTT
CASTpA2[2]	AH014526	Sense: AACGGACTTCTCCAAAAGCA Antisense: CGTACCTGACTCATCGCACTT

[1]Abbreviated mRNA transcript identifiers for *CALP1* = calpain 1; *CALP3* = calpain 3; *CAST* = 'total' calpastatin; *RPLP0* = Ribosomal Protein, Large, P0; *CASTII* = type II calpastatin splice variant; *CASTIII (ex3)* = type III calpastatin splice variant containing exon 3; *CASTIII (ex2-ex4)* = type III calpastatin splice variant lacking exon 3; *CASTpA1* = calpastatin proximal polyadenylation variant; and *CASTpA2* = calpastatin distal polyadenylation variant.

[2]The antisense primer corresponds to the underlined sequence in the Adapter-oligodT primer in Table 2.

Quantitative assessment of alternatively polyadenylated CAST *transcripts*

The level of *CAST* mRNA transcripts terminated at the proximal **(pA1)** and distal (**pA2**) polyadenylation sites were measured directly using a quantitative PCR methodology that we devised and validated, which employed principles inherent to the 3′RACE technique (Frohman et al., 1988). Direct quantification of the *CAST* proximal (pA1) and distal (pA2) polyadenylation variants was achieved by performing qPCR on cDNA that had been reverse transcribed with an oligodT primer containing a 5′ adaptor sequence (Table 2). The pA1 transcripts were quantified using a sense primer (pA1fwd; Table 3; Fig. 1), located within 100 bp of the proximal (pA1) polyadenylation site, in combination with an antisense primer (Table 3), which matched a portion of sequence at the 5′ end of the Adaptor-oligodT primer (Table 2). Similarly, the pA2 transcripts were quantified with a sense primer (pA2fwd; Table 3), located within 100 bp of the distal (pA2) polyadenylation site, and an antisense primer (Table 3), which matched a portion of sequence at the 5′ end of the Adaptor-oligodT primer (Table 2). The distance between the pA1 and pA2 polyadenylation sites in the 3′ UTR of the bovine *CAST* gene is approximately 1,100 bp (Fig. 1). This distance prevented the pA1 forward primer from amplifying transcripts terminated at the pA2 polyadenylation site. Essentially, the larger 1,100 bp amplicon was out-competed by the significantly smaller 100 bp amplicon that was produced by the pA1 forward primer and the adaptor sequence affixed to cDNA terminated at the pA1 polyadenylation site. The same cDNA synthesis and real-time PCR procedures as described previously were used to quantitatively measure the mRNA transcript levels of the *CAST* polyadenylation variants.

Data processing, normalization and statistical analyses

Real-time PCR data was processed and normalized using an in-house computer software package, qEXPRESS (G. S. Nattrass, unpublished data), which was modeled on the qBase software package (Hellemans et al., 2007). Tab delimited text files from each real-time PCR run were exported from the SDS 2.3 software (Applied Biosystems) and imported into qEXPRESS, where the data was processed in the same manner as the qBase software. In brief, the reaction efficiency of each gene assay was determined from the standard curve and applied to a delta Ct quantification model to calculate the relative quantities between samples. The reaction efficiencies for assays used in this study ranged between 90 and 102%. Inter-run calibrations were performed using the common set of 4 pooled cDNA samples which were present on all plate runs for each gene. The relative quantification data was then normalized within qEXPRESS to a reference gene (Ribosomal Protein, Large, P0; ***RPLP0***). The stability of the *RPLPO* gene and hence its suitability as reference gene was confirmed via a NormFinder (Andersen et al., 2004) analysis using the Genex software package (MultiD, Goteborg, Sweden).

Statistical analyses of the normalized data were performed with a General Linear Model (GLM) in SAS (v9.2; SAS Inst. Inc., Cary, NC). The relative quantification data for each gene were assessed against the following fixed components: kill group (1 or 2 [WA]; 3 or 4 [NSW]), HGP implant (with or without), sex (Steer or Heifer), Breed (Angus or Brahman) and marker genotype (0, 1 or 2). The favorable allele for *CAST*, *CALP*3 and *CALP*1 was assigned the marker Genotype 2. In the case of *CAST* where the marker genotype of all the Angus cattle was 2, the statistical analysis was conducted with the marker genotype nested within breed. First- and second-order interactions were conducted on the model and the nonsignificant effects ($P > 0.05$) were sequentially removed. The statistical analysis performed on each gene initially included all the SNP for the 3 genes as fixed effects. The 4 SNP: *CAST (CAST:c.2832A > G), CAPN1–4751 (CAPN1:g.6545C > T), CAPN1–316 (CAPN1:c.947C > G)* and *CAPN3 (CAPN3:c.1538+225G > T)* were sequentially removed from the statistical model if they were not significant. The

Table 4. Significance (*P*-values) of the terms fitted in the linear models[1]

Dependent variable[2]	Terms fitted (*P*-values)											
	K	B	S	H	CAPN1(B)	CAST(B)	S*H	B*S	B*H	K*B	K*H	CAST(B)*S
CALP1[3]	< 0.0001	0.06	.	0.68	0.0018	.	.	.	0.018	.	.	.
CALP3[3]	0.03	0.21	.	.	.	.	.	.	.	< 0.0001	.	.
"total" *CAST*[3]	0.004	0.02	.	0.01	.	.	.	.	.	0.005	.	.
CASTIIIex2-ex4[3]	< 0.0001	0.0006	.	0.002	.	0.03	.	.	.	0.002	.	.
CASTIIIex3[3]	0.01	< 0.0001	< 0.0001	0.01	.	.	.	0.004	0.02	.	0.02	.
CASTII (replicate 1)[3]	0.008	< 0.0001	0.41	0.71	.	< 0.0001	0.03	.	.	.	.	.
CASTII (replicate 2)[3]	0.006	< 0.0001	.	.	.	< 0.0001	.	.	.	.	.	.
CASTII (replicate 3)[4]	< 0.0001	0.0002	0.34	0.96	.	0.0018	0.043	.	.	.	.	.
CASTpA1[4]	< 0.0001	0.1	0.21	0.0023	.	0.0007		.	.	.	.	0.006
CASTpA2[4]	0.0009	0.87	0.47	0.0002	.	.	.	0.016	.	.	.	.
CASTpA1:CASTpA2[4]	< 0.0001	0.0025	< 0.0001	.	.	0.0002	.	.	.	.	.	.

[1]K = Killgroup, B = Breed, S = Sex, H = HGP, *CAPN1*(B) = *CAPN1–4751* nested within Breed, *CAST*(B) = *CAST:c.2832A > G* nested within Breed, S*HGP = Sex*HGP, B*S = Breed*Sex, B*H = Breed*HGP, K*B = Killgroup*Breed, K*H = Killgroup*HGP, *CAST*(B)*S = *CAST*(B)*Sex.

[2]Abbreviated mRNA transcript identifiers for *CALP1* = calpain 1; *CALP3* = calpain 3; *CAST* = 'total' calpastatin; *RPLP0* = Ribosomal Protein, Large, P0; *CASTII* = type II calpastatin splice variant; *CASTIII (ex3)* = type III calpastatin splice variant containing exon 3; *CASTIII (ex2-ex4)* = type III calpastatin splice variant lacking exon 3; *CASTpA1* = calpastatin proximal polyadenylation variant; and *CASTpA2* = calpastatin distal polyadenylation variant.

[3]The initial phase of the experiment screened 152 cattle from NSW and 191 cattle from WA.

[4]The second phase of the experiment screened 213 cattle from NSW and 191 cattle from WA.

normalized relative expression level of each gene is presented as least square means (LS means) ± SEM.

Two approaches were employed to statistically analyze the significance of the proximal (pA1) and distal (pA2) alternatively polyadenylated *CAST* transcripts: 1) the pA1 and pA2 transcripts were normalized against the *RPLP0* reference gene in the same manner as the other qPCR data; and 2) a ratio of the pA1 to pA2 transcripts within each sample was calculated using non-normalized qPCR data. The use of a ratio to measure the relative abundance of the polyadenylated *CAST* transcripts was therefore independent of the amount of input RNA/cDNA analyzed for each sample. This complementary strategy was used as an alternative approach to further demonstrate that transcriptional regulation of the *CAST* polyadenylation variants was not unduly influenced by normalization to the *RPLP0* reference gene.

RESULTS

Kill group

Cattle within the NSW and WA herds were slaughtered at different abattoirs and the cattle within each herd were slaughtered in 2 separate groups 2 to 3 d apart. The impact that a variety of hard-to-control variables such as time off food, transport distance and different preslaughter management and stressors may have had on the expression of the *CAPN1*, *CAPN3* and *CAST* genes are evident between the 4 kill groups (Table 4). Although kill group had a significant influence on the mRNA levels of the calpain and calpastatin genes, the inclusion of this variable in all the statistical models has helped to minimize any confounding of results due to these influences.

CAPN1 *mRNA transcripts*

The *CAPN1–4751* polymorphism which is located in intronic DNA between exons 17 and 18 was associated with differences in *CAPN1* mRNA transcript levels. Brahman cattle with 2 favorable *CAPN1–4751* alleles had higher CAPN1 mRNA levels than cattle with 0 favorable alleles by 9% ($P = 0.03$; Tables 4 and 5). Angus cattle with 1 favorable allele had lower *CAPN1* mRNA levels than Brahman cattle with 1 favorable allele by 9% ($P = 0.03$; Tables 4 and 5). There was no association between *CAPN1* mRNA levels and the CAPN1–316 polymorphism, which is located in exon 9 and is responsible for an amino acid substitution. CAPN1 mRNA levels in Brahman cattle were not influenced by the HGP implants, whereas Angus cattle with a HGP implant had 6% higher CAPN1 mRNA levels than non-implanted Angus cattle ($P = 0.02$; Tables 4 and 7).

"Total" CAST *mRNA transcripts*

A region of the *CAST* mRNA (Fig. 1; exons 27 and 28) that has not been reported to undergo alternative splicing and thus is common to all *CAST* mRNA transcripts was targeted with a qPCR assay so that "total" *CAST* mRNA in skeletal muscle could be quantified. Notably, there was no association between "total" *CAST* mRNA levels and the *CAST:c.2832A > G* SNP, which is located in the 3′UTR. Levels 5% higher of "total" *CAST* mRNA were detected in the LLM of HGP-implanted cattle than in non-implanted cattle ($P = 0.01$; Table 4 and 7).

Table 5. Least squares (LS) means for the Breed × Genotype effects. Relative mRNA levels of genes measured in the LLM of Brahman and Angus cattle that were significantly associated with either the CAPN1–4751 or *CAST:c.2832A > G* DNA marker. The mRNA levels of the *CAPN1*, *CASTIIIex2-ex4*, and *CASTII* genes were normalized against the *RPLP0* reference gene, while the *CASTpA1* and *CASTpA2* mRNA variants were analyzed as a within sample ratio (non-normalized). For *CAPN1*, the LS mean value in Angus cattle with 1 copy of the favorable allele was arbitrarily set to 1.00 with the other LS means within the same row expressed relative to this value. For *CASTIIIex2-ex4*, *CASTII* (replicates 1–3) and *CASTpA1:CASTpA2*, the LS mean value in Brahman cattle with 0 copies of the favorable allele was arbitrarily set to 1.00 with the other LS means within the same row expressed relative to this value

Dependent variable[1]	Genotype	Brahman, 0 copies	Brahman, 1 copy	Brahman, 2 copies	Angus, 1 copy	Angus, 2 copies
CAPN1[2]	*CAPN1–4751*	1.06 ± 0.01^{bc}	1.09 ± 0.02^{ac}	1.15 ± 0.04^{a}	1.00 ± 0.04^{b}	1.09 ± 0.03^{abc}
CASTIIIex2-ex4[2]	*CAST:c.2832A > G*	1.00 ± 0.03^{a}	0.96 ± 0.03^{ab}	0.93 ± 0.02^{b}	.	0.85 ± 0.03^{c}
CASTII (replicate 1)[2]	*CAST:c.2832A > G*	1.00 ± 0.03^{a}	0.83 ± 0.05^{b}	0.75 ± 0.03^{b}	.	0.52 ± 0.04^{c}
CASTII (replicate 2)[2]	*CAST:c.2832A > G*	1.00 ± 0.04^{a}	0.85 ± 0.05^{b}	0.77 ± 0.03^{b}	.	0.52 ± 0.05^{c}
CASTII (replicate 3)[3]	*CAST:c.2832A > G*	1.00 ± 0.03^{a}	0.91 ± 0.04^{ab}	0.86 ± 0.03^{b}	.	0.76 ± 0.04^{c}
CASTpA1:CASTpA2[3]	*CAST:c.2832A > G*	1.00 ± 0.02^{a}	1.00 ± 0.02^{a}	0.91 ± 0.02^{b}	.	0.89 ± 0.02^{b}

[a–c]Within rows, LS mean values without a common superscript differ significantly at $P < 0.05$.

[1]Abbreviated mRNA transcript identifiers for *CALP1* = calpain 1; *RPLP0* = Ribosomal Protein, Large, P0; *CASTII* = type II calpastatin splice variant; *CASTIII (ex2-ex4)* = type III calpastatin splice variant lacking exon 3; *CASTpA1* = calpastatin proximal polyadenylation variant; and *CASTpA2* = calpastatin distal polyadenylation variant.

[2]The initial phase of the experiment screened 152 cattle from NSW and 191 cattle from WA.

[3]The second phase of the experiment screened 213 cattle from NSW and 191 cattle from WA.

CAST *type I, II, and III mRNA 5′ splice variants in heart and skeletal muscle*

The relative abundance of the type I, II, and III *CAST* 5′ splice variants was assessed using 1 heart and 6 skeletal muscle samples (LLM, infraspinatus, semimembranosus, semispinalis, semitendinosus and psoas major) from a Hereford yearling (results not shown). Quantifiable levels of the type I variant could not be accurately measured in heart or skeletal muscle. This was due primarily to the high GC content of the type I exon. We therefore focused the remainder of this study on the type II and type III *CAST* mRNA variants. The type II transcript was detected at approximately 100-fold higher levels in the heart sample than in the 6 skeletal muscles, whereas the type III variant was transcribed at lower levels in heart than in the skeletal muscle samples. Based on published evidence for alternative splicing of exon 3, we developed qPCR assays that could distinguish type III transcripts which either contained exon 3 (*CASTIII-ex3*) or lacked exon 3 (*CASTIII-ex2ex4*). In skeletal muscle, the *CASTIII-ex2ex4* mRNA transcript was found to be the predominant isoform, being approximately 25 times more abundant than the *CASTIII-ex3* mRNA transcript (results not shown). Moreover, the *CAST* type II transcript and the *CASTIII-ex3* transcript were present in the 6 skeletal muscle types at comparable levels.

CAST *type II mRNA transcript*

The mRNA expression of the type II *CAST* variant exhibited a strong association with the *CAST:c.2832A > G* SNP, both within and between breed ($P < 0.0001$; Table 4 and 5). There was 14% higher type II *CAST* mRNA in the LLM of Brahman cattle with 0 favorable alleles compared with Brahman cattle with 2 favorable alleles ($P < 0.0001$; Table 4 and 5). Brahman cattle that had 1 favorable allele of the *CAST:c.2832A > G* SNP had 9% lower type II *CAST* mRNA levels than Brahman cattle with 0 favorable alleles ($P = 0.0017$). Angus cattle with 2 favorable alleles had type II *CAST* mRNA 11% lower than Brahman cattle with 2 favorable alleles ($P < 0.0001$).

CAST *typeIII-ex2-ex4 and type III-ex3 mRNA transcripts*

In the LLM of Angus and Brahman, the *CASTIII-ex2ex4* variant which lacks exon 3 was, again, more abundant than the *CASTIII-ex3* variant which contained exon 3. The *CASTIII-ex2ex4* variant was affected by the HGP implants. Cattle with the HGP implants had 8% higher *CASTIII-ex2ex4* mRNA levels than nonHGP implanted cattle ($P = 0.002$; Table 4 and 7). A similar trend as detected for the *CAST* type II variant and the *CAST:c.2832A > G* SNP was also detected for the *CASTIII-ex2ex4* mRNA transcript ($P = 0.03$). The *CASTIII-ex2ex4* mRNA transcript was higher in the LLM of Brahman cattle with 0 favorable alleles by 7% compared with Brahman cattle with 2 favorable alleles ($P = 0.009$; Table 4 and 5). Brahman cattle with 2 favorable alleles had 7% higher *CASTIII-ex2ex4* mRNA levels than Angus cattle with 2 favorable alleles ($P = 0.035$; Table 4 and 5).

The *CASTIII-ex3* mRNA transcript was not associated with the *CAST*:c.2832A > G SNP. However, there were interactions between breed and sex ($P = 0.004$; Table 4) and breed and HGP implant ($P = 0.02$; Table 4). In Angus cattle there were 47% higher levels of the

Table 6. Least squares (LS) means for the Breed × Genotype × Sex effects. Relative mRNA levels of the *CASTpA1* variant measured in the LLM of Brahman and Angus were normalized against the *RPLP0* reference gene. The LS mean value for *CASTpA1* in Brahman cattle with 0 copies of the favorable *CAST:c.2832A > G* allele was arbitrarily set to 1.00 with the other LS means expressed relative to this value

Dependent variable[1]	Genotype	Breed × Genotype (number of favorable alleles)	Heifer	Steer
CASTpA1[2]	*CAST:c.2832A > G*	Brahman (0)	1.00 ± 0.04^{ab}	1.02 ± 0.02^{a}
		Brahman (1)	0.93 ± 0.1^{abcde}	0.95 ± 0.03^{bcd}
		Brahman (2)	0.90 ± 0.04^{cd}	0.89 ± 0.02^{d}
		Angus (2)	1.00 ± 0.05^{abc}	0.78 ± 0.03^{e}

[a–e]LS mean values without a common superscript differ significantly at $P < 0.05$.

[1]Abbreviated mRNA transcript identifier for *CASTpA1* = calpastatin proximal polyadenylation variant; and *RPLP0* = Ribosomal Protein, Large, P0.

[2]The second phase of the experiment screened 213 cattle from NSW and 191 cattle from WA.

CASTIII-ex3 mRNA in heifers than in steers, whilst in Brahman cattle *CASTIII-ex3* mRNA levels were 16% higher in steers than in heifers (Table 7). In Brahman cattle, *CASTIII-ex3* mRNA levels were not influenced by the HGP implants, but nonHGP implanted Angus cattle had 20% higher *CASTIII-ex3* mRNA levels than Angus cattle with the HGP implants ($P = 0.007$; Table 7).

Alternatively polyadenylated CAST *mRNA transcripts*

The mRNA levels of the *CAST* pA1 transcript were associated with the *CAST*:c.2832A > G SNP (Table 4 and 6) whereas the levels of the *CAST* pA2 transcript did not show any association with the *CAST*:c.2832A > G SNP. In Angus cattle, the mRNA level of the *CAST* pA1 transcript was higher in heifers than steers by 22% ($P = 0.0002$; Table 4 and 6), but there were no differences between Brahman heifers and steers with the same number of favorable alleles. Brahman heifers with 2 favorable *CAST*:c.2832A > G alleles had 10% and 12% less *CAST* pA1 transcript compared with Brahman heifers and steers, respectively, with 0 favorable *CAST*:c.2832A > G alleles (Table 4 and 6). Brahman steers with 2 favorable *CAST*:c.2832A > G alleles had 13%, 11% and 6% less *CAST* pA1 transcript compared with Brahman steers and heifers, respectively, with 0 favorable *CAST*:c.2832A > G alleles, and Brahman steers, respectively, with 1 favorable *CAST*:c.2832A > G allele (Table 4 and 6). The HGP-implanted cattle had 8% higher levels of the pA1-terminated *CAST* transcript ($P < 0.001$) and 9% higher levels of the pA2-terminated *CAST* transcript ($P < 0.001$) than nonHGP implanted cattle (Table 4 and 7).

The within sample ratio of *CAST* mRNA transcripts terminated at the proximal (pA1) polyadenylation site vs. the distal (pA2) polyadenylation site also revealed significant differences associated with the *CAST:c.2832A > G* SNP (Table 4) and sex (Table 4). In relation to sex, there was a 12% higher pA1:pA2 *CAST* transcript ratio in heifers compared with steers ($P < 0.001$; Table 7). The pA1:pA2 *CAST* mRNA transcript ratio was not statistically different between Angus and Brahman cattle with 2 favorable *CAST:c.2832A > G* alleles. However, the ratio of pA1:pA2 *CAST* mRNA transcripts in Brahman cattle with 2 favorable *CAST:c.2832A > G* alleles was 10% lower than Brahman cattle that had 1 favorable allele ($P < 0.01$; Table 5) or had 0 favorable alleles ($P < 0.001$; Table 5).

DISCUSSION

In livestock, postmortem proteolysis in skeletal muscle is one of the key biological processes influencing meat tenderization. The rate and extent of proteolysis of the myofibrillar proteins has a direct bearing on the eating quality of meat, especially in cattle. The importance of the calpain and CAST proteins in skeletal muscle proteolysis is well established, with the activities of these proteins being directly responsible for differences in meat tenderness. It is therefore not surprising that genetic associations have been reported for meat tenderness in cattle carrying polymorphisms in the calpain and calpastatin genes (Barendse, 2002; Page et al., 2002; White et al., 2005; Barendse et al., 2008). The reproducibility of these findings across studies in different continents suggests that the allelic variants in these genes with favorable consequences for meat tenderness may be directly influencing the biological activity of their respective gene. As the causal nature of these mutations is currently unknown, this study sought to assess whether the reported polymorphisms in the calpain and calpastatin genes were associated with alterations in mRNA transcript levels. The demonstration in this study of transcript levels of the *CAPN1* and *CAST* genes showing associations with their respective gene markers provides a possible biological insight into the genetic basis for phenotypic variation in muscle shear force and consumer assessed tenderness in the LLM of Brahman cattle (Cafe et al., 2010b; Robinson et al., 2012).

The objective meat quality assessments reported by Cafe et al. (2010b), on the same Brahman cattle used in the present study, highlight the additive effects of the *CAST* and *CAPN3* markers on meat tenderness in cattle with 2

Table 7. Least squares means for HGP (Hormone Growth Promotant), Sex, Sex*HGP, Sex*Breed, Breed*HGP. Relative mRNA levels measured in the LLM of Brahman and Angus were normalized against the *RPLP0* reference gene

Dependent variable[1]	HGP (No)[2]	HGP (Yes)		
"total" *CAST*[3]	1.00 ± 0.02[b]	1.05 ± 0.02[a]		
CASTpA1[4]	1.00 ± 0.02[b]	1.08 ± 0.02[a]		
CASTpA2[4]	1.00 ± 0.02[b]	1.09 ± 0.02[a]		
CASTIIIex2-ex4[3]	1.00 ± 0.02[b]	1.08 ± 0.02[a]		
Dependent variable[3]	Steer[2]	Heifer		
CASTpA1:CASTpA2[4]	1.00 ± 0.02[b]	1.12 ± 0.03[a]		
Dependent variable[1]	Steer, HGP (Yes)[2]	Steer, HGP (No)	Heifer, HGP (Yes)	Heifer, HGP (No)
CASTII (replicate 1)[3]	1.00 ± 0.05[b]	1.16 ± 0.05[a]	1.21 ± 0.09[a]	1.10 ± 0.09[ab]
CASTII (replicate 3)[4]	1.00 ± 0.04[ab]	1.08 ± 0.04[a]	1.04 ± 0.06[ab]	0.95 ± 0.06[b]
Dependent variable[1]	Heifer, Brahman[2]	Steer, Brahman	Heifer, Angus	Steer, Angus
CASTpA2[4]	1.00 ± 0.03[b]	1.12 ± 0.02[a]	1.06 ± 0.05[ab]	1.01 ± 0.04[b]
CASTIIIex3[3]	1.00 ± 0.04[b]	0.84 ± 0.03[c]	1.41 ± 0.08[a]	0.96 ± 0.05[b]
Dependent variable[1]	Brahman, HGP (No)[2]	Brahman, HGP (Yes)	Angus, HGP (No)	Angus, HGP (Yes)
CALP1[3]	1.00 ± 0.02[b]	1.04 ± 0.02[a]	1.00 ± 0.03[ab]	0.94 ± 0.03[b]
CASTIIIex3[3]	1.00 ± 0.03[c]	1.00 ± 0.03[c]	1.40 ± 0.06[a]	1.17 ± 0.06[b]

[a–c]Within rows, LS mean values without a common superscript differ significantly at $P < 0.05$.

[1]Abbreviated mRNA transcript identifiers for *CALP1* = calpain 1; *CAST* = 'total' calpastatin; *RPLP0* = Ribosomal Protein, Large, P0; *CASTII* = type II calpastatin splice variant; *CASTIII (ex3)* = type III calpastatin splice variant containing exon 3; *CASTIII (ex2-ex4)* = type III calpastatin splice variant lacking exon 3; *CASTpA1* = calpastatin proximal polyadenylation variant; and *CASTpA2* = calpastatin distal polyadenylation variant.

[2]The LS mean value in these columns was arbitrarily set to 1.00 with the other LS means within the same row expressed relative to this value.

[3]The initial phase of the experiment screened 152 cattle from NSW and 191 cattle from WA.

[4]The second phase of the experiment screened 213 cattle from NSW and 191 cattle from WA.

favorable alleles. For cattle with 4 favorable alleles compared with 0 favorable alleles for the *CAST* and *CAPN3* markers, reductions in shear force of the 7 d aged LLM of 9.3 N and 12.2 N were observed respectively for the WA and NSW herds. In the NSW herd there were sufficient cattle with extreme genotypes of the 3 markers to include the *CAPN1–4751* genotypes. A combination of the 2, 2, 1 copies of the beneficial *CAST*, *CAPN3* and *CAPN1–4751* alleles improved beef tenderness by 15.8 N compared with cattle in the same herd that had the 0, 0, 0 allelic combination. Moreover, the additive improvements in meat tenderness in Brahmans were within the threshold levels that consumers could detect (Robinson et al., 2012), indicating that alterations in the combined biological functions of the *CAST*, *CAPN3* and *CAPN1* genes might well be responsible for the detected phenotypic differences in meat eating quality.

The difficulty in sourcing sufficient Brahman cattle with 2 favorable alleles of the *CAPN1–316* and *CAPN1–4751* genotypes, meant that this study was primarily designed to examine the effects of the *CAST* and *CAPN*3 genotypes. The relationship between 0 copies (unfavorable), 1 copy (heterozygous) and 2 copies (favorable) of the *CAST* and *CAPN*3 gene markers and their mRNA transcript levels was assessed in detail. The relationship between the *CAPN1* gene marker and its mRNA transcript was also assessed, but given the limited number of Brahman cattle with 2 favorable alleles, the significance of these findings should not be overstated. However, unlike *CAPN3*, where the allelic status of the *CAPN3:c.1538+225G > T* gene marker clearly did not influence *CAPN*3 mRNA levels, *CAPN1* mRNA levels were associated with the *CAPN1–4751* marker. What is noteworthy from this finding is that of the 2 calpain markers that were assessed, the *CAPN1–4751* marker is located in intronic DNA between exons 17 and 18 while the *CAPN1–316* marker is located in exon 9 and is associated with an amino acid substitution. Therefore, since the *CAPN1–4751* marker is located in a noncoding region of the *CAPN1* gene, the observation of higher *CAPN1* mRNA levels in cattle with more copies of the favorable allele may point to the possibility that the *CAPN1–4751* polymorphism is linked to regulatory sequences in the *CAPN1* gene which have an influence on its mRNA transcription.

In the case of calpain 3, the absence of any transcriptional modulation by 0, 1 or 2 copies of the *CAPN3JK* polymorphism suggests that the amino acid substitution caused by this mutation may alter the action of the calpain 3 protein, which contributes as yet unknown positive effects on meat tenderness. Favorable *CAPN1–316* alleles were noted to improve consumer assessment of meat tenderness of rump but not LLM steaks (Robinson et al., 2012), although Cafe et al. (2010b) reported that Brahman cattle with 1 favorable *CAPN1–316* had lower shear force of 7-d aged LLM samples in NSW and lower compression force of 7-d aged LLM samples in WA.

A comprehensive assessment of the transcriptional activity of the *CAST* gene was undertaken utilizing a range of qPCR assays which measured "total" *CAST* mRNA levels as well as a variety of 5′ splice variants that are transcribed from different promoters. In a subsequent qPCR experi-

ment, alternatively polyadenylated calpastatin transcripts which possess different length 3′UTR were also quantified. The findings of these studies showed that the decreased mRNA levels of the type II and type III (ex2-ex4) *CAST* splice variants and decreased *CAST* mRNA transcripts terminated at the proximal (pA1) polyadenylation site were significantly associated with the favorable *CAST:c.2832A > G* SNP (Barendse, 2002), which is located in the 3′UTR of the *CAST* gene. Taken together, these results support the notion that the *CAST:c.2832A > G* polymorphism may be in linkage disequilibrium with regulatory sequences which have a role in the post-transcriptional regulation of *CAST* mRNA transcripts, leading to reduced levels of calpstatin protein in the LLM of cattle carrying the favorable allele. Alternatively, the *CAST:c.2832A > G* polymorphism may be associated with long-range regulators, either an enhancer, silencer or insulator, within upstream introns that directly interact with the type II and type III promoters (Dean, 2011).

The bovine *CAST* gene spans ~135 kb of genomic DNA and has 4 promoters (Raynaud et al., 2005a). Three of the promoters generate transcripts (type I, II, and III) with a coding sequence that differs almost exclusively at the first exon. The fourth promoter synthesizes a truncated protein which has a translation start site in exon 14t, and is expressed in a tissue-specific manner, mainly in the testis. The distinct N-terminal protein sequences of the type I, II, and III *CAST* variants are thought to play a pivotal role in conferring unique biological properties, since the remainder of the *CAST* protein coding sequence is almost identical between the 3 splice variants. The abundance of the type I, II, III and IV CAST proteins differs significantly between bovine heart, diaphragm, skeletal muscle and testis (Raynaud et al., 2005b), reflecting the likely functional importance of the different N-terminal portions of *CAST* in these tissues. In the current study we have examined the type II and type III transcripts in 6 different bovine skeletal muscle types and in heart and determined marked differences in the relative abundance of mRNA levels of the *CAST* 5′ splice variants between skeletal and cardiac muscle. In addition, we also followed-up on previous findings that indicated alternative splicing of exon 3 (Geesink et al., 1998), and were able to show that the predominant *CAST* transcript in bovine skeletal muscle is a type III variant that lacks exon 3. These findings emphasize the complexity associated with the transcriptional regulation of the *CAST* gene, and given that additional splice variants have also been described in exons 4, 5, 6, 8, and 12 (Takano et al., 1999; Goll et al., 2003; De Tullio et al., 2007), it is likely that other potential exon combinations exist within the type III variant. These as-yet identified *CAST* isoforms may account for the absence of a genetic association between "total" *CAST* mRNA levels and the *CAST:c.2832A > G* marker, as there may be additional isoforms present in skeletal muscle like the *CASTIII-ex3* variant which had a contrasting transcriptional profile to the *CAST* type II and *CASTIII-ex2ex4* mRNA variants.

Another intriguing feature of the bovine *CAST* gene is that it produces 3 polyadenylation variants (Raynaud et al., 2005b) while 2 polyadenylation variants have been reported in the porcine ortholog (Parr et al., 2001). The presence of multiple polyadenylation signals in the 3′UTR of the *CAST* gene is significant because these signals truncate the mRNA transcript to varying extents. In the case of the bovine *CAST* gene, termination of the mRNA transcript at the proximal pA1 site as opposed to the distal pA2 site removes around 1,100 bp of 3′UTR sequence. By shortening the length of the 3′UTR, the availability of motifs for RNA binding proteins and microRNAs is reduced and this has the potential to lead to alterations in the level of protein translation (Di Giammartino et al., 2011). In general, shorter 3′UTR are considered to be more stable than longer 3′UTR hence they have a greater half-life and typically produce more protein (Mayr and Bartel, 2009; Hogg and Goff, 2010). The overall importance of alternative polyadenylation in regulating gene expression was largely overlooked until recently when more than 50% of genes were shown to utilize multiple polyadenylation sites and notably, genome-wide changes in the length of the 3′UTR were tightly associated with cellular proliferation and differentiation (Sandberg et al., 2008; Ji and Tian, 2009). Moreover, patterns of alternative splicing and polyadenylation appear to be coordinately regulated across tissues, suggesting that promoters may play a role in RNA processing (Wang et al., 2008; Mapendano et al., 2010).

In the context of linkage disequilibrium, the significant association between the *CAST:c.2832A > G* marker and the *CAST* type II and *CASTIII-ex2ex4* mRNA transcripts was difficult to rationalize, given that in cattle the average r^2 for SNP loci separated by 100 kb is only 0.15–0.2 (McKay et al., 2007). Since the *CAST:c.2832A > G* marker is located 134 kb and 86 kb, respectively, from the promoters that control the transcription of the type II and type III *CAST* variants, it seemed unlikely that linkage disequilibrium would extend over this distance or that linked polymorphisms would be having similar long-range regulatory effects on 2 distinct promoters. By drawing on evidence from Winter et al. (2007) for a link between promoter selection and termination of transcription, it was hypothesized that the *CAST* type II and *CASTIII-ex2ex4* transcript variants may preferentially utilise the proximal polyadenylation site over the distal polyadenylation site. Winter et al. (2007) showed that mRNA termination of the X-linked ***MID1*** gene at different polyadenylation sites is predetermined by the choice of promoter that the *MID1* gene uses to initiate transcription. Given the proximity of the *CAST:c.2832A > G* polymorphism to the pA1 polyadenylation site, it seemed reasonable to speculate that *CAST:c.2832A > G* or a linked

SNP in the vicinity was either influencing the usage of the pA1 polyadenylation site or affecting the mRNA stability of transcripts terminated at the pA1 polyadenylation site. After devising a qPCR-based strategy that enabled direct measurement of *CAST* transcripts terminated at the pA1 and pA2 sites, we confirmed that cattle with the favorable *CAST* allele had lower levels of pA1-terminated *CAST* mRNA transcripts than cattle with the unfavorable allele, while levels of the pA2-terminated *CAST* mRNA transcript were not influenced by the *CAST:c.2832A > G* marker.

The expression level of the calpastatin protein in the LLM samples from the same group of cattle as used in the current study has been assessed (M. B. McDonagh, NSW Department of Primary Industries, unpublished data). In this work, cattle with 2 copies of the favorable *CAST:c.2832A > G* allele had significantly lower levels of the CAST protein than cattle with 2 copies of the unfavorable *CAST:c.2832A > G* allele. The similarities between the findings for CAST mRNA levels and protein expression in LLM of these cattle, leads us to speculate that the *CAST* marker or other regulatory sequences in linkage disequilibrium with the *CAST* maker are associated with a post-transcriptional regulatory mechanism which reduces the number of pA1-terminated *CAST* transcripts in the LLM, leading to lower levels of CAST protein expression. As "total" *CAST* mRNA and pA2-terminated *CAST* mRNA levels were not associated with the *CAST* marker, but the *CAST* type II, *CASTIIIex2ex4* and *CASTpA1* mRNA transcripts were associated with the *CAST* marker, we speculate that a post-transcriptional regulatory mechanism involving a truncated 3′UTR in some but not all *CAST* mRNA transcripts may be linked to the observed reduction in "total" calpastatin protein levels. In general, a consequence of alternative polyadenylation is that transcripts which possess shorter 3′UTR express higher amounts of protein than mRNA transcripts from the same gene which have longer 3′UTR. Therefore, given that CAST pA1- and pA2-terminated mRNA transcripts were generally present in the LLM in similar amounts, the 10 to 13% decline in the level of pA1-terminated *CAST* mRNA transcripts between Brahman with 2 favorable and Brahman with 0 favorable alleles may actually account for a significant reduction in CAST protein expression in the LLM since the pA1-terminated transcripts are likely to be responsible for synthesizing a greater fraction of the CAST protein in the LLM than the pA2-terminated *CAST* mRNA transcripts.

As we have no direct evidence to link the reduction in pA1-terminated transcripts to lower CAST protein expression in the LLM we cannot rule out other potential regulatory mechanisms which may be influencing the activity of the *CAST* type II and type III promoters, such as long-range regulatory elements in the intronic regions upstream of the 3′UTR which either enhance or suppress transcription (Dean, 2011). Alternatively, the *CAST:c.2832A > G* polymorphism or a linked flanking polymorphism may introduce or ablate a microRNA binding site which lies within the vicinity of the pA1 polyadenylation site, although no evidence for this possibility was found when putative microRNA binding sites were searched for within the *CAST* 3′UTR. Therefore, follow-up studies to this work are required which comprehensively characterize the pattern of linkage disequilibrium associated with the *CAST:c.2832A > G* polymorphism in surrounding regions of the bovine calpastatin gene, and in vitro studies are also required to assess whether the *CAST:c.2832A > G* polymorphism or other linked polymorphisms in the 3′UTR have a direct role in the post-transcriptional regulation of the bovine calpastatin gene.

Conclusions

The transcriptional differences detected in this study for the *CAPN1* and *CAST* genes are associated with SNP that have been shown to account for a significant proportion of phenotypic variation in meat tenderness and eating quality in beef cattle (Cafe et al., 2010b; Robinson et al., 2012). These findings suggest that the *CAPN1_4751* and *CAST:c.2832A > G* polymorphisms are linked to regulatory sequences in the bovine genome which are exerting biological effects either directly or indirectly on their respective genes, leading to alterations in the level and type of mRNA transcripts that are transcribed, which ultimately influences the translation of the CAPN1 and CAST proteins in the LLM. A comprehensive assessment of known 5′ splice and polyadenylation variants of the *CAST* gene has uncovered a post-transcriptional regulatory mechanism which potentially involves the combined regulatory effects of different promoters and variable length 3′UTR. Furthermore, the proximity of the *CAST:c.2832A > G* SNP to the distal (pA1) polyadenylation site in the *CAST* gene and the association between this polymorphism and levels of the pA1-terminated *CAST* mRNA transcripts in the LLM, leads us to speculate that *CAST:c.2832A > G* may be linked to regulatory sequences which are imparting post-transcriptional regulatory control over calpastatin protein levels in the LLM via a mechanism that either reduces the levels or stability of pA1-terminated *CAST* mRNA transcripts.

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