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
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 CAPN1 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 CAPN3, CAST and CAPN1 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 CAPN1, CAPN3 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 K2EDTA (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 Brigliolo 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.

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 mid-morning. 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.

### 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 synthesized 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

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Oligonucleotide sequence (5'–3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CALP1cdna</td>
<td>CATCCGCATTTCATAG</td>
</tr>
<tr>
<td>CALP3cdna</td>
<td>TGGCATCGTCTGGAAA</td>
</tr>
<tr>
<td>CASTcdna</td>
<td>TCGGCTTGGCCTGGG</td>
</tr>
<tr>
<td>RPLP0cdna</td>
<td>ATGGACACCCAAAGAA</td>
</tr>
<tr>
<td>CASTIIcdna</td>
<td>GCGGAGGGGCGATGG</td>
</tr>
<tr>
<td>CASTIIIdna</td>
<td>TCTGGTACACCGACCTT</td>
</tr>
<tr>
<td>CASTIII ex5–6cdna</td>
<td>TGGGCTCTGTTGTCCTTTTG</td>
</tr>
<tr>
<td>OligodT-VN</td>
<td>TTTTTTTTTTTTTTTTTTTTTTTTTTTTT</td>
</tr>
<tr>
<td>Adapter-oligodT2</td>
<td>GACATCGTACCTGTATCATGCACTTT</td>
</tr>
</tbody>
</table>

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.
transcripts, the same reverse transcription reagents and procedure was applied but the reactions were primed with an oligo(dT) 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, 5 μl of 1:20 diluted cDNA was combined with 30 μl 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 **CAPN1**, **CAPN3** 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.
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 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 RPLP0 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, CALP3 and CALP1 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:c.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
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 is 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).

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

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>K</th>
<th>B</th>
<th>S</th>
<th>H</th>
<th>CAPN1(B)</th>
<th>CAST(B)</th>
<th>S*H</th>
<th>B*S</th>
<th>B*H</th>
<th>K*B</th>
<th>K*H</th>
<th>CAST(B)*S</th>
</tr>
</thead>
<tbody>
<tr>
<td>CALP1²</td>
<td>&lt; 0.0001</td>
<td>0.06</td>
<td>.</td>
<td>0.68</td>
<td>0.0018</td>
<td>.</td>
<td>.</td>
<td>0.018</td>
<td>.</td>
<td>.</td>
<td>&lt; 0.0001</td>
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<tr>
<td>CALP3³</td>
<td>0.03</td>
<td>0.21</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>&lt; 0.0001</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>“total” CAST³</td>
<td>0.004</td>
<td>0.02</td>
<td>.</td>
<td>0.01</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>0.005</td>
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<tr>
<td>CASTIIex2-ex4⁴</td>
<td>&lt; 0.0001</td>
<td>0.0006</td>
<td>.</td>
<td>0.002</td>
<td>.</td>
<td>0.03</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>0.002</td>
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<tr>
<td>CASTIIex3³</td>
<td>0.01</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
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<td>.</td>
<td>.</td>
<td>.</td>
<td>0.004</td>
<td>0.02</td>
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<tr>
<td>CASTII (replicate 1)³</td>
<td>0.008</td>
<td>&lt; 0.0001</td>
<td>0.41</td>
<td>0.71</td>
<td>.</td>
<td>&lt; 0.0001</td>
<td>0.03</td>
<td>.</td>
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<td>CASTII (replicate 2)³</td>
<td>0.006</td>
<td>&lt; 0.0001</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>&lt; 0.0001</td>
<td>.</td>
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<td>.</td>
</tr>
<tr>
<td>CASTII (replicate 3)³</td>
<td>&lt; 0.0001</td>
<td>0.0002</td>
<td>0.34</td>
<td>0.96</td>
<td>.</td>
<td>0.0018</td>
<td>0.043</td>
<td>.</td>
<td>.</td>
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<tr>
<td>CASTpA1⁴</td>
<td>&lt; 0.0001</td>
<td>0.1</td>
<td>0.21</td>
<td>0.0023</td>
<td>.</td>
<td>0.0007</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>0.006</td>
<td>.</td>
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<tr>
<td>CASTpA2⁴</td>
<td>0.0009</td>
<td>0.87</td>
<td>0.47</td>
<td>0.0002</td>
<td>.</td>
<td>.</td>
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<td>.</td>
<td>0.016</td>
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<tr>
<td>CASTpA1:CASTpA2⁴</td>
<td>&lt; 0.0001</td>
<td>0.0025</td>
<td>&lt; 0.0001</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>0.0002</td>
<td>.</td>
<td>.</td>
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<td>.</td>
</tr>
</tbody>
</table>

¹K = 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*Sex = Breed*Sex, B*H = Breed*HGP, K*B = Killgroup*Breed, K*H = Killgroup*HGP, CAST(B)*S = CAST(B)*Sex.

²Abbreviated 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.

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

⁴The second phase of the experiment screened 213 cattle from NSW and 191 cattle from WA.
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, CASTIII-ex2-ex4, and CASTII genes were normalized against the RPLP0 reference gene, while the CASTpA1 and CASTpA2 mRNA variants were analyzed as within a 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 CASTIII-ex2-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.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Genotype</th>
<th>Brahman, 0 copies</th>
<th>Brahman, 1 copy</th>
<th>Brahman, 2 copies</th>
<th>Angus, 1 copy</th>
<th>Angus, 2 copies</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASTP1</td>
<td>CAPN1–4751</td>
<td>1.06 ± 0.01abc</td>
<td>1.09 ± 0.02abc</td>
<td>1.15 ± 0.04a</td>
<td>1.00 ± 0.04b</td>
<td>1.09 ± 0.03abc</td>
</tr>
<tr>
<td>CASTIII-ex2-ex4</td>
<td>CAST:c.2832A &gt; G</td>
<td>1.00 ± 0.03a</td>
<td>0.96 ± 0.03ab</td>
<td>0.93 ± 0.02b</td>
<td>0.85 ± 0.03c</td>
<td>0.52 ± 0.04c</td>
</tr>
<tr>
<td>CASTII (replicate 1)</td>
<td>CAST:c.2832A &gt; G</td>
<td>1.00 ± 0.03a</td>
<td>0.83 ± 0.05b</td>
<td>0.75 ± 0.03b</td>
<td>0.72 ± 0.03b</td>
<td>0.52 ± 0.05c</td>
</tr>
<tr>
<td>CASTII (replicate 2)</td>
<td>CAST:c.2832A &gt; G</td>
<td>1.00 ± 0.04a</td>
<td>0.85 ± 0.05b</td>
<td>0.77 ± 0.03b</td>
<td>0.72 ± 0.03b</td>
<td>0.76 ± 0.04c</td>
</tr>
<tr>
<td>CASTII (replicate 3)</td>
<td>CAST:c.2832A &gt; G</td>
<td>1.00 ± 0.03a</td>
<td>0.91 ± 0.04ab</td>
<td>0.86 ± 0.03b</td>
<td>0.86 ± 0.03b</td>
<td>0.76 ± 0.04c</td>
</tr>
<tr>
<td>CASTpA1:CASTpA2</td>
<td>CAST:c.2832A &gt; G</td>
<td>1.00 ± 0.02a</td>
<td>1.00 ± 0.02a</td>
<td>0.91 ± 0.02b</td>
<td>0.89 ± 0.02b</td>
<td>0.89 ± 0.02b</td>
</tr>
</tbody>
</table>

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 (CASTII-ex3) or lacked exon 3 (CASTII-ex2ex4). In skeletal muscle, the CASTII-ex2ex4 mRNA transcript was found to be the predominant isoform, being approximately 25 times more abundant than the CASTII-ex3 mRNA transcript (results not shown). Moreover, the CAST type II transcript and the CASTII-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-ex2-ex4 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
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 tenderness. 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

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**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.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Genotype</th>
<th>Breed × Genotype (number of favorable alleles)</th>
<th>Heifer</th>
<th>Steer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASTpA12</td>
<td>CAST:c.2832A &gt; G</td>
<td>Brahman (0)</td>
<td>1.00 ± 0.04&lt;sup&gt;a/b&lt;/sup&gt;</td>
<td>1.02 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brahman (1)</td>
<td>0.93 ± 0.1&lt;sup&gt;b/c/d/e&lt;/sup&gt;</td>
<td>0.95 ± 0.03&lt;sup&gt;b/d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brahman (2)</td>
<td>0.90 ± 0.04&lt;sup&gt;c/d&lt;/sup&gt;</td>
<td>0.89 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Angus (2)</td>
<td>1.00 ± 0.05&lt;sup&gt;a/b/c&lt;/sup&gt;</td>
<td>0.78 ± 0.03&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a–e</sup>LS mean values without a common superscript differ significantly at P < 0.05.

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

2The 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 \textit{CAST} and \textit{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 \textit{CAPN1–4751} genotypes. A combination of the 2, 2, 1 copies of the beneficial \textit{CAST}, \textit{CAPN3} and \textit{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 \textit{CAST}, \textit{CAPN3} and \textit{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 \textit{CAPN1–316} and \textit{CAPN1–4751} genotypes, meant that this study was primarily designed to examine the effects of the \textit{CAST} and \textit{CAPN3} genotypes. The relationship between 0 copies (unfavorable), 1 copy (heterozygous) and 2 copies (favorable) of the \textit{CAST} and \textit{CAPN3} gene markers and their mRNA transcript levels was assessed in detail. The relationship between the \textit{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 \textit{CAPN3}, where the allelic status of the \textit{CAPN3:c.1538+225G > T} gene marker clearly did not influence \textit{CAPN3} mRNA levels, \textit{CAPNI} mRNA levels were associated with the \textit{CAPN1–4751} marker. What is noteworthy from this finding is that of the 2 calpain markers that were assessed, the \textit{CAPN1–4751} marker is located in intronic DNA between exons 17 and 18 while \textit{CAST} is located in intronic DNA between exons 17 and 18 while \textit{CAST} is located in intronic DNA between exons 17 and 18 while \textit{CASTII} (ex3) = type III calpastatin splice variant containing exon 3; \textit{CASTIII (ex2-ex4)} = type III calpastatin splice variant lacking exon 3; \textit{CASTpA1} = calpastatin proximal polyadenylation variant; and \textit{CASTpA2} = calpastatin distal polyadenylation variant.

### 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 \textit{RPLP0} reference gene

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>HGP (No)</th>
<th>HGP (Yes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>“total” \textit{CAST}\textsuperscript{3}</td>
<td>1.00 ± 0.02\textsuperscript{b}</td>
<td>1.05 ± 0.02\textsuperscript{a}</td>
</tr>
<tr>
<td>\textit{CASTpA1}\textsuperscript{4}</td>
<td>1.00 ± 0.02\textsuperscript{b}</td>
<td>1.08 ± 0.02\textsuperscript{a}</td>
</tr>
<tr>
<td>\textit{CASTpA2}\textsuperscript{4}</td>
<td>1.00 ± 0.02\textsuperscript{b}</td>
<td>1.09 ± 0.02\textsuperscript{a}</td>
</tr>
<tr>
<td>\textit{CASTIII (ex2-ex4)}\textsuperscript{3}</td>
<td>1.00 ± 0.02\textsuperscript{b}</td>
<td>1.08 ± 0.02\textsuperscript{a}</td>
</tr>
<tr>
<td>Dependent variable \textsuperscript{1}</td>
<td>Steer\textsuperscript{2}</td>
<td>Heifer</td>
</tr>
<tr>
<td>\textit{CASTpA1:CASTpA2}\textsuperscript{4}</td>
<td>1.00 ± 0.02\textsuperscript{b}</td>
<td>1.12 ± 0.03\textsuperscript{a}</td>
</tr>
<tr>
<td>Dependent variable \textsuperscript{1}</td>
<td>Brahman, HGP (No)\textsuperscript{2}</td>
<td>Angus, HGP (Yes)</td>
</tr>
<tr>
<td>\textit{CALP1}\textsuperscript{3}</td>
<td>1.00 ± 0.02\textsuperscript{b}</td>
<td>1.04 ± 0.02\textsuperscript{a}</td>
</tr>
<tr>
<td>\textit{CASTIII (ex3)}\textsuperscript{3}</td>
<td>1.00 ± 0.03\textsuperscript{c}</td>
<td>1.00 ± 0.03\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Within rows, LS mean values without a common superscript differ significantly at \( P < 0.05 \).

\textsuperscript{1}Abbreviated mRNA transcript identifiers for \textit{CALP1} = calpain 1; \textit{CAST} = “total” calpastatin; \textit{RPLP0} = Ribosomal Protein, Large, P0; \textit{CASTII} = type II calpastatin splice variant; \textit{CASTIII (ex3)} = type III calpastatin splice variant containing exon 3; \textit{CASTIII (ex2-ex4)} = type III calpastatin splice variant lacking exon 3; \textit{CASTpA1} = calpastatin proximal polyadenylation variant; and \textit{CASTpA2} = calpastatin distal polyadenylation variant.

\textsuperscript{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.

\textsuperscript{3}The initial phase of the experiment screened 152 cattle from NSW and 191 cattle from WA.

\textsuperscript{4}The second phase of the experiment screened 213 cattle from NSW and 191 cattle from WA.
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 calpastatin 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. 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 r² 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 MIDI gene at different polyadenylation sites is predetermined by the choice of promoter that the MIDI 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 pastatin protein levels in the LLM via a mechanism that 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.

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.

LITERATURE CITED

Post-transcriptional regulation of calpastatin


