Further testing of the effect of a calpain-1 variant on meat tenderness in cattle

N.G. CULLEN, C.A. MORRIS, B.A. VEENVLIET[1], S.M. HICKEY, P.M. DOBBIE and T. WILSON[2]

AgResearch, Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand.

ABSTRACT

We have previously reported that a single-nucleotide polymorphism (SNP) on the large subunit of micro-calpain (the calcium-activated neutral protease-1 gene) is associated with differences in tenderness, as measured in cooked steaks from the *longissimus dorsi* of animals from the AgResearch Jersey-Limousin Beef DNA-marker trial. The effect was significant at the intermediate stages of the tenderising process and had little effect on initial or ultimate tenderness, so that tenderising was accelerated in animals having one or two copies of the favourable variant. We have now analysed tenderness and genotype data from 310 additional animals, comprising Hereford-cross and pure Angus breeds slaughtered under varying conditions of electrical stimulation and aged at a range of temperatures. Results in all cases support the original finding: animals with two copies of the 'C' allele (the SNP on Exon-9 encoding alanine at amino acid number 316) have more tender meat (decreased shear force) at the intermediate stages than animals with two copies of the 'G' allele (encoding glycine). Combining measurements from three intermediate cook times, mean shear forces from 'CG' were less than from 'GG' genotypes by 0.80 ± 0.29 kg ($P < 0.01$), and mean shear forces from 'CC' were less than from 'CG' genotypes by 1.19 ± 0.36 kg ($P < 0.001$). These results show that a genotype substitution from 'GG' to 'CC' is associated with a difference in shear force in *longissimus dorsi* steaks of approximately 20% at intermediate aging times.

Keywords: cattle; meat; tenderness; micro-calpain; SNP.

INTRODUCTION

Micro-calpain is a protein associated with the tenderisation process in meat at and after *rigor mortis*. It consists of two subunits of different size and the large subunit is encoded by the gene known as calpain-1 (calcium-activated neutral protease-1). Results published by the United States Department of Agriculture and AgResearch (Smith *et al.,* 2000; Page *et al.,* 2002; Cullen *et al*., 2003) have demonstrated that single-nucleotide polymorphisms (SNPs) on calpain-1 are associated with differences in the tenderness of cooked steaks from the *longissimus dorsi* of cattle, at various times after *rigor mortis.* These published results referred to cattle which were the progeny of Piedmontese x Angus sires in the USA and Jersey x Limousin sires in New Zealand. Recently we have collected data from more animals of different breeds to ascertain whether the same relationship holds across breeds. To this end, data were collected from Hereford-cross industry heifers and steers, and from heifers and bulls in an Angus research herd. In spite of unbalanced allele frequencies, we confirmed the previously reported association in these two breeds.

MATERIALS AND METHODS

Design

Hereford-cross:

A total of 214 'Hereford Prime' cattle obtained from industry sources were slaughtered at the Ruakura Abattoir in 14 groups (from 10 to 21 animals per group), between July 2003 and September 2004. The animals were predominantly heifers (186 cf. 28 steers); they were also predominantly Hereford-crosses, with Friesian probably being the main dam breed. It was difficult to identify pure Herefords from coat colour as some Friesians carry the 'red' gene, and dams with some Jersey blood will also produce red animals. Breed was therefore not fitted in any analysis; this group of animals will be referred to as 'Hereford-cross'. These animals were killed under commercial conditions with electrical stimulation. DNA was collected at slaughter and 196 animals were genotyped for an Exon-9 SNP previously identified in calpain-1 (Page *et al.* (2002); details are given below). Records from three animals were discarded subsequently as they had high muscle pH readings, so that data for 193 animals were included in the analyses (167 heifers and 26 steers).

Angus:

A total of 126 bulls and heifers born in 2002 in AgResearch's Puberty trial at Tokanui Station (Morris *et al*., 2000) were slaughtered in five groups in May-June 2004. Bulls were slaughtered at the Greenlea Meats plant in Hamilton and the heifers at the Ruakura Abattoir. The bulls were killed under commercial conditions with electrical stimulation followed by hot-boning, while the heifers were not electrically stimulated. A total of 117 animals were genotyped for the Exon-9 SNP of calpain-1 and included in the analyses.

[1] AgResearch, Invermay Agricultural Centre, Private Bag 50034, Mosgiel, New Zealand
[2] AgResearch Molecular Biology Unit, Department of Biochemistry, University of Otago, Dunedin, New Zealand

Muscle measurements

For the Hereford-cross cattle, the carcass sides were placed in a commercial chiller. Initial pH was recorded on the *longissimus dorsi* (striploin) within about 30 minutes of slaughter and pH was then monitored at intervals until *rigor mortis* (pH < 5.5*)*. The right striploin was removed at *rigor mortis*, and six steak portions were cut from the striploin. One steak was put on ice for immediate evaluation of shear force at *rigor mortis* whilst the other five were held at -1ºC and aged for various times (until approximately 1, 3, 7, 14 and 28 days *post rigor*). For the Angus cattle, the striploin was removed from the hot-boned left side (bulls), or from the right side immediately after the carcass scales (heifers). For both sexes, the striploin was held at 15ºC in a controlled-temperature cabinet until *rigor mortis*. Six steak portions were then cut from each striploin; the first steak was processed for shear force on reaching *rigor mortis*, and the other five steaks were aged at 15ºC for various times (until approximately 0.5, 1, 2, 3 and 7 days *post rigor*).

Each steak was processed for shear force by placing it inside a plastic cooking bag, heating in a boiling water-bath to an internal temperature of 75ºC, then removing and cooling rapidly in ice to an internal temperature of 2ºC. Shear-force measurements were then recorded using a MIRINZ tenderometer (Fraserhurst & MacFarlane, 1983), taking the average from measurements of ten 1 cm x 1 cm cores, aligned with the fibres running longitudinally along the core. The measures of average shear force on the six steak samples are referred to as COOK1….COOK6.

PCR methods

A 150-nucleotide region surrounding amino acid number 316 in the calpain-1 gene was amplified using a primary PCR reaction. The forward and reverse primers were
ACGTTGGATGGCAGGTCAGTGGCCGCCA and
ACGTTGGATGGGCTGCTCACCAGAACTCC,
respectively. Forty-five cycles of PCR were performed at a $MgCl_2$ concentration of 2.5 mM and an annealing temperature of 56°C, producing a primary PCR product which was dephosphorylated using shrimp alkaline phosphatase. A mass-extend reaction was then used to identify the nucleotide variation from the individual samples. The primer GCTCCTCGGAGTCCAACG binds adjacent to the SNP site in the sequence amplified by the primary PCR, and this primer was then mass-extended to produce allele-specific molecular products. The product was loaded onto a Sequenom chip and analysed using a Biflex-3 mass spectrometer.

Genotyping

All animals were genotyped for an Exon-9 SNP at amino acid number 316 in the calpain-1 gene (Page *et al.*, 2002). Segregation at this locus had been shown for the nucleotides (alleles) 'C' and 'G', where the 'C' allele encodes alanine and the 'G' allele encodes glycine. DNA samples were analysed with the mass spectrometer, which identified the 'C' and 'G' alleles corresponding to the calpain-1 amino acid #316 by mass, producing peaks of 5813.8 and 6143 daltons, respectively.

Statistical analyses

Results for each of the shear force traits defined above were analysed using JMP (SAS Institute Inc., 1995), fitting relevant fixed effects for each breed group (for Hereford-cross: slaughter group (1-14), sex, and herd of origin (1-24); for Angus: selection line (1-5), and slaughter group (1-5; this also accounted for sex)). Data were first analysed within breed group (Hereford-cross or Angus) to obtain estimates of genotype differences (between the heterozygote and one homozygote). Genotype frequencies within each breed group were unbalanced (see later). Estimates for all three genotypes were obtained from the combined data set, fitting breed group as an extra fixed effect.

TABLE 1: Genotype and allele frequencies by breed for the 310 animals in analyses of shear force at each cook time.

		Genotypes			Allele frequency
Breed	n	CC	CG	GG	%C
Hereford cross	193	5	80	108	23
Angus	117	57	55	5	72

RESULTS

Genotype frequencies for the Exon-9 SNP are shown in Table 1. The two breed groups were almost complementary in their frequency of the 'C' allele. These frequencies closely match the results obtained from our Meat & Wool New Zealand project to ascertain allele frequencies in New Zealand cattle breeds, if most of the Hereford-cross animals are assumed to be Hereford x Friesian crosses. The small numbers of 'CC' genotypes for Hereford cross and 'GG' genotypes for Angus precluded our obtaining reliable estimates for these subclasses, so the estimates in Tables 2 and 3 are entered in brackets. As the Angus bulls and heifers were killed at different plants under differing electrical stimulation procedures, the two sexes were analysed separately first. There was no significant sex by genotype interaction, so the combined results are also presented in Table 3. For the Hereford-

TABLE 2: Hereford-cross: effects of genotype (least-square mean) on shear force (kg force), with residual standard deviation (RSD), overall mean, standard error of the difference (SED) for the comparison quoted, probability values and the genotype difference expressed as a proportion of the mean. Shear forces were determined for cook times 1 to 6 (0, 1, 3, 7, 14 and 28 days *post rigor,* respectively) and the average of cook times 2, 3 and 4.

Genotype	Cook 1	Cook 2	Cook 3	Cook 4	Cook 5	Cook 6	Ave 234
GG, n = 108	13.6	12.9	9.97	8.29	6.96	6.52	10.4
GC, n = 80	13.3	11.8	9.10	7.54	6.54	6.35	9.47
(CC), n = 5	(13.0)	(11.9)	(9.66)	(7.01)	(4.77)	(5.23)	(9.52)
RSD	1.93	2.67	2.31	2.00	1.23	1.18	2.00
Overall mean[1]	13.1	11.5	9.02	7.66	6.63	6.12	9.41
SED (GG- GC)	.30	.42	.36	.31	.19	.22	.31
P (GG- GC)	ns	**	*	*	*	ns	**
Diff/mean	.020	.096	.097	.098	.064	.028	.097

[1] Low overall means are explained by the imbalance in subclass numbers.

cross cattle, the difference in shear force between the GG and GC genotypes was significant for four of the six cook times, and the combined class (averaging cook data from times 2-4) showed a 9.7% difference in shear force ($P < 0.01$). We also analysed the Hereford-cross heifer data alone, and obtained a genotype effect for 'GG minus GC' of 9.5% of the mean ($P < 0.01$), very similar to the result with heifer and steer data combined. For the Angus cattle overall, the difference in shear force between the GC and CC genotypes was significant for three of the six cook times, and the combined sexes (averaging cook data from times 2-4) showed an 11.6% difference in shear force ($P < 0.01$). In both breed groups, the calpain-1 SNPs failed to show a significant effect on shear force for COOK1 (*rigor mortis*) and COOK6 (7 days at 15°C, Angus; 28 days at -1°C, Hereford-cross).

We then analysed the two breed groups of data together, fitting breed group as an additional fixed effect; the interaction between breed group and genotype was not significant. The combined genotype effects are presented in Table 4 for each cook and the average of cook data from times 2, 3 and 4. Overall, there were significant effects of 0.80 ± 0.29 kg or 7.9% for GG minus GC ($P < 0.01$) and 1.19 ± 0.36 kg or 11.7% for GC minus CC ($P < 0.001$) on COOKs 2-4, amounting to a total effect of 19.6% (GG minus CC).

Ratios of residual standard deviation to overall mean at the six cook times averaged 21.3% for the Hereford-cross samples and 19.0% for the Angus samples. The variances accounted for by this Exon-9 SNP at COOKs 2-4 were 7 and 18 % of the phenotypic variation, respectively.

DISCUSSION

There were two different trial designs in this study. The Hereford-cross commercial cattle were electrically stimulated and steaks were aged after *rigor mortis* at -1°C, as in one of the normal commercial practices. The Angus cattle were an experimental line, and the heifers were not electrically stimulated; the steaks from the Angus bulls and heifers were aged at 15°C. This study was not a breed comparison; rather it was an exercise which made use of different processing conditions for the carcass and the aging conditions for the meat, with two groups of cattle. Did the CC-, CG- and GG-genotype effects on tenderness exist under different conditions of processing and aging? Our results suggest that the genotype effects were consistent across the two designs, although differences in genotype frequency among the breed groups (unknown at the beginning of this study) meant that there were fewer subclass numbers for some of the contrasts than originally intended.

FIGURE 1: Calpain-1 genotype effects on shear force, as a measure of tenderness, recorded over six time points, using data combined from Hereford-cross and Angus cattle (kg force units; standard error bars are shown for each mean).

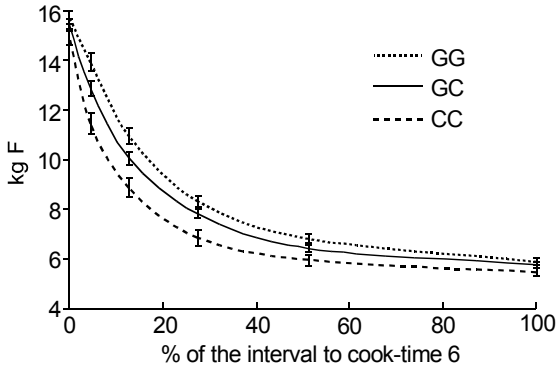

TABLE 3: Angus: effects of genotype (least-square mean) on shear force (kg force), with residual standard deviation (RSD), overall mean, standard error of the difference (SED) for the comparison quoted, probability values and the genotype difference expressed as a proportion of the mean. Shear forces were determined for cook times 1 to 6 (0, 0.5, 1, 2, 3 and 7 days *post rigor,* respectively) and the average of cook times 2, 3 and 4; results were derived from bulls, heifers and a combined analysis.

Sex	Genotype	Cook 1	Cook 2	Cook 3	Cook 4	Cook 5	Cook 6	Ave 234
Bulls								
(n=59)	(GG), n = 4	(18.1)	(17.2)	(15.1)	(9.06)	(6.46)	(5.25)	(13.8)
	GC, n = 29	17.9	16.0	13.2	9.60	6.64	5.37	12.9
	CC, n = 26	16.8	15.0	12.2	8.22	6.39	5.24	11.8
	RSD	1.74	2.65	2.72	2.68	1.65	.894	2.26
	Mean	17.7	15.8	13.7	9.41	6.63	5.49	13.0
	SED (GC-CC)	.48	.73	.75	.74	.46	.25	.63
	P (GC- CC)	*	ns	ns	+	ns	ns	+
	Diff/mean	.060	.065	.074	.145	.039	.024	.088
Heifers								
(n=58)	(GG), n = 1	(19.7)	(16.4)	(12.8)	(9.97)	(7.75)	(5.03)	(13.1)
	GC, n = 26	17.5	14.2	10.0	7.10	6.23	5.31	10.4
	CC, n = 31	17.7	12.0	8.36	6.43	5.87	5.05	8.94
	RSD	2.45	2.53	2.13	1.46	1.10	.648	1.86
	Mean	17.6	13.1	9.23	6.80	6.15	5.27	9.72
	SED (GC-CC)	.68	.71	.60	.41	.31	.18	.52
	P (GC- CC)	ns	**	**	ns	ns	ns	**
	Diff/mean	.010	.168	.179	.098	.059	.050	.155
Both								
(n=117)	(GG), n = 5	(18.8)	(17.0)	(13.8)	(9.60)	(7.19)	(5.11)	(13.5)
	GC, n= 55	17.5	15.2	11.5	8.46	6.51	5.30	11.7
	CC, n = 57	17.1	13.6	10.2	7.49	6.18	5.07	10.4
	RSD	2.12	2.58	2.43	2.15	1.40	.785	2.06
	Mean	17.6	14.5	11.5	8.11	6.39	5.38	11.4
	SED (GC-CC)	.41	.51	.48	.42	.27	.15	.40
	P (GC- CC)	ns	**	**	*	ns	ns	**
	Diff/mean	.027	.115	.115	.119	.052	.042	.116

TABLE 4: Combined Hereford-cross and Angus analysis: overall mean shear force (kg force) and genotype effects expressed as percentages of the overall mean, along with probability values for genotype effects at cook times 1 to 6 and the average of times 2 to 4.

Genotype	Cook 1	Cook 2	Cook 3	Cook 4	Cook 5	Cook 6	Ave 234
mean	14.8	12.7	9.95	7.78	6.54	5.79	10.1
GG – GC, %	2.0	8.4	9.2	6.6	6.4	2.4	7.9
P	ns	**	**	+	*	ns	**
GC – CC, %	3.2	11.2	11.8	12.3	7.2	5.0	11.7
P	ns	**	**	**	*	ns	***

Coefficients of variation for the two designs were similar, from which we concluded that it was feasible to pool the data (21.3% for the Hereford-crosses and 19.0% for the Angus cattle). Cooking times (days after *rigor mortis*) were broadly equivalent in the two designs, after allowing for the differences in temperatures.

Results from the Jersey-Limousin QTL trial (Cullen *et al*., 2003) showed that the Exon-9 polymorphism had a significant effect on shear force at intermediate cook times, and the heterozygotes were

roughly intermediate between the two homozygotes. Individual breed-gender analyses from the additional Hereford-crosses and Angus cattle in the present study, and then the combined results from these two groups, showed the same result as in the Jersey-Limousin trial. The analysis for the Angus bulls was not so convincing as the other data in Tables 2 and 3, but the genotype differences in shear force for the bulls followed the same direction; the reason for the smaller differences is not clear. These are the only bulls we have analysed to date.

A plot of the decline in shear force for each of the three genotypes from the combined data set is shown in Figure 1. The three intermediate cooks (COOKs 2, 3 and 4) were significant ($P < 0.01$), and showed the largest differences between genotypes. Initial tenderness (COOK1) and ultimate tenderness (COOK6) were not significantly affected by genotype in our data. In the case of *Bos indicus* cattle or their crosses, Australian studies have also shown a significant effect of the calpastatin gene on shear force (Genetic Solutions, 2005), but this gene effect may not be as important in *Bos taurus* cattle. However, in spite of the encouraging results for calpain-1 in *Bos taurus* cattle it would still be useful to find effects on shear force from further genes, so that more of the genetic variation in tenderness may be explained and potentially controlled under commercial conditions, alongside known processing factors (e.g. as reviewed by Ferguson *et al*., 2001). Genotyping to determine any breed difference in tenderness would require a knowledge of all the relevant genes.

CONCLUSIONS

These results are further confirmation that a 'G' to 'C' polymorphism on Exon-9 at amino acid 316 of calpain-1 is the causative mutation for meat tenderness, or is very closely linked to it.

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REFERENCES

Cullen, N. G.; Page, B. T.; Morris, C. A.; Hickey, S. M.; Dobbie, P. M. 2003: A single-nucleotide polymorphism on Calpain-1 is associated with meat tenderness in cattle. *Proceedings of the New Zealand Society of Animal Production 63:* 53-56

Ferguson, D. M.; Bruce, H. L.; Thompson, J. M.; Egan, A. F.; Perry, D.; Shorthose, W. R. 2001: Factors affecting beef palatability - farmgate to chilled carcass. *Australian journal of experimental agriculture 41:* 879-891

Fraserhurst, L.; MacFarlane, P. 1983: A device for measuring the tenderness of meat. N.Z. Patent 190945

Genetic Solutions 2005: http://www.geneticsolutions.com.au/files/GeneSTAR/pdf/GeneNOTE_7.pdf

Morris, C. A.; Wilson, J. A.; Bennett, G. L.; Cullen, N. G.; Hickey, S. M.; Hunter, J. C. 2000: Genetic parameters for growth, puberty, and beef cow reproductive traits in a puberty selection experiment. *New Zealand journal of agricultural research 43:* 83-91

Page, B. T.; Casas, E.; Heaton, M. P.; Cullen, N. G.; Hyndman, D. L.; Morris, C. A.; Crawford, A. M.; Wheeler, T. L.; Koohmaraie, M.; Keele, J. W.; Smith, T. P. L. 2002: Evaluation of single-nucleotide polymorphisms in *CAPN1* for association with meat tenderness in cattle. *Journal of animal science 80:* 3077-3085

SAS.1995: JMP Version 3, SAS Institute, Cary, NC, USA.

Smith, T. P. L.; Casas, E.; Rexroad, C. E.; Kappes, S. M.; Keele, J. W. 2000: Bovine *CAPN1* maps to a region of BTA29 containing a quantitative trait locus for meat tenderness. *Journal of animal science 78:* 2589-2594