Research Article

Association of *CAPN1* and *CAST* gene polymorphisms with meat tenderness in *Bos taurus* beef cattle from Argentina

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Abstract

The activity of the calpains/calpastatin proteolytic system is closely related to the postmortem tenderization of meat. We investigated the association between beef tenderness and single nucleotide polymorphism (SNP) markers on the *CAPN1* gene (SNP316, alleles C/G; SNP530 alleles A/G) and the *CAST* gene 3' untranslated region (SNP2870, alleles A/G). We sampled nine slaughter groups comprising 313 steers which had been reared in beef production systems in Argentina between 2002 and 2004 from crosses between Angus, Hereford and Limousin cattle. Minor allele frequencies for the markers were 0.27 to 0.46 (C), 0.02 to 0.18 (A), and 0.24 to 0.53 (A), respectively. The presence of *CAPN1* markers had significant effects on meat shear force but no detectable effects were demonstrated for the *CAST* marker. The shear force of meat from steers with the SNP316 CC genotype was 11% lower than for the SNP316 CG genotype and 17% lower than for the SNP316 GG genotype. There were very few steers with the SNP530 AA genotype and, contrary to previous studies, meat from steers with the SNP530 GG genotype showed an 11.5% higher shear force than that from steers with the SNP530 GA genotype. Final body weight, carcass weight and rib eye area were not affected by any of the markers. These results support the concept that *CAPN1* variants are associated with tenderness across a wide range of beef production systems.

Key words: beef cattle, *CAPN1*, *CAST*, genetic markers, meat quality.

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Introduction

Several genetic markers associated with differences in beef tenderness have been reported in the last few years. These markers target two genes corresponding to the most important proteolytic system of skeletal muscle, the *calcium-activated neutral protease* gene (*CAPN1*) encoding the large subunit of μ-calpain and the *calpastatin* gene (*CAST*) encoding a specific inhibitor of the calpains (Page *et al.*, 2002; White *et al.*, 2005; Schenkel *et al.*, 2006). Genetic marker technology is a promising tool for genetic improvement in livestock. However, single genes are not free from potential interactions with environmental factors that could modulate their effect on a given trait of economic importance, as happens when breeds and even individual animals within breeds are compared. For this reason, new markers should be validated in each production system before widespread use by the industry. This is especially important in the case of beef quality traits, because the properties of beef are strongly affected by nongenetic factors such as animal nutrition, slaughter age and weight, animal handling and carcass manipulation, among others.

The beef industry in Argentina has some unique features that clearly distinguish it from other countries. Consumers have a strong preference for beef cuts from low-weight, young animals, because they associate these characteristics with a higher probability of the beef being more tender. As a consequence, the local market discriminates against the heaviest categories of finished steers. Also, car-

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casses are sold soon after slaughter so there is little time left for beef aging, a process that favors beef tenderization.

Most studies reporting significant effects of either *CAPN1* or *CAST* alleles on beef tenderness have been based on the evaluation of aged beef from typical feedlot steers, with the exception of results from New Zealand reported by Morris *et al.* (2006). Both the animal category and the management of beef after slaughter described in most of these studies are not common to the beef industry in Argentina. Therefore, the objective of this work was to confirm the association of markers in the *CAPN1* and *CAST* genes with beef tenderness, in cattle from typical beef production systems of Argentina. A secondary objective was to produce an appraisal of allele frequencies in a local population.

Materials and Methods

Animal resources and phenotypic information

In this study we used a cattle population that has been involved in various studies to evaluate supplementation strategies and/or crossbreeding systems at the Experimental Station of the National Institute of Agricultural Technology (Instituto Nacional de Tecnología Agropecuaria (INTA), Balcarce, Argentina). All the animals received a grass-based diet that was combined with different supplements (grains, hays and silages) to modify growth rate and length of the fattening period. We sampled nine slaughter groups containing a total of 313 steers which were 15 to 18 months old and had been reared between 2002 and 2004 (Table 1). The decision to sample this experimental population instead of other commercial cattle populations was based on the availability of reliable phenotypic information in terms of management and the genetic background of the animals. The steers belonged to different genetic groups and included purebred *Bos taurus* Angus and Hereford steers from the experimental herds maintained at the Balcarce Station, with other steers coming from a rotational crossbreeding scheme involving the same breeds. Females from the original crossbred population were split in two

groups that were recurrently mated to either Angus or Hereford sires. At the time this study was conducted, the proportion of either Angus or Hereford genes in each group was 80% or above. There were also F_1 Angus-Hereford/Hereford Angus crossbreds and reciprocal backcrosses and a group of steers produced by mating Limousin (*B. taurus*) sires to Angus-Hereford crossbred cows (LX). All the animals came from the herds maintained at INTA with the exception of Limousin sires, which were bought at private ranches. In order to allow an approximate estimation of allele frequencies, the steers were grouped in the following way: steers that were at least 75% Angus (AX), steers that were at least 75% Hereford (HX), Angus-Hereford (AH) steers and Limousin (LX) crossbred steers. The genetic structure of the sample is detailed in Table 1.

Each contemporary group was slaughtered at a private abattoir when at least 50% of the steers in a given group had a backfat thickness (BFT) of 5 mm between the 12 and 13th ribs, estimated by ultrasound measurements. Final live body weight (BW) and rib eye area (REA, estimated by ultrasound) were recorded before slaughter. At the time of slaughter, mean live weight was 332 ± 33 kg and backfat thickness was 5.2 ± 1.5 mm as averaged across slaughter groups. Carcasses were weighed and the hot carcass weight (HCW) recorded and then placed in a chiller for 24 h at 5 °C without previous electrical stimulation, after which a block of steaks corresponding to the 11, 12 and 13th ribs was removed from each cold carcass and kept at -20 °C until they were thawed for study. Analytical determinations were performed at the University of Buenos Aires Meat Laboratory. Beef tenderness was estimated indirectly as Warner-Bratzler shear force (WBSF) measured on samples taken from the *Longissimus lumborum* muscle. Steaks (2.5cm-thick) were thawed overnight at room temperature for 24 h and external fat, peripheral connective tissue and other muscles were removed from each steak and samples placed in plastic bags which were then immersed in a water bath at 70 °C for 50 min. The cooked steaks were cooled under running tap water for 40 min, the bags drained and

Table 1 - Slaughter groups, showing number of steers (n), final body weight (BW), hot carcass weight (HCW), rib eye area (REA), backfat thickness (BFT) and Warner-Bratzler Shear Force (WBSF) for each genetic group (AX, HX, HA, LX). Values represent mean ± standard deviation (SD).

Slaughter group	n	Genetic group*	BW (kg)	HCW (kg)	REA (cm^2)	BFT (mm)	WBSF (kg)
1	25	AX	340 ± 30	184 ± 21	48.1 ± 4.0	6.1 ± 1.5	8.05 ± 1.38
2	25		343 ± 20	187 ± 11	46.3 ± 5.2	6.3 ± 1.6	9.32 ± 1.96
3	27	AX, HX, HA, LX	331 ± 47	170 ± 25	46.8 ± 7.5	5.3 ± 1.1	10.52 ± 3.98
4	27		328 ± 34	168 ± 19	44.0 ± 6.2	5.0 ± 1.3	10.58 ± 3.09
5	25		320 ± 39	168 ± 21	43.2 ± 5.7	4.6 ± 1.0	11.13 ± 3.66
6	37	AX	346 ± 24	185 ± 18	44.5 ± 5.3	5.0 ± 1.3	8.14 ± 1.72
7	78	AX, HX, LX, HA	328 ± 35	170 ± 21	44.7 ± 7.4	4.4 ± 1.1	7.89 ± 2.26
8	31	HX	328 ± 32	175 ± 20	44.5 ± 4.6	4.4 ± 1.1	11.92 ± 3.05
9	38	AX	333 ± 21	182 ± 13	47.5 ± 4.2	6.5 ± 1.8	8.82 ± 2.88

*AX: ≥ 75% Angus - ≤25% Hereford; AH: 50% Angus - 50% Hereford; HX: ≥75% Hereford - ≤25% Angus; LX: Limousin x Hereford-Angus.

the cuts gently blotted dry with a paper towel. Five 2.5 cm-diameter cores were removed from each steak parallel to the muscle fibers and sheared at their mid-point using a 50 kg compression load cell and a Warner-Bratzler V-notch blade mounted on an Instron model 4442 testing machine (Canton, MA, USA) at a crosshead speed of 50 mm min^{-1}. A single peak-shear force measurement was obtained for each core and these results were averaged to obtain a single WBSF value for each sample.

Markers and genotyping

For each sample, DNA was isolated from a 160-mg sample removed from the frozen steaks following the standard procedures of Maniatis *et al.* (1982), except for the use of a modified cell lysis buffer (50 mM Tris-HCl, 25 mM EDTA pH 7.5, N-Laurylsarcosine 2% (w/v), 0.05 M DL-dithiothreitol). After phenol/chloroform extraction and ethanol precipitation DNA was resuspended in 10 mM Tris HCl.

We used two previously described markers on the *CAPN1* gene (Page *et al.*, 2002) which had been named according to the position of the amino acid substitutions produced by each single nucleotide polymorphism (SNP) as SNP316 (alleles C/G) on *CAPN1* exon 9 and SNP530 (alleles A/G) on *CAPN1* exon 14. Previous research (Page *et al.*, 2002) has shown that alleles C and G have a more favorable effect on beef tenderness over the alternative G and A alleles.

The polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method was designed to distinguish SNP alleles both on the *CAPN1* and *CAST* genes. Primers for PCR amplification were designed based on reported bovine *CAPN1* sequences (GenBank AF252504 and AF248054). For SNP316 a 709 bp fragment was amplified with the primers CCAGGGCCAGATGG TGAA (forward) and CGTCGGGTGTCAGGTTGC (reverse) and digested with the *Btg*I restriction enzyme, while for SNP530 a 787 bp fragment was amplified with the primers AGCGCAGGGACCCAGTGA (forward) and TC CCCTGCCAGTTGTCTGAAG (reverse) and digested with the *Ava*II restriction enzyme. The PCR amplification was performed in a total volume of 25 μL containing 50 pmol of each primer, 1.5 mM MgCl$_2$, 200 μM of each dNTP (Promega Corporation, Madison, WI), 1X PCR-buffer, 2.5 units of *Taq* DNA Polymerase (Invitrogen Life technologies, Brazil) and 120 ng of DNA. The following PCR program was used: 35 cycles of 45 s at 95 °C, 45 s at the annealing temperature and 45 s at 72 °C, with a first step at 95 °C for 5 min and a final extension for 5 min at 72 °C. Annealing temperatures were 62.5 °C for SNP316 and 64 °C for SNP530. After amplification, 16 ng of the PCR product of each SNP was digested with 10 units of the corresponding restriction enzyme (New England Biolabs, Beverly, MA) for 4 h at the temperature suggested for each enzyme by the manufacturer. The digestion product was then electrophoresed in 1.5% agarose gels at 60V for 1 h 15 min and photographed under UV light.

A bioinformatic approach was followed to develop a new marker on the *CAST* gene. We downloaded 97 Expressed Sequence Tags (EST) corresponding to the bovine *CAST* gene from GenBank and aligned them with the CAP3 software (Huang and Madan, 1999). The resulting cluster derived from several overlapping and highly similar sequences (contig) was searched for polymorphisms with the AutoSNiP software (Barker *et al.*, 2003) at the Plant Genetics and Genomics web site, Victorian AgriBiosciences Centre, Australia. A conservative criterion was applied because ESTs are usually low-quality sequences and there are many sequencing errors that could be wrongly considered real polymorphisms. In order to declare an SNP, both alternative alleles should appear at least twice in the same position of the contig and this minimum frequency was increased together with the number of sequences in the contig (Barker *et al.*, 2003). Only three polymorphisms in the 3' untranslated region (UTR) of the gene fulfilled this requirement, with the polymorphism considered most reliable based on the number of ESTs with the alternative alleles of the putative SNP being chosen for our study. This polymorphism was named SNP2870 according to its position on the EST contig. Based on the reported bovine *CAST* sequence (GenBank AF159246), a 275 bp fragment encompassing SNP2870 was amplified with the primers AAT TTTTAAAAATTGCCTTCAGTTGGGAG (forward) and AAGAAACATCAAACACAGTCCACAAGTCTA (reverse). The PCR product was digested with the *Tsp*509I restriction enzyme that distinguishes the alternative alleles (A/G). The PCR-RFLP amplification, digestion and electrophoresis proceeded as described above, except that the annealing temperature was 62 °C.

Statistical analyses

Allele frequencies were compared by chi-square analysis. The BW, HCW, REA and WBSF data were analyzed using a model that included the genetic marker and slaughter group fixed effects and the genetic marker x slaughter group interaction. For the analysis of WBSF, percentage lipid content in meat (overall mean = 2.71 ± 1.4%) was initially included as a covariate but it had no significant effect so it was removed from the final model. Pedigree information was available for only a few of the slaughter groups so it was not included in the analyses.

Previous trials showed a slight but significant effect of the number of days that the steaks remained frozen (range: 40 to 202 days, overall mean = 113 ± 57 days) on WBSF (-0.0145 ± 0.003 kg/day). Therefore, this variable was included as a covariate in the model. All statistical analyses were carried out with the SAS program version 8.1 (SAS, 1998).

Because the genetic background was not uniform across the slaughter groups, both effects were confounded.

Preliminary analysis with a subset of the phenotypic data showed that slaughter group was the most important source of variation in WBSF, while genetic groups made a minor contribution to differences on that trait.

Results

Genotype and allele frequencies

The allele and genotype frequencies of each marker are listed in Table 2. Because most of the animals were crossbreds, allele frequencies for each breed could not be thoroughly estimated. Allele frequencies at SNP316 were significantly different between the AX and HX groups, the former having a higher frequency of the C allele. The A allele at SNP530 seemed to be rare among British breeds.

Table 2 - Genotype and allele frequencies of the *CAPN1* gene SNP316 and SNP530 markers and the *CAST* gene SNP2870 marker.

Marker	AX	HA	HX	LX
		Genetic group*[†]		
SNP316				
CC	31	5	2	3
CG	98	19	33	15
GG	45	11	33	18
Frequency of C	0.46[a]	0.41[ab]	0.27[c]	0.29[bc]
SNP530				
AA	3	1	-	-
AG	46	4	3	13
GG	125	30	65	23
Frequency of G	0.85[a]	0.91[a]	0.98[b]	0.82[a]
SNP2870				
AA	46	4	1	2
AG	92	19	31	20
GG	36	12	36	14
Frequency of A	0.53[a]	0.39[b]	0.24[c]	0.33[bc]

*AX: ≥75% Angus - ≤25% Hereford; AH: 50% Angus - 50% Hereford; HX: ≥75% Hereford - ≤25% Angus; LX: Limousin x Hereford-Angus.
[†]In the same row, allele frequencies with different letters are significantly different (chi-square test, $p < 0.05$).

For the newly discovered *CAST* SNP2870, the group with a predominantly Angus background (AX) had a higher frequency of the A allele, whereas the opposite was true for the Hereford-based group (HX).

Association analyses

There was a significant effect of both the *CAPN1* SNP316 and SNP530 polymorphisms on WBSF (Table 3). For SNP316, the WBSF value for the CC genotype was 10% lower than for the CG genotype and 14.6% lower than for the GG genotype. There were only four steers with the SNP530 AA genotype, so these steers were not included in the association analysis. Beef from steers with the GG genotype at this marker had an 11.5% higher WBSF than beef from steers with the GA genotype. Interestingly, other studies conducted under different conditions (Page *et al.*, 2002; White *et al.*, 2005) established that G was the most favorable allele in terms of beef tenderness. The *CAST* SNP2870 polymorphism showed no significant detectable effect on WBSF. It remains to be demonstrated whether this marker has any association with tenderness in aged beef. There were no detectable effects of any marker on BW, HCW or REA. Mean values of these traits for each slaughter group are presented in Table 1.

Discussion

Despite the relevance of beef tenderness as a selection objective in cattle, virtually no selection has been practiced on this trait. In fact, the difficulties encountered in measuring measure beef quality traits make them suitable for marker-assisted selection strategies. Thus, the validation of marker effects becomes a crucial step towards their application in a breeding program. In this paper we report the results of a validation study involving markers targeting a proteolytic system of skeletal muscle closely connected to the process of beef tenderization.

Extreme frequencies of any allele at a given locus make selection highly inefficient, independently of its effect on the mean of a quantitative trait (Falconer and Mackay, 1996), so that consideration of allele frequencies is an important part of the marker validation process. The

Table 3 - Mean Warner-Bratzler Shear Force (WBSF) values and standard error (SE) of the mean for genotypic classes based on individual polymorphisms on the *CAPN1* and *CAST* genes. Genotype AA at the SNP530 locus was not included in the analysis due to the low number of animals in this class.

	CAPN1 gene						*CAST* gene		
	SNP316 marker			SNP530[2] marker			SNP2870 marker		
Genotype	n	WBSF ± SE (kg)*	Genotype	n	WBSF ± SE (kg)*	Genotype	n	WBSF ± SE (kg)*
CC	41	7.86 ± 0.65[a]	-	-	-	AA	53	9.11 ± 0.75[a]
CG	165	8.73 ± 0.51[ab]	AG	66	7.98 ± 0.62[a]	AG	162	8.91 ± 0.53[a]
GG	107	9.21 ± 0.54[b]	GG	243	8.90 ± 0.47[b]	GG	98	8.68 ± 0.56[a]

[*]In the same column, means with different letters are significantly different ($p < 0.05$).

broad pattern of allele frequencies across genetic groups (Table 2) for *CAPN1* markers is consistent with other results from research herds (Page *et al.*, 2004). In our study Angus steers showed a higher frequency of the *CAPN1* SNP316 C allele than Hereford steers, similar to that reported by Page *et al.* (2004) and Morris *et al.* (2006). While the *CAPN1* SNP530 A allele seemed to be very uncommon among British breeds it tended to have a higher frequency in the continental breeds as suggested by the results for LX crosses, these results being in agreement with those of (Page *et al.*, 2004). Our new SNP2870 marker for the *CAST* gene showed an allele frequency pattern resembling that of SNP316, with the allele that predominated in the Hereford-based groups being at a lower frequency in the Angus-based group (Table 2).

With the exception of Limousin sires, all the animals that contributed to the genetic background of the population studied belonged to INTA experimental herds and represent two distinct genetic pools. Both the Angus and Hereford herds have been kept closed since their creation in the late 1960s. The Hereford herd has been under selection since 1986 with the objective of increasing preweaning growth rate while keeping birth weight constant (Melucci and Mezzadra, 2003), but no selection has ever been practiced in the Angus herd. It is worth noting that both Angus and Hereford cattle from these herds are smaller than cattle from registered herds. Special attention was paid when animals were chosen to create both herds, in order to resemble the genetic background of commercial herds at that time. However, most commercial herds have undergone changes that have probably altered allele frequencies for some genes. Unfortunately, there is no information available about the genetic trends for this particular set of mutations. A comprehensive validation program conducted by the National Beef Cattle Evaluation Consortium in the United States provides information about frequencies for the most popular markers for beef tenderness currently in the market. For SNP316, the frequency of the G allele was above 0.76 for all the sampled breeds and crosses of *B. taurus* breeds. This value is close to those reported by Page *et al.* (2004) for most crosses and consistent with results reported here for HX but not AX. It would be interesting to confirm whether these differences are due to genetic trends or sampling effects and genetic drift.

The WBSF values from the present study (Table 1) were higher than many others reported in the literature (Shackelford *et al.*, 1999; King *et al.*, 2003). Both the lack of an aging period and the cooking method could have contributed to obtaining such high values. It has been demonstrated that chilling and cooking rates and the interaction between these factors can have dramatic effects on beef tenderness (King *et al.*, 2003). Moreover, the highest WBSF values are usually obtained 24 h postmortem, and coincidentally that was the sampling point chosen in the present study. Also, slight differences between experimental protocols for WBSF determination may exist among laboratories. Although our WBSF values cannot be compared to those reported elsewhere, it was considered that relative comparisons between genetic classes within the same study were still valid.

In the present study the toughest meat seemed to be associated with G alleles at both SNP316 and SNP530 (Table 3). Previous research suggested that GA would be the two-marker haplotype (SNP316/SNP530) with the highest WBSF values (Page *et al.*, 2002; White *et al.*, 2005). A new SNP (alleles C/T) on intron 17 of *CAPN1* has been recently described (Page *et al.*, 2002; White *et al.*, 2005) and the C allele at this locus is expected to be more favorable to tenderness. Extending the genotyping to include the new SNP in future studies together with the definition of haplotypes, could allow for better classification of carcasses in terms of their WBSF. No formal proof of causality has been presented for any of the described polymorphisms on *CAPN1*. The strength of their statistical association with tenderness depends on the degree of linkage with a putative polymorphism affecting calpain activity, yet to be discovered. The present knowledge suggests that the C allele at SNP316 has the strongest linkage disequilibrium with the putative tenderness allele. Therefore, a good definition of haplotypes could help in the identification of gametes carrying the favorable allele of the actual polymorphism affecting beef tenderness.

The SNP2887 on the *CAST* gene corresponds to base 2870 in the GenBank sequence AF159246 and another previously described SNP in the *CAST* 3' UTR is in position 2959 of the same sequence (Barendse, 2002), a difference of only 89 bp. However, at least three haplotypes exist according to the EST contig (AG, AA and GA), suggesting that linkage disequilibrium is not complete. The 3' UTR has gained attention due to its importance in regulation of gene function (Hughes, 2006), with many regulatory motifs being only 8 bp long (Xie *et al.*, 2005). Also, the most favorable allele of the marker described by Barendse (2002) seems to be at high frequency in taurine populations (Casas *et al.*, 2006; Morris *et al.*, 2006). For these reasons, it was considered worthwhile to validate the effects of the new polymorphism on beef tenderness. However, this marker had no significant effect on WBSF. Two other polymorphisms on the *CAST* gene have been successfully associated with differences in beef tenderness (Barendse, 2002; Schenkel *et al.*, 2006). Comparison of studies evaluating markers for beef tenderness consistently suggests that polymorphisms on *CAST* have a weaker effect on WBSF compared to those on *CAPN1*. It remains to be demonstrated whether the lack of association in the present study was due to the marker being in equilibrium with a putative functional mutation or the need of measuring WBSF in aged meat for the effect to be detectable. Although the new marker on the *CAST* gene had no effect on WBSF, it was still useful to demonstrate the efficacy of the methodology used to detect the polymorphism. Comparison of EST from

different sources confirmed to be an inexpensive yet powerful method to detect variability at the gene level among cattle populations.

Although care was taken to follow the same protocol during slaughter and sampling of the carcasses across the entire study, the most important source of variation on WBSF was always the slaughter group. Similar results have been observed in other studies (Schenkel *et al.*, 2006). In our study, the slaughter group effect cannot be explained by the genetic background alone, stressing the relevance of environmental effects on beef quality traits, especially the management of animals before slaughter and handling and the storage of carcasses. Additional care is needed in future studies to detect and control as many sources of variation as possible in order to conduct these types of genetic evaluations more precisely.

If the allele frequencies estimated in our study were representative of those in the commercial population, it would be advisable to select cattle favoring the SNP316 C allele. The SNP530 A allele was present at very low frequencies and was usually found on GA haplotypes, while the CA haplotype seems to be rare in British breeds (Page *et al.*, 2004; White *et al.*, 2005). Thus a SNP316 C allele will be most likely tagging the CG haplotype and genotyping of SNP530 would add little information at least in Angus, Hereford and crosses between these breeds.

In summary, our results showed that there is a detectable effect of *CAPN1* markers on tenderness, even in beef that has not been aged. However, the *CAST* marker showed no detectable effect on tenderness under the conditions tested. None of the markers tested showed any effects on live weight, carcass weight or rib eye area. Therefore, selection favoring beef tenderness would not produce any undesired correlated responses. The results are promising and warrant further research to apply this technology to the beef industry.

Acknowledgments

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Internet Resources

Plant Genetics and Genomics, Victorian AgriBiosciences Centre, Australia, http://hornbill.cspp.latrobe.edu.au/.

National Beef Cattle Evaluation Consortium, USA, http://www.nbcec.org/nbcec/index.html (September 2006).

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