Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Effect of leptin gene polymorphisms on growth, slaughter and meat quality traits of grazing Brangus steers

P.M. Corva[1], G.V. Fernández Macedo[1], L.A. Soria[2], J. Papaleo Mazzucco[3], M. Motter[2], E.L. Villarreal[3], A. Schor[4], C.A. Mezzadra[3], L.M. Melucci[1] and M.C. Miquel[2]

[1]Departamento de Producción Animal, Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata, Balcarce, Argentina
[2]Área de Genética, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Buenos Aires, Argentina
[3]Instituto Nacional de Tecnología Agropecuaria, Balcarce, Argentina
[4]Laboratorio de Carnes, Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina

Corresponding author: P.M. Corva
E-mail: pcorva@balcarce.inta.gov.ar

Genet. Mol. Res. 8 (1): 105-116 (2009)
Received November 11, 2008
Accepted December 1, 2008
Published February 3, 2009

ABSTRACT. Leptin is a hormone that affects the regulation of feed intake, energy balance and body composition in mammals. Several polymorphisms in the bovine leptin gene have been associated with phenotypic variance of these traits. We evaluated two known single nucleotide polymorphisms (SNPs) in the leptin gene of 253 grazing Brangus steers. Brangus is a 5/8 Angus-3/8 Brahman composite. Data were collected during two consecutive growth/fattening cycles from two farms in southeast Buenos Aires province, Argentina. One of the markers is in the promoter region of the gene (SNP1) and the other is a non-synonymous polymorphism in exon 2 (SNP2). The traits that we evaluated were live weight gain in the spring, gain in backfat thickness in the spring, final live weight, final ultrasound backfat thickness, final ultrasound rib eye area, carcass weight and length, carcass yield, kidney

fat, kidney fat percentage, backfat thickness, rib eye area, and intramuscular fat percentage. Both markers affected some meat traits; though the only significant associations were of SNP1 with ultrasound rib eye area and of SNP2 with carcass yield and backfat thickness. Under the same conditions as in the present study, leptin markers could be of help only as part of a larger genotyping panel including other relevant genes.

Key words: Beef cattle; Brangus; Leptin; Body composition; Beef quality

INTRODUCTION

The leptin factor has gained much attention recently as a key regulator of biological processes that are related to very important productive traits in beef cattle, such as feed intake, fat content and meat quality (Houseknecht et al., 1998; Geary et al., 2003). In turn, polymorphisms in the corresponding gene have been proposed as predictors of relative differences among individuals for those traits (Nkrumah et al., 2004; Schenkel et al., 2005). In fact, markers in the leptin gene are already part of commercial genotyping panels designed for Marker Assisted Selection (M.A.S.) in beef cattle.

However, association studies involving markers in the leptin gene have shown inconclusive results. It could be argued that the inconsistency is due to differences in environmental factors such as nutrition and also to genetic background of the animals, which are potential sources of genetic x environment interactions. Nutritional level, both in terms of quantity and quality of nutrients, becomes an important constraint in production systems that strongly depend on direct grazing.

Regarding the genetic background of the animals, zebu breeds and their crosses represent an irreplaceable resource in many countries because of their rusticity and adaptation. This is the case in the subtropical region of Argentina, where livestock activities are progressively increasing due to the competition with agriculture in temperate areas. Independent of their relevance, zebu breeds and their crosses are underrepresented in association studies involving leptin.

There is a growing interest for M.A.S. technology in Argentina, but farmers would prefer markers to be validated in local conditions before widespread use of the technology. For this reason, we evaluated the association of two markers in the leptin gene with weight gain and carcass composition traits in grazing steers of the Brangus breed, which is a 5/8 Angus-3/8 Brahman composite.

MATERIAL AND METHODS

Animals and phenotypic information

Brangus steers were obtained through a collaborative agreement between the intervening research institutions and the Argentine Brangus Association. The study was conducted during two consecutive cycles (2004/2005 and 2005/2006) in two different locations of the southeast of Buenos Aires Province. For the first cycle, 60 weaned calves from three different farms (20 steers each) were received at the Experimental Station of INTA (National Institute of Agricultural Technology) in Balcarce, Argentina, between April 27 and May 17, 2004. The second cycle was conducted at the San Claudio Experimental Farm of the University of Buenos Aires in Carlos Casares, ap-

proximately 400 km from Balcarce. For this cycle, six farms provided 193 weaned calves (10 to 48 steers each) that were received at San Claudio between May 10 and September 2, 2005. Average initial body weights were similar between cycles: 226 ± 27 kg on June 3, 2004 and 231 ± 33 kg on October 6, 2005, respectively. In both cycles, all animals received a grass-based diet. For the first cycle only, steers were supplemented in winter with corn silage, corn grain and sunflower meal.

Live weight and ultrasound backfat thickness were recorded at arrival and then at 28-day intervals until they left for slaughter. In order to analyze weight gain and changes in backfat thickness on pastures, an interval that made both cycles comparable had to be defined. Coincidentally, steers had the highest daily weight gains in a comparatively short period during spring in both cycles (data not shown). Despite the differences in starting dates and length of the two cycles, an interval was identified (September to November) for which the dates on which animals were weighed each year were very close. For each of these periods, daily weight gain was calculated as the regression of live weight on days. Gain in backfat thickness was estimated in a similar fashion.

Steers from both cycles were progressively sent to a private abattoir in Balcarce as they reached a backfat thickness of at least 6 mm between the 12 and 13th ribs, estimated by ultrasound. There were three slaughter groups in 2005 (March 30, April 28 and May 12) and six groups in 2006 (between July 21 and December 13). Final body weight and rib eye area (by ultrasound) were recorded before slaughter. Weights of carcass and kidney fat, carcass length, rib eye area, and backfat thickness were recorded at the slaughter plant. Carcass yield was calculated as (hot carcass weight*100) / final weight. Weight of kidney fat was expressed as an absolute value and also as a percentage of hot carcass weight.

Carcasses were placed in a chiller for 24 h at 10-12°C, without previous electrical stimulation. The day after slaughter a block of steaks corresponding to the 11, 12 and 13th ribs was removed from the 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. Intramuscular fat was extracted according to protocol 920.39 of AOAC (1992) and expressed as g/100 g fresh tissue. The number of records and means for the analyzed traits are shown in Table 1.

Table 1. Number of records, mean and standard deviation (SD) for traits included in the association analyses.

Trait	N	Mean	SD
LWDG (kg/day)	248	1.070	0.188
BFDG (mm/day)	250	0.016	0.014
FW (kg)	247	447.5	48.4
USBFT (mm)	245	6.55	1.59
USREA (cm^2)	246	58.3	8.8
CW (kg)	247	248.4	29.7
CY (%)	247	55.45	2.00
KF (kg)	247	3.149	1.219
KFP (%)	247	1.24	0.41
CL (cm)	247	123.8	4.4
BFT (mm)	247	3.31	1.37
REA (cm^2)	246	67.1	8.7
IMF (%)	244	2.49	0.95

LWDG = live weight gain in spring; BFDG = gain in backfat thickness in spring; FW = final live weight; USBFT = final ultrasound backfat thickness; USREA = final ultrasound rib eye area; CW = carcass weight; CY = carcass yield; KF = kidney fat; KFP = kidney fat percentage; CL = carcass length; BFT = backfat thickness; REA = rib eye area; IMF = intramuscular fat percentage.

Selection of markers and genotyping

DNA was isolated from a blood sample collected from the jugular vein, following standard procedures (Maniatis et al., 1982) with the exception that a modified cell lysis buffer (50 mM Tris-HCl, 25 mM EDTA, pH 7.5, 2% N-laurylsarcosine, 0.05 M DL-dithiothreitol) was used. After phenol/chloroform extraction and ethanol precipitation, DNA was resuspended in 10 mM Tris-HCl.

Several polymorphisms in the leptin gene have been described (Buchanan et al., 2002; Lagonigro et al., 2003; Nkrumah et al., 2005). For the present validation study, we decided to evaluate those markers that could be more informative in Brangus in terms of allele frequencies and substitution effects.

The Brangus breed is a composite with contribution of genetic material from *Bos indicus* breeds. Before the first results from the bovine HapMap project were available, *Bos indicus* breeds were underrepresented in genomic databases. Therefore, before selecting the markers for the association study, we decided to sequence the promoter and most of the coding sequence of the gene in order to identify new polymorphisms in Brangus that could be contributed by their *Bos indicus* ancestors. For this purpose, we assembled a panel of nine Brahman, two Brangus and five Nellore bulls. Also, the promoter sequence was compared in a sample of 32 Brangus steers.

The leptin gene has three exons and two introns. Exon 1 is very short (34 bp) and is part of the 5' UTR. Therefore, it was not considered for sequencing. A 465-bp fragment corresponding to bases 877 to 1342 of Genbank sequence U50365 spanning exon 2 and a 495-bp fragment corresponding to bases 2961 to 3456 of the same sequence spanning exon 3 were amplified using primers designed by Konfortov et al. (1999). Polymerase chain reaction (PCR) conditions in both cases were 60°C annealing temperature, 2 mM $MgCl_2$.

A phylogenetic footprinting approach was used to define the most relevant region upstream of the leptin gene with promoter activity. A fragment of 3000 bp upstream of the transcription start site was retrieved from the Ensemble Genome Browser (http://www.ensembl.org) for each of the following species: human, chimpanzee, macaque, mouse, rat, bison, and bovine. These sequences were aligned with the MULAN software (Ovcharenko et al., 2005) and the multiple alignment was visualized with mVISTA (http://www.gsd.lbl.gov/vista/). Based on this analysis, a 553-bp fragment was sequenced. Primers for PCR (forward: 5'-ATTTTGCGGGAGCACGTTCC-3' and reverse: 5'-TCCGGGGCTCCATGCCTG-3') were designed with the Primer3 software (www.genome.wi.mit.edu) using Genbank sequence AJ571671 as a reference. PCR conditions were 64°C annealing temperature and 2 mM $MgCl_2$. PCR products corresponding to the promoter, exon 2 and exon 3 of the leptin gene were purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences/GE Healthcare Biosciences, UK). Each PCR amplicon was sequenced in a MegaBace1000 system (GE Healthcare) with the MegaBace Sequence Analyzer Cimarron 312 software. The same forward primers from PCR were used for sequencing. The TFSEARCH program (http://www.cbrc.jp) that searches the TRANSFAC database was used to detect transcription factor-binding sites (TFBS) in the set of promoter sequences.

Because no new relevant single nucleotide polymorphisms (SNPs) were identified through sequencing, we decided to genotype the Brangus population for two previously described SNPs. One was a nonsynonymous SNP in exon 2 (Buchanan et al., 2002) with significant effects in several association studies involving the leptin gene. For the sake of comparison, we also included one of the polymorphisms located upstream of the transcription start site (SNP UASMS2, position -2530) that had significant association with production traits in some evaluations (Nkrumah et al.,

2005) but not in others (Schenkel et al., 2005). Both SNPs (named SNP1 and SNP2, respectively) were genotyped with a tetra-primer amplification refractory mutation system-PCR method (Ye et al., 2001). This method employs two primer pairs to amplify the two different alleles of an SNP in a single PCR. Primers were designed with software available at http://cedar.genetics.soton.ac.uk/public_html/primer1.html. Primer sequences and PCR conditions appear in Table 2.

Table 2. Primers and conditions for the genotyping of single nucleotide polymorphisms (SNP) in the leptin gene by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR).

Marker	Primer sequence	Annealing temperature	$MgCl_2$	Amplicon size (bp)
SNP1	Forward inner primer (C allele) 5′-gcccagggactcagcggttgcaacaaac-3′	74°C (touchdown PCR)	1.5 mM	116
	Reverse inner primer (T allele) 5′-cggctcccgagcccaagctctagagcata-3′			150
	Forward outer primer 5′-tgcaatgtgcaagcttctcactgtggcagc-3′			209
	Reverse outer primer 5′-tggtacagtggatgagaatccgcctgcca-3′			
SNP2	Forward inner primer (T allele) 5′-tgtcttacgtggaggctgtgcccagct-3′	70°C (touchdown PCR)	2.5 mM	131
	Reverse inner primer (C allele) 5′-agggttttggtgtcatcctggacctttcg-3′			164
	Forward outer primer 5′-gacgatgtgccacgtgtggtttcttctgt-3′			239
	Reverse outer primer 5′-cggttctacctcgtctcccagtccctcc-3′			

Statistical analyses

Allele frequencies were compared by the chi-square test. Live weight gain in spring (LWDG), gain in backfat thickness in spring (BFDG), final live weight (FW), final ultrasound backfat thickness, final ultrasound rib eye area (USREA), carcass weight (CW), carcass length (CL), carcass yield (CY), kidney fat (KF), kidney fat percentage (KFP), backfat thickness (BFT), rib eye area (REA), and intramuscular fat percentage (IMF) data were analyzed with a model that included the fixed effects of year, farm of origin nested within year and genetic marker. Initial live weight was included as a covariate for the analysis of LWDG and BFDG. Separate analyses were conducted with either one or two SNPs in the model. The probability of each two-marker haplotypes given the genotype of each SNP was estimated with the PHASE software (Stephens et al., 2001). Haplotypes (0, 1, or 2 copies) were included as covariates in the model in place of SNP genotypes. Because specifying three haplotypes as covariates directly defines the fourth haplotype, the most frequent haplotype (CT) was not included.

Information about paternity was not available for all steers. Moreover, the sire effect was confounded with farm of origin. Therefore, the sire was not included as a factor in the model.

RESULTS

Sequence analysis

Taniguchi et al. (2002) reported a strong promoter activity for the first 250 bp

above the transcription start site of the bovine leptin gene. Our multiple alignment and sequence comparison indicated that a region not longer than 500 bp upstream of the leptin gene was highly conserved among species (sequence homology above 50%) and probably most closely involved in the regulation of expression. Therefore, we focused our analysis on that region for our search of new polymorphisms.

The sequences of promoter, exon 2 and exon 3 were obtained from 16 bulls (9 Brahman, 2 Brangus and 5 Nellore). The promoter was also sequenced in 32 Brangus steers. The results of sequence analyses are summarized in Table 3. These results suggest that there is high similarity between previously published sequences from *Bos taurus* breeds and those from *Bos indicus*. In the 5'-UTR of the bovine leptin gene, a new SNP (G/A alleles) was found at position -201 (contig position 105642, accession number NW_001494939). Two other SNP were found in introns (contig positions 117932 and 118124, respectively). There were also three new SNP in exons (contig positions 118061, 120130 and 120158, respectively), but all of them originated conservative substitutions.

The analysis with TFSEARCH of promoter sequences from GenBank indicated the existence of one putative SREBP-1 (sterol regulatory element-binding protein) motif (CAGGTG) between nucleotides -199 and -204 in some sequences that was not detected in our own sequences, that were either CAGGCG or CAGACG. No SREBP-1 binding sites were reported by Taniguchi et al. (2002) in the bovine leptin promoter. The discrepancy could be attributed to the simultaneous existence of SNPs at positions -200 (Gen-

Table 3. Single nucleotide polymorphisms (SNP) in the leptin gene identified in Brangus, Brahman and Nellore bulls.

Gene location	Reported alleles	Contig position[1]	Ref ID SNP[2]	Protein residue	Alleles[3] (This study)		
					Brangus	Brahman	Nellore
5'-UTR[4]	G/-	105430	rs29004469	-	G/-	G/-	G/-
	T/C	105552	rs29004470	-	T/C	T/C	T/C
	G/T	105562	rs29004471	-	G	G/T	G/T
	G/A	105572	rs29004472	-	G/A	G	G/A
	G/A	105633	rs29004473	-	G	G	G/A
	G[5]	105642	-	-	G	G/A	G/A
	T/C	105643	rs29004474	-	C	C	C
	C/T	105674	rs29004475	-	C/T	T	C/T
	C/T	105697	rs29004476	-	C/T	C	C/T
	C/G	105739	rs29004477	-	C/G	C	C/G
Intron 1	C/T	117824	rs29004484	-	C/T	C	C
	G/C	117847	rs29004485	-	G/C	G/C	G
	G[5]	117932	-	-	G/A	G/A	G
Exon 2	A/T	117973	rs29004487	7 Tyr>Trp	A/T	A	A
	C/T	118026	rs29004488[6]	25 Arg>Cys	C/T	C/T	C/T
	G[5]	118061	-	36 Lys>Lys	G/A	G	G/A
Intron 2	G/A[5]	118124	-	-	G/A	G	G/A
Exon 3	C/T	119949	rs29004508	80 Ala>Val	C	C	C
	A/G	120006	Unpublished[7]	119 Asn>Ser	A	A	A
	C/T	120106	rs29004509	132 Gly>Gly	C/T	C	C
	T/C	120109	rs29004510	133 Val>Val	T/C	T	T/C
	T/C	120121	rs29004511	137 Ala>Ala	T/C	T	T/C
	C[5]	120130	-	140 Tyr>Tyr	C/T	C	C/T
	C[5]	120158	-	150 Leu>Leu	C/T	C/T	C
	C/T	120205	rs29004512	165 Pro>Pro	C/T	C	C/T

[1]Position refers to a genomic contig (Accession NW_001494939) corresponding to bovine chromosome 4; [2]Reference SNP (refSNP) Cluster Report on NCBI Reference Assembly; [3]Alleles found in each breed; [4]5' Untranslated region (5'-UTR); [5]This study; [6]SNP2 (Buchanan et al., 2002); [7]Orrú et al. (2006).

Bank rs29004474) and -201 (this study). For this reason, it was concluded that the new SNP found at -201 was a weak candidate to have an influence on gene expression; therefore, it was not considered for further study. The previously reported C/G polymorphism at position -105 (Liefers et al., 2004), which could be of interest because it is also located on a putative TFBS, was seen in a single Brangus bull. Genotyping of 32 Brangus steers for this marker indicated that they were all homozygous CC. In summary, the sequence analyses did not reveal any new relevant SNP for inclusion in the association analysis.

Genotype and allele frequencies

Genotypic frequencies for the two markers on the leptin gene are presented in Table 4. There were no differences between cycles in allele frequencies. Frequencies for alleles at SNP2 were balanced (0.504 for C and 0.496 for T, respectively) whereas at SNP1 the C allele was predominant over the T allele (0.697 and 0.303, respectively). Genotype frequencies were in Hardy-Weinberg equilibrium in the first cycle, but not in the second one. Trends in allele frequencies were reflected in the two-marker haplotype frequencies, for which the corresponding estimated values were 0.338 for CC, 0.360 for CT, 0.169 for TC, and 0.134 for TT.

Table 4. Genotypic frequencies for markers in the promoter (SNP1) and exon 2 (SNP2) of the bovine leptin gene, discriminated by cycle.

Genotype	2004/2005		2005/2006		Total	
	N	%	N	%	N	%
SNP1						
CC	27	45.7	109	57.6	136	54.6
CT	24	40.7	51	26.7	75	30.1
TT	8	13.6	30	15.7	38	15.3
Total	59		190		249	
SNP2						
CC	17	28.3	37	19.5	54	21.6
CT	31	51.7	113	59.4	144	57.6
TT	12	20.0	40	21.1	52	20.8
Total	60		190		250	

Association analyses

Cycle and farm of origin had highly significant effects ($P < 0.01$) on every analyzed trait, with a few exceptions. The effect of cycle was significant ($P < 0.05$) for IMF, whereas it had no effect on BFDG or REA. Farm of origin had significant effects on LWDG, BFDG, CY, carcass length, and BFT but had no effect on IMF. The gene pools represented by the different groups of sires were probably the most influential factor contributing to the farm of origin effect.

Considered overall, there was little influence of the polymorphisms in the leptin gene on productive and quality traits of grazing Brangus steers (Tables 5 and 6, showing results from single marker analysis only).

LWDG and BFDG were analyzed in a comparatively short interval (approximately three months, from September to November) that corresponded to the highest growth rates of the entire fattening period of both cycles. No significant effect of any marker was detected for these traits.

The SNP located at position -2530 (SNP1) had a significant effect on USREA, with CT being higher than CC. That same genotypic class tended to be heavier and leaner at slaughter (Tables 5 and 6). SNP1 was also significant for USREA in the two-marker analysis and consistently, there was a significant haplotype effect for this trait (b = 2.53 ± 1.14 cm^2 for TC). At slaughter, the CT genotype tended to have heavier carcasses and higher CY and REA. The effect of SNP1 on USREA could be taken *a priori* as a direct effect of the gene on muscle growth. Taking into account the known effects of leptin on metabolism, a probably better explanation is that because steers were slaughtered at an approximately constant body composition, those with the CC genotype were slightly heavier and had more muscle than their counterparts.

The marker in exon 2 (SNP2) had a significant effect on CY and BFT only. The CT genotype, which had a higher CY than the TT group, also tended to have lower LWDG in spring and lower carcass KF and KFP. Surprisingly, the genotypic class that tended to be leaner based on KF and KFP values, had higher carcass BFT. This result could be partially explained by the influence of mechanical hide pulling that tends to remove subcutaneous fat during the process. For BFT only, the effect remained when both markers were included simultaneously in the model. In the haplotype analysis, CC was associated with higher BFT when compared to the most abundant CT haplotype (b = 0.40 ± 0.15 mm).

Table 5. Associations of single nucleotide polymorphisms (SNP) in the promoter (SNP1) and exon 2 (SNP2) of the leptin gene with live weight gain (LWDG) and gain in backfat thickness (BFDG) in spring, final live weight (FW), final ultrasound backfat thickness (USBFT), and final ultrasound rib eye area (USREA) of grazing Brangus steers.

Trait	SNP1 least square means								
	CC			CT			TT		
	N	Mean	SE	N	Mean	SE	N	Mean	SE
LWDG (kg/day)	132	1.031	0.02	75	1.025	0.022	37	1.029	0.032
BFDG (mm/day)	134	0.014	0.0014	75	0.016	0.0016	37	0.017	0.0024
FW (kg)	134	439.9	4.6	75	446.0	5.4	37	436.2	8.0
USBFT (mm)	133	6.39	0.15	75	6.23	0.18	37	6.68	0.25
USREA (cm^2)	132	55.9[a]	0.79	75	58.9[b]	0.93	37	57.7[ab]	1.38
Trait	SNP2 least square means								
	CC			CT			TT		
	N	Mean	SE	N	Mean	SE	N	Mean	SE
LWDG (kg/day)	53	1.051	0.026	141	1.012	0.018	51	1.055	0.026
BFDG (mm/day)	54	0.015	0.0019	142	0.016	0.0013	51	0.014	0.0019
FW (kg)	54	447.0	6.5	142	440.0	4.4	51	442.2	6.6
USBFT (mm)	54	6.28	0.21	141	6.52	0.18	50	6.18	0.22
USREA (cm^2)	54	58.4	1.1	141	57.5	0.8	51	55.8	1.2

Trait means with different letters are significantly different ($P < 0.05$). SE = standard error of the mean.

Table 6. Associations of single nucleotide polymorphisms (SNP) in the promoter (SNP1) and exon 2 (SNP2) of the leptin gene with carcass weight (CW), carcass yield (CY), carcass length (CL), kidney fat (KF), kidney fat percentage (KFP), backfat thickness (BFT), rib eye area (REA), and intramuscular fat percentage (IMF) of grazing Brangus steers.

Trait	SNP1 least square means								
	CC			CT			TT		
	N	Mean	SE	N	Mean	SE	N	Mean	SE
CW (kg)	134	241.1	2.6	75	245.6	3.1	37	238.9	4.6
CY (%)	134	54.74	0.17	75	55.00	0.19	37	54.68	0.29
CL (cm)	134	123.3	0.44	75	124.0	0.52	37	123.0	0.78
KF (kg)	134	2.881	0.104	75	2.782	0.124	37	2.648	0.183
KFP (%)	134	1.16	0.04	75	1.09	0.04	37	1.08	0.06
BFT (mm)	134	3.03	0.13	75	3.06	0.15	37	2.73	0.22
REA (cm^2)	133	66.42	0.85	75	67.97	1.00	37	67.61	1.49
IMF (%)	131	2.63	0.10	75	2.63	0.11	37	2.62	0.17
Trait	SNP2 least square means								
	CC			CT			TT		
	N	Mean	SE	N	Mean	SE	N	Mean	SE
CW (kg)	54	245.2	3.8	142	242.5	2.5	51	240.9	3.8
CY (%)	54	54.79^{ab}	0.23	142	55.02^{a}	0.16	51	54.4^{b}	0.24
CL (cm)	54	123.7	0.63	142	123.4	0.42	51	123.7	0.64
KF (kg)	54	2.800	0.148	142	2.766	0.100	51	3.013	0.152
KFP (%)	54	1.11	0.05	142	1.10	0.03	51	1.20	0.05
BFT (mm)	54	3.22^{a}	0.17	142	3.13^{a}	0.12	51	2.55^{b}	0.18
REA (cm^2)	54	67.8	1.2	141	67.0	0.8	51	66.7	1.2
IMF (%)	52	2.59	0.14	141	2.63	0.09	51	2.68	0.14

Trait means with different letters are significantly different ($P < 0.05$). SE = standard error of the mean.

DISCUSSION

Sequence analysis

A comparative analysis of the leptin promoter was reported by Liefers et al. (2005). We repeated the analysis adding more species to the comparison in order to increase the resolution in the identification of conserved regions.

New polymorphisms were discovered in the bovine leptin gene through sequencing (Table 3). However, none of them introduced changes in the corresponding protein sequence or modified TFBS. The comparative sequence analysis confirmed that the most conserved region of the leptin promoter corresponds to the first 500 bp upstream of the transcription start site. From all the polymorphisms previously described in the leptin promoter, only one seems to be located within a TFBS. A C/G SNP at -105 reported by Liefers et al. (2004) is supposed to affect the binding affinity in an SP1 binding site, in such a way that differential expression between alternative genotypes is noticeable (Adamowicz et al., 2006). However, we genotyped this SNP in 32 Brangus steers and found that they were all homozygous for the C allele. Therefore, it was not further considered for the association study.

It can be concluded that the leptin gene has an essential role in metabolism across species, and most mutations affecting either expression level or protein sequence are not tolerated by natural selection.

Association analysis

The marker identified as SNP1 in this paper was originally described by Nkrumah et al. (2005). The reported frequency of the T allele ranged from 0.11 to 0.26 in a heterogeneous sample of composite breeds. This allele was associated with higher serum leptin, higher final weight and backfat thickness and more marbling, among other effects.

Buchanan et al. (2002) originally evaluated the polymorphism designated here as SNP2 in beef cattle. They found a significant association of the T allele with carcass fatness. This allele had a higher frequency in British breeds compared to Continental breeds (0.58 in Angus, 0.34 in Charolais). Later, Nkrumah et al. (2004) reported an association of the T allele with higher carcass grades but lower carcass lean percentage, with no effect on feed intake or feed efficiency. In a sample of crossbred steers with varied genetic background, the frequency of T was between 0.42 and 0.71. A similar effect of SNP2 on carcass quality traits was obtained by Kononoff et al. (2005).

The same markers considered in the present study were evaluated by Schenkel et al. (2005) in crossbred cattle with different proportions of Angus, Charolais, Limousin, and Simmental. The frequencies of the T allele were between 0.22 to 0.34 for SNP1 and 0.41 to 0.54 for SNP2. Interestingly, in that study, SNP2 but not SNP1 had significant associations with carcass traits. When allele frequencies at SNP2 across studies are considered, there seems to be a trend towards a higher frequency of the T allele in early fattening breeds, while there is no clear distinction for SNP1. Another interesting feature of SNP2 is the type of gene action. For many composition and quality traits, relative comparisons among genotypic classes have shown no consistent distinction between additivity and dominance (Nkrumah et al., 2004; Schenkel et al., 2005; Di Stasio et al., 2007).

Most association studies involving leptin have focused on carcass composition and beef quality traits; Di Stasio et al. (2007) reported one of the few studies that included growth traits. In a sample of 59 Blonde d'Aquitaine bulls, the C allele at SNP2 had a favorable effect on weight gain.

Although there is no information about allele frequencies in zebu breeds, frequencies reported here are within the ranges cited in the literature for both markers. Haplotype frequencies were highly consistent with previous results, with CC and CT being the most abundant combinations.

Contrary to previous experimental results involving leptin markers in beef cattle, the effects of leptin polymorphisms on grazing Brangus steers reported here were modest at the most. Several explanations could be suggested for the lack of effect of markers in the leptin gene reported here.

Average daily weight gain in this study was on the order of 700 g/day, well below the growth potential of the animals. Growth rates of this magnitude are not uncommon in grazing systems. In these conditions, nutritional environment poses a strong constraint to the manifestation of genetic variation of any kind affecting growth and body composition.

Beside the influence of growth rate, both the initial and final weights of the fattening period (that increased from 220 to 450 kg, approximately) are below those of other production systems. Barendse et al. (2005) pointed out that this could be another source of discrepancies among studies.

Different breeds and crosses have been included in previous association analyses in-

volving leptin. The product of the leptin gene is part of a complex signaling network regulating energy balance and body composition. Manifestation of measurable phenotypic effects involving leptin metabolism could probably require epistatic interactions, which in turn lead to a genetic background effect. Just to give one example, polymorphisms in the leptin receptor have been significantly associated with production traits in beef cattle (Schenkel et al., 2006).

Differences in circulating leptin levels have been reported among Angus, Brahman and Brangus (Thomas et al., 2002), with the British breed having the highest. However, this kind of results has not been linked to variation at the genomic level. The only results evaluating the leptin gene in Brangus correspond to those reported by Almeida et al. (2003). In that study, there was a significant association of linked anonymous restriction fragment length polymorphisms and microsatellites with calving interval.

Bos indicus breeds (mostly Brahman and, in a very small proportion, Nellore) make a nominal contribution of 37.5% to the genetic pool of Argentine 3/8-Brangus cattle, which is the most broadly adopted variety within the composite breed. A very intensive selection process takes place to obtain registered Brangus seedstock. Therefore, the actual contribution of *Bos indicus* to the different bloodlines that exist within the breed is unknown. Moreover, a non-random contribution of each breed (either Angus or zebu) could take place in specific regions of the genome due to selection.

As the research on leptin metabolism progresses, new modulating factors are being identified. Recently, photoperiod has been established as a factor affecting the expression of leptin and leptin receptor genes in dairy cattle (Bernabucci et al., 2006). Photoperiod could be another factor contributing to the lack of agreement among different association studies in beef cattle as well. Further research is needed to discriminate between genetic and environmental factors affecting association studies such as the one reported here with Brangus cattle.

We are aware of the fact that a larger sample size would have provided higher statistical power to our association study. However, the experiment was conducted mainly for the validation of two previously known markers. Differences among genotypic classes, even those detected as statistically significant, were small and of little relevance for application in M.A.S. Under the same conditions as in the present study, leptin markers could be of help only as part of a larger genotyping panel including other relevant genes.

ACKNOWLEDGMENTS

Research supported by the National Agency of Science and Technology (ANPCyT) of Argentina, Grant PICTR 00177.

REFERENCES

Adamowicz T, Flisikowski K, Starzynski R, Zwierzchowski L, et al. (2006). Mutation in the Sp1 motif of the bovine leptin gene affects its expression. *Mamm. Genome* 17: 77-82.

Almeida SEM, Almeida EA, Moraes JCF and Weimer TA (2003). Molecular markers in the LEP gene and reproductive performance of beef cattle. *J. Anim. Breed. Genet.* 120: 106-113.

AOAC (Association of Official Analytical Chemists) (1992). Official Methods of Analysis of the Association of Official Analytical Chemistry. 15th edn. Association of Official Analytical Chemists, Washington, 139-140.

Barendse W, Bunch RJ and Harrison BE (2005). The leptin C73T missense mutation is not associated with marbling and fatness traits in a large gene mapping experiment in Australian cattle. *Anim. Genet.* 36: 86-88.

Bernabucci U, Basirico L, Lacetera N, Morera P, et al. (2006). Photoperiod affects gene expression of leptin and leptin receptors in adipose tissue from lactating dairy cows. *J. Dairy Sci.* 89: 4678-4686.

Buchanan FC, Fitzsimmons CJ, Van Kessel AG, Thue TD, et al. (2002). Association of a missense mutation in the bovine leptin gene with carcass fat content and leptin mRNA levels. *Genet. Sel. Evol.* 34: 105-116.

Di Stasio L, Brugiapaglia A, Galloni M, Destefanis G, et al. (2007). Effect of the leptin c.73T>C mutation on carcass traits in beef cattle. *Anim. Genet.* 38: 316-317.

Geary TW, McFadin EL, MacNeil MD, Grings EE, et al. (2003). Leptin as a predictor of carcass composition in beef cattle. *J. Anim. Sci.* 81: 1-8.

Houseknecht KL, Baile CA, Matteri RL and Spurlock ME (1998). The biology of leptin: a review. *J. Anim. Sci.* 76: 1405-1420.

Konfortov BA, Licence VE and Miller JR (1999). Re-sequencing of DNA from a diverse panel of cattle reveals a high level of polymorphism in both intron and exon. *Mamm. Genome* 10: 1142-1145.

Kononoff PJ, Deobald HM, Stewart EL, Laycock AD, et al. (2005). The effect of a leptin single nucleotide polymorphism on quality grade, yield grade, and carcass weight of beef cattle. *J. Anim. Sci.* 83: 927-932.

Lagonigro R, Wiener P, Pilla F, Woolliams JA, et al. (2003). A new mutation in the coding region of the bovine leptin gene associated with feed intake. *Anim. Genet.* 34: 371-374.

Liefers SC, Veerkamp RF, te Pas MF, Delavaud C, et al. (2004). A missense mutation in the bovine leptin receptor gene is associated with leptin concentrations during late pregnancy. *Anim. Genet.* 35: 138-141.

Liefers SC, Veerkamp RF, te Pas MF, Delavaud C, et al. (2005). Leptin promoter mutations affect leptin levels and performance traits in dairy cows. *Anim. Genet.* 36: 111-118.

Maniatis T, Fritsch EF and Sambrook J (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York.

Nkrumah JD, Li C, Basarab JB, Guercio S, et al. (2004). Association of a single nucleotide polymorphism in the bovine leptin gene with feed intake, feed efficiency, growth, feeding behaviour, carcass quality and body composition. *Can. J. Anim. Sci.* 84: 211-219.

Nkrumah JD, Li C, Yu J, Hansen C, et al. (2005). Polymorphisms in the bovine leptin promoter associated with serum leptin concentration, growth, feed intake, feeding behavior, and measures of carcass merit. *J. Anim. Sci.* 83: 20-28.

Orrù L, Napolitano F, Catillo G, De Matteis G, et al. (2006). High-throughput genotyping by DHPLC of the bovine leptin exon 3 reveals a novel single nucleotide polymorphism that results in an amino acid substitution. Proceedings of the 8th World Congress on Genetics Applied to Livestock Production, Belo Horizonte, Abstract 22-53.

Ovcharenko I, Loots GG, Giardine BM, Hou M, et al. (2005). Mulan: multiple-sequence local alignment and visualization for studying function and evolution. *Genome Res.* 15: 184-194.

Schenkel FS, Miller SP, Ye X, Moore SS, et al. (2005). Association of single nucleotide polymorphisms in the leptin gene with carcass and meat quality traits of beef cattle. *J. Anim. Sci.* 83: 2009-2020.

Schenkel FS, Miller SP, Moore SS, Li C, et al. (2006). Association of SNPs in the leptin and leptin receptor genes with different fat depots in beef cattle. In: 8th World Congress on Genetics Applied to Livestock Production, August 13-18. Instituto Prociência, Belo Horizonte.

Stephens M, Smith NJ and Donnelly P (2001). A new statistical method for haplotype reconstruction from population data. *Am. J. Hum. Genet.* 68: 978-989.

Taniguchi Y, Itoh T, Yamada T and Sasaki Y (2002). Genomic structure and promoter analysis of the bovine leptin gene. *IUBMB Life* 53: 131-135.

Thomas MG, Enns RM, Hallford DM, Keisler DH, et al. (2002). Relationships of metabolic hormones and serum glucose to growth and reproductive development in performance-tested Angus, Brangus, and Brahman bulls. *J. Anim. Sci.* 80: 757-767.

Ye S, Dhillon S, Ke X, Collins AR, et al. (2001). An efficient procedure for genotyping single nucleotide polymorphisms. *Nucleic Acids Res.* 29: E88.