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Polymorphisms Related to Bovine Leptin Gene and Association with Productive and Reproductive Traits in Nellore Heifers

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ABSTRACT

The identification of genes and polymorphisms in female zebu related to the leptin gene that may be involved in better productive and reproductive performances is relevant to the livestock and economic sector. Thus, the objective of this study was to evaluate the SNP markers (T954M and C305T) and the BM1500 microsatellite and to analyze their associations with the traits of body weight, age at first calving (AFC), calving interval (CI), and reproductive efficiency (RE) in Nellore heifers. DNA extraction was performed, followed by the amplification of all markers. Association analyses were carried out with each marker and each traits separately. The result showed that no significant association was found between the reproductive traits and all markers and between body weight and both SNP markers. For the BM1500 microsatellite, the effect of the allele 138 (p=0.0383) on body weight was observed, with the growth curve for this allele being related to the lower asymptotic weight. Therefore, the BM1500 microsatellite marker was the only one with a significant association with a productive trait providing information about the condition of body weight in Nellore heifers.

Keywords: body weight; growth curve; zebu cattle

INTRODUCTION

In Zebu cattle, traits related to female sexual precocity are economically important, because they provide the larger disponibility of animals in the herds which leads to a greater profitability for the production sector (Santana *et al.*, 2012). Traits such as age at first calving (AFC) and calving interval (CI) are parameters used in evaluation of reproductive efficiency (RE) of females bovine (Lacerda *et al.*, 2018). The onset of reproductive activity is indicated by puberty which is dependent of several factors such as race, environmental, food availability, and body weight. Regarding body weight, females of zebu cattle tend to reach the maturity of their reproductive system when the body weight is around half of the adult weight (Estill, 2014).

Puberty occurs when body energy stores are in adequate conditions and the signaling to the brain indicating such sufficient energy is provided by leptin (Wylie, 2011). Leptin is a peptide hormone produced primarily by adipocytes that play a role in body condition (Kowalski *et al.*, 2014). Polymorphisms related to the leptin gene (LEP) and its receptor (LEPR) have been associated with several traits of economic relevance in the livestock sector (Buchanan *et al.*, 2002; Komisarek & Dorynek, 2006; Ferraz *et al.*, 2009; Oliveira *et al.*, 2013; Silva *et al.*, 2014).

The leptin gene is directly and indirectly associated with the productive and reproductive traits, so it is relevant to investigate molecular markers that can seek for associations with economically important traits. Therefore, the aim of this study was to evaluate two single nucleotide polymorphism (SNP) markers, T945M e C305T, located in the leptin gene and in the leptin receptor gene, respectively, and the BM1500 microsatellite marker, located near to the stop codon of the leptin gene, then seek for the associations with AFC, CI, RE, and body weight traits in Nellore heifers.

MATERIALS AND METHODS

Animals and Phenotypes

Phenotype data from the Nellore heifers (*Bos taurus indicus*) used in this study were obtained from a commercial herd from São Jorge de Maracay Farm (Iguatemi/MS, Brazil). A total of 138 heifers, born between 1996 and 2007, were maintained exclusively with pasture (*Brachiaria* spp.) and mineral supplementation.

The live weight from birth up to 48 months of age was evaluated considering the several measurements obtained, characterizing a set of repeated measurements or longitudinal data. We also analyzed reproductive traits such as the age at first calving (AFC), calving in-

terval (CI), and reproductive efficiency (RE). The RE was calculated referring to Wilcox (1957) as follows:

$$RE\ (\%) = (365*(N-1)*100) / D$$

where RE (%) is the reproductive efficiency; N is the total number of parturitions; D is the number of days from the first to the last parturition; and 365 is the interval (days) between parturition desired.

DNA Extraction and Genotyping

DNA extraction was performed using aliquots of 300 μL of blood samples following the methodology described by Crispim *et al.* (2012). DNA quality and concentration were determined using DS-11 spectrophotometer (DeNovix®).

The DNA samples were genotyped for the following markers: SNP T945M (Komisarek & Dorynek, 2006), SNP C305T (Buchanan *et al.*, 2002) and BM1500 microsatellite (Fitzsimmons *et al.*, 1998). Polymerase chain reaction amplifications and enzymatic reactions were performed in a thermal cycler BIORAD®, model MyCycler™ Thermal Cycler.

The forward (5'GCAACTACAGATGCTCTAC TTTTGT3') and reverse (5'CAGGGAAATTTCCCTCAA GTTCAA3') primers were used to amplify a 400 bp fragment of the SNP T945M, based on sequences of the bovine leptin receptor gene available in GenBank (AN: AJ580801.1). The amplification reaction contained 6 μL of ultra-pure water, 1.5 μL (10 pM/μL) of each primer, 13 μL of the PCR Master MIX (ThermoScientific®, USA), and 2 μL of the genomic DNA (50 ng/μL). The amplification protocol consisted of initial denaturation (94ºC for 5 min), followed by 35 cycles (denaturation at 94ºC for 30 s, annealing at 56ºC for 30 s, and extension at 72ºC for 40 s) and final extension at 72ºC for 4 min. The fragments were digested in reaction containing 5 μL of ultra-pure water, 1 μL of 10X buffer, 3 μL of restriction enzyme Taq*I* (ThermoScientific®, USA), and 5 μL of PCR product at 65°C for 4 h.

The forward (5'ATGCGCTGTGGACCCCTGTA TC3') and reverse (5'TTCCGGAAGGTCCAGAGATG ACACCA3') primers were used to amplify a fragment of 94 bp of the SNP C305T, based on sequences of the bovine leptin genes available in GenBank (AN: AY138588.1). The amplification reaction contained 6 μL of ultra-pure water, 2.0 μL (10 pM/μL) of each primer, 12.5 μL of the PCR Master MIX (ThermoScientific®, USA), and 2.5 μL (50 ng/μL) of genomic DNA. The amplification conditions consisted of initial denaturation (94°C for 4 min) followed by 35 cycles (denaturation at 94°C for 45 s, annealing at 56°C for 55 s, and extension at 72°C for 50 s) and final extension at 72°C for 5 min. The fragments were digested in reaction containing 13 μL of ultra-pure water, 1.5 μL of the buffer, 2 μL of restriction enzyme Kpn2*I* (ThermoScientific®, USA), and 12 μL of the amplified product at 37°C for 6 h.

Finally, the forward (5'GATGCAGCAGACCAAG TGG3') and reverse (CCCTTGCTAGAACCCAGG3') primers were used to amplify the BM1500 microsatellite available in GenBank (AN: G18568.1). The amplification reaction contained 7.5 μL of ultra-pure water, 1.5 μL (10 pM/μL) of each primer, 12.5 μL of the PCR Mix (ThermoScientific®, USA), and 2.0 μL (50 μg/μL) of the genomic DNA. The amplification conditions showed an initial denaturation step (94°C for 3 min), followed by 25 cycles (denaturation at 94°C for 45 s, annealing at 57°C for 30 s, and extension at 72°C for 45 s); and final extension at 72°C for 4 min.

The fragments obtained from the enzymatic digestion for both SNP were analyzed in 2% agarose gel electrophoresis stained with ethidium bromide (0.05 μg/mL), and the alleles for the BM1500 microsatellite were submitted to 7% polyacrylamide gel electrophoresis stained with silver nitrate.

Populations and Statistical Analyses

The population parameters examined (allele and genotype frequencies, expected (H_E) and observed (H_O) heterozygosity, polymorphic information content (PIC), and Hardy-Weinberg equilibrium (HWE) test were calculated using the CERVUS v.3.0 software (Kalinowski *et al.*, 2007).

The statistical analyses were performed using the SAS® (Statistical Analysis System) University Edition software (available online). The association analyses between the reproductive traits (AFC, CI, and RE) and the molecular markers considered as fixed effects of the year of birth of the cow and each genotype of the molecular markers individually (SNP T945M and C305T and BM1500 microsatellite), as well as the random effect of sire, using the mixed model in the PROC MIXED procedure.

In the association analyses between the markers and the repeated measures of weight were considered as fixed effects of the year of birth of the cow, the age of the animal at the weighing date, the genotypes of each marker individually (both SNP), and the random effect of sire nested to each animal.

The association analysis for the BM1500 microsatellite was performed considering the absence and presence of the alleles in each genotype (0 for absence and 1, 2, and 3 for presence, respectively, for the alleles 138, 147, and 149). Numbers of copies of each allele were also evaluated in both the homozygous and heterozygous forms (0, 1, and 2 copies) and the absence and presence of the alleles in homozygosis (0 and 1, respectively).

The fixed and random effects for the BM1500 microsatellite were the same used for the SNP markers. The PROC MIXED procedure from SAS® software was used to analyze the mixed model, considering the age of the animal at the weighing date as a repeated measure in the REPEATED function. The TOEP (Toeplitz) variance structure was the most adequate for the data set analyzed.

The non-linear Gompertz model (Laird, 1965) was applied in the analysis with the weight data and the BM1500 microsatellite in order to obtain the growth curve parameters using the Marquardt method (Marquardt, 1963). The nonlinear regression by the NLIN procedure of the SAS® software was used to get the fit to the reduced model (average curve) and the complete model (curve fit for each allele separately). The

Gompertz model was calculated as follows:

$$y_t = C^{(-b^{(-at)})} + \varepsilon_t$$

where *y* represents the average weight of the animals as a function of age (*t*); *C* is the asymptotic or adult weight, when $t \rightarrow \infty$; *b* is an integration constant without biological interpretation with the role of modeling the growth curve to a sigmoidal format, from birth to adulthood of the animal; *a* is the rate or maturity index, which measures the variation in the speed of growth, being an indicator of the speed in which the animal reaches the asymptotic weight; and ε is the random error associated with each weighing (Silva *et al.*, 2011; Marinho *et al.*, 2013). After obtaining the curves for both models, the Lack of Fit test was performed to test the fit of the complete model in relation to the reduced model.

RESULTS

Population Analyses

The SNP T945M presented two allelic variations (C and T alleles) and three fragment sizes, 400 bp resulting from amplification (TT genotype) and 375 and 25 bp (CC genotype), obtained after the enzymatic digestion using the restriction endonuclease Taq*I*. The heterozygous CT genotype showed three different fragment sizes: 400, 375, and 25 bp. The C allele was the most frequent, along with the CC genotype, found in 73% of the population (Table 1).

Two allelic variations were also observed for the SNP C305T and the sizes of the fragments obtained after amplification and enzymatic digestion with Kpn2*I* endonuclease were 94 bp (TT genotype) and 75 and 19 bp (CC genotype), respectively. The heterozygous CT genotype showed the three fragment sizes and was found in 29% of the population.

Three alleles and five genotypes were obtained for the BM1500 microsatellite. The allele 147 was the most frequent (73%), as well as the CC genotype, present in 55% of the population (Table 1). The lowest allele frequency was observed for the 138 allele (6%) and any homozygous genotype was observed for this allele.

The PIC values ranged from 0.22 (SNP T945M) to 0.37 (BM1500 microsatellite) and the Hardy-Weinberg Equilibrium test was not significant for any marker.

Association Analyses

No significant association was observed between all the markers and the reproductive traits (Table 2). The analysis of the mixed model between both SNP markers and the weight data showed that no significant effect for T945M (p=0.1641) and C305T (p=0.0681) was observed. On the other hand, in the evaluation of BM1500 microsatellite and the weight data, a significant association was found for the allele 138 (p=0.0383). The parameters of the Gompertz model obtained by the fit of the nonlinear model of the weight-age data related to the alleles of the BM1500 microsatellite were presented in Table 3.

The comparison of fit of growth curves of the completed and reduced model by the Lack of Fit test presented significant effect (p=0.0128), therefore a significant difference was observed for the fit of the curves of both models (Figure 1). The Figure 1 showed the growth curves for the completed and reduced model for the weight-age data of Nellore heifers in relation to the alleles obtained for the BM1500 microsatellite marker. The curve for allele 138 showed the lowest growth for weight during the 48 months of observation and the Lack of Fit test was significant, meaning that the curve for the allele 138 was also significant, so this allele was associated with the lower weight in the heifers.

DISCUSSION

The highest allele frequency (85%) for T945M was observed for the C allele (Table 1). Silva *et al.* (2012) and Pinto *et al*. (2011) reported in their study with Nellore bulls a similar frequency for the same allele i.e., 88% and 89% respectively. The genotype frequencies for the CC genotype were higher in both studies.

For the C305T, there was a predominance of the C allele (82%) over the T allele (0.18) and higher frequency for the CC genotype (0.67), results that are similar with the study of Oliveira *et al.* (2013) that used animals from

Table 1. Number of individuals (N), allelic and genotypic frequencies, expected (H_E) and observed heterozygosity (H_O), polymorphic information content (PIC) and Hardy-Weinberg Equilibrium (HWE) for a population of Nellore heifers

Marker	N	Allele frequency	Genotypic frequency	H_O	H_E	PIC	HWE
T945M	101	C = 0.85 T = 0.15	CC = 0.73	0.24	0.25	0.22	NS
	34		CT = 0.24				
	3		TT = 0.02				
C305T	93	C = 0.82 T = 0.18	CC = 0.67	0.29	0.29	0.25	NS
	41		CT = 0.29				
	4		TT = 0.03				
BM1500	0	138 = 0.06 147 = 0.73 149 = 0.21	138/138 = 0.00	0.4	0.41	0.37	NS
	12		147/138 = 0.08				
	76		149/138 = 0.02				
	3		147/147 = 0.55				
	40		149/147 = 0.30				
	7		149/149 = 0.05				

Note: NS= not significant.

Table 2. Estimated average and standard deviations for the of age at first calving (AFC), calving interval (CI) and reproductive efficiency (RE) for the genotypes of the SNP T945M and C305T and for the alleles of the BM1500 microsatellite in a population of Nellore heifers

Marker	Genotype/ Allele	AFC	CI	RE
T945M	CC	29.81 ± 0.64	420.05 ± 10.61	84.88 ± 2.77
	CT	30.84 ± 0.87	427.11 ± 15.32	85.88 ± 3.66
	TT	29.57 ± 2.14	435.93 ± 41.32	76.25 ± 8.35
	p value	0.3244	0.8162	0.5122
C305T	CC	29.85 ± 0.69	424.81 ± 11.34	85.06 ± 2.91
	CT	30.04 ± 0.76	415.40 ± 12.76	84.51 ± 3.20
	TT	30.22 ± 1.84	437.60 ± 28.51	87.13 ± 7.21
	p value	0.9446	0.6143	0.9251
BM1500*				
Allele 138	0	28.35 ± 3.64	447.20 ± 60.10	78.23 ± 15.55
	1	28.07 ± 4.12	473.73 ± 68.79	77.75 ± 13.09
	p value	0.9518	0.4279	0.2034
Allele 147	0	28.18 ± 3.19	448.96 ± 60.76	82.57 ± 10.52
	2	28.33 ± 3.72	449.81 ± 61.48	77.83 ± 15.56
	p value	0.4539	0.3369	0.1607
Allele 149	0	28.18 ± 3.79	448.78 ± 59.10	77.53 ± 15.81
	3	28.56 ± 3.49	451.26 ± 65.02	79.33 ± 14.35
	p value	0.749	0.8203	0.4983
Allele copy 138*	0	28.35 ± 3.64	447.20 ± 60.10	78.23 ± 15.55
	1	28.07 ± 4.12	473.73 ± 68.79	77.75 ± 13.09
	p value	0.9518	0.4279	0.2034
Allele copy 147	0	28.18 ± 3.18	448.96 ± 60.76	82.57 ± 10.52
	1	28.54 ± 3.81	458.47 ± 69.72	78.06 ± 14.89
	2	28.18 ± 3.68	444.28 ± 55.52	77.68 ± 16.09
	p value	0.7552	0.1945	0.3112
Allele copy 149	0	28.18 ± 3.80	448.78 ± 59.10	77.53 ± 15.81
	1	28.60 ± 3.49	451.38 ± 64.69	78.77 ± 14.70
	2	28.35 ± 3.81	450.68 ± 71.83	82.67 ± 12.42
	p value	0.8412	0.8776	0.6196
Allele 147 vs 147	0	28.48 ± 3.69	456.44 ± 67.39	78.81 ± 14.28
	1	28.18 ± 3.68	444.28 ± 55.52	77.68 ± 16.09
	p value	0.8358	0.2953	0.2972
Allele 149 vs 149	0	28.32 ± 3.68	449.66 ± 60.71	77.93 ± 15.41
	1	28.34 ± 3.81	450.68 ± 71.83	82.67 ± 12.42
	p value	0.57	0.7232	0.3784

Note: *The copy of the allele 138 appears only in heterozygosity, so the average values obtained for the reproductive traits are the same for the 138 allele, as there is only one copy.
Obs.: The association analyses for the BM1500 microsatellite was performed using its alleles, so 0 is the absence of the allele and 1, 2 and 3 is the presence for the alleles 138, 147 and 149, respectively. The numbers of copies of each allele are 1 and 2 for homozygote and heterozygote genotype, respectively.

Table 3. Asymptotic weight (*C*), integration constant (*b*) and maturation rate (*a*) and their standard deviations for the Gompertz model adjusted for weight-age data, from birth to 48 months age in Nellore heifers

	Parameters		
	C	*b*	*a*
Average Curve (reduced model)	436.8 ± 3.85	2.9342 ± 0.07	0.2807 ± 0.007
Allele curve for alleles 147 and 149	443.0 ± 4.14	2.9003 ± 0.07	0.2720 ± 0.007
Allele curve for allele 138	386.8 ± 9.34	3.4349 ± 0.34	0.3798 ± 0.029

a commercial herd of Nellore cattle. Buchanan *et al.* (2002) found frequencies of 0.54 and 0.46 for the C and T alleles, respectively, in a study performed with taurine breed and these authors also found a higher frequency for the C allele.

Fitzsimmons *et al.* (1998) studied the BM1500 mic-

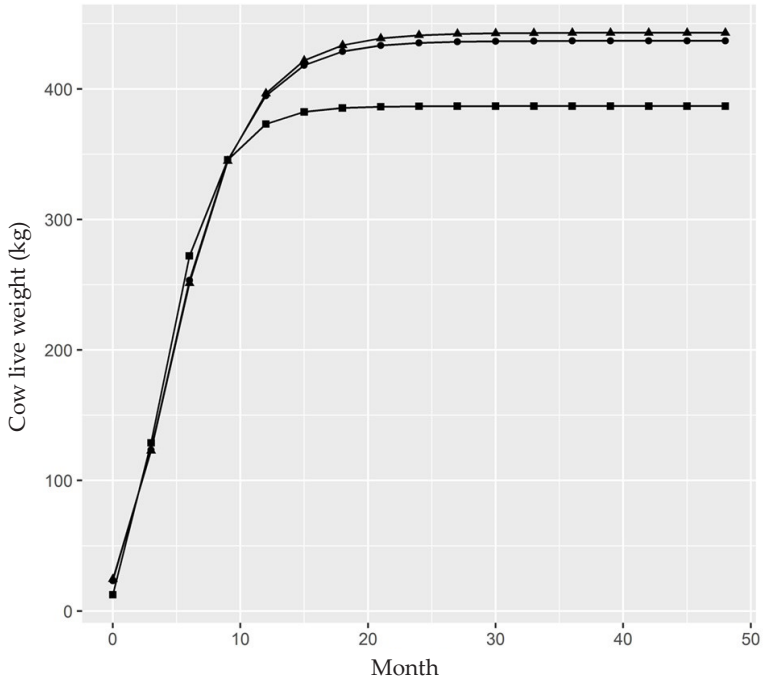

Figure 1. Growth curve of reduced model (BM1500 microsatellite marker) and growth curves of completed model (allele 138 and other alleles – 147 e 149) for weight measurement, from birth up to 48 months old, in Nellore heifers. (●= reduced model; ▲= other alleles; ■= allele 138).

rosatellite in taurine breed and observed a higher allele frequency for the 138 allele (47%). This result differs from the present study where the 138 allele showed the lowest frequency (Table 1) and the genotype 138/138 was not observed. Silva *et al.* (2014) studied this marker in Nellore males and also found a lower frequency for the 138 allele (8%), but the higher genotype frequency was observed for genotype 149/149 (70%), differently from this study that found a higher frequency genotype to 147/147 (55%).

The differences observed for the allelic and genotypic frequencies when compared with previous studies for the same markers are relevant, once studies in the literature were developed mostly with taurine bull.

The classification of Botstein *et al.* (1980) for PIC considers three categories, low (PIC<0.25), moderate (0.25<PIC<0.50), and high (PIC>0.50) informative. Both SNP markers presented low to moderate values of PIC and the BM1500 microsatellite presented a moderate informative PIC value. Despite being a marker with a high degree of polymorphic information, the PIC observed (0.37) may be justified by the size of the sample group.

The herd presented a non-significant result for the Hardy-Weinberg equilibrium test for all markers. The farm used the traits as selection indices based on the Nellore Brazil Program from the National Association of Researchers and Breeders (ANPC) and among the traits, several of them was related to body weight. Although the selection has been occurred in the herd for over 30 years, the frequencies observed for the markers were not influenced, therefore there were no deviations in the Hardy-Weinberg equilibrium.

Regarding the association analyses, both SNP markers and the BM1500 microsatellite had no influence on reproductive traits (Table 2). The body weight also showed no correlation with both SNP markers, however the BM1500 microsatellite presented a significant effect of the allele 138. This allele was associated with a lower weight in heifers and as observed in Figure 1, the curve for the allele 138 was lower when compared to the curve for the other alleles, in relation to the average asymptotic weight. It was observed that around the tenth month of the growth curves showed different behavior so the performance of the heifers was affected by the environment conditions and was not directly influenced by the maternal effects (Boligon *et al.*, 2019).

These results corroborate with the population analysis (Table 1), where the frequencies found for the allele 138 were lower than the other ones and the genotype frequency for this allele was null. This fact indicates that during the selection process applied in this herd, the heifers with lower body weight were being eliminated because they did not present economic interest related to body weight. As a consequence, the allele was also eliminated because it was related to the lower body weight in females.

The microsatellite marker BM1500 is located approximately 3.6 Kb downstream of the leptin gene stop codon on bovine chromosome 4 (Buchanan *et al.*, 2002). This condition has been associated with different fat triats in beef bulls (Fitzsimmons *et al.*, 1998), body weight in Velásquez breed (Montoya *et al.*, 2009), and carcass traits (Silva *et al.*, 2014).

Leptin is a hormone mainly produced by white adipose tissue and is responsible for giving information to the brain about the body's fat store (Wylie, 2011), because the increase of body weight triggers the increase of adipose tissue, so more adipocytes induces the release

of more leptin in the bloodstream that is detected by brain receptors (Catunda *et al.*, 2014). The major role of the leptin in bovines is related to the onset of puberty that only occurs in adequate conditions of body energy store. Therefore, leptin displays an important role in reproduction because the maturity of reproductive system is dependent of puberty (Wylie, 2011; Symonds *et al.*, 2016).

The reproductive parameters used in this research are puberty dependent because the maturity of reproductive system only occurs after puberty (Wylie, 2011). The population of bovine heifers analyzed comes from a commercial herd where the selection was based on reproductive phenotypes, so the results found for AFC, CI, and RE reflect several years of selection applied in the animals, not being found significant association of these traits with the markers.

Despite the association with a trait that is not interesting from an economic point of view, the BM1500 marker may be used in marker assisted selection processes along with the other markers that also has significant associations with productive traits in beef cattle, in order to contribute to the better efficiency of the herd.

CONCLUSION

Both SNP markers did not have associations with the traits evaluated. The allele 138 for the BM1500 microsatellite was associated with lower body weight in Nellore heifers and it could be used as a genetic marker related to body weight.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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