Influence of leptin gene polymorphism on marbling percentage in Simmental cross breed cattle *(Bos Taurus)*

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ABSTRACT

Single nucleotide polymorphisms (SNP's) are the most frequent type of variation in the genomes of mammals, and they provide powerful tools for genetic selection. Therefore, they can be used to improve different quality traits by means of marker identification. The objective of this study was to detect polymorphisms in the obese gene of Chinese Simmental bulls, that are associated with variations in intramuscular fat content. Therefore, DNA from 51 Simmental bulls was collected and sequenced. Sequenced products were analyzed for SNP's and compared to the known bovine genome. Five SNP's were found in the leptin gene. There was a C-T substitution at location 14697 of leptin gene exon 2 resulting in a change in amino acid from Arganine (R) to Cysteine (C). In exon 3 an A-G substitution at location 18095, C-T substitution at location 18268, T-C substitution at 19130 and a G-T substitution at 19263 has been determent, however, because these SNP's are located in the non-coding region of the ob-gene, mutations do not result in amino acid substitutions. Two different statistical analyses were carried out: associations of both genotype and haplotypes with intra muscular fat content were evaluated. No significant differences in intra muscular fat content and individual leptin SNP genotypes were observed, nor was there any tendency towards significance. Also, there was no significant effect between different genotypes. These results are comparable as well as conflicting with similar published studies. Therefore, more research is needed before a reliable answer can be formulated for the of leptin gene SNP's in relation to IMF.

INTRODUCTION

Since we are capable to identify variations at DNA level, the study of molecular genetics made major steps forward. Especially at the level of single nucleotide polymorphisms (SNP's), which are mainly responsible for the variation of individuals. The interest in DNA variances that are responsible for specific traits in our livestock has been studied extensively. Although most of these traits are affected by many genes with small effects, the major gene model, described by Russel Lande (*1981*), suggests that even a few genes can provide large phenotypical differences. Therefore, these specific genes can be of great value when it comes to genetic improvement by means of marker identification *(Vivek Choudhary et al., 2005)*.

In the beef cattle industry, specific attention for breeding is crucial to achieve an increase of product quality. Therefore, gaining knowledge about the genetic variances that are responsible for the intramuscular fat content (IMF), commonly called marbling, could permit us to influence our beef production by using genetic selection. In our livestock, the IMF content is proven to be an important marker for meat quality *(Dubesky et al., 1997)*. Nowadays people become more and more aware of what they eat and there is more interest in meat containing less fat. However, it may be at the expense of flavour and tenderness. In a study where beef flavour and tenderness was scored, both scores markedly increased with increasing IMF content *(Thompson, 2004)*.

One way to measure IMF content is with scoring the marbling percentage. Marbling of meat refers to the white specks of adipose tissue between the bundles of muscle, which can be measured using an

ultrasound device. This is a quick, non-invasive, sensitive and easy method, making it an appropriate method to determine differences in total body fat amount between individual animals *(Greiner et al., 2003, Whittaker et al., 1992).* The marbling percentage, the amount of the muscle that consists of fat depositions, is in close relation to juiciness, aroma and tenderness of beef *(Abel et al., 2007).* The hormone that is closely related to the regulation of the amount of IMF content is leptin *(Dubey et al., 2007)*. Leptin, a protein product of the obese (ob-) gene, is a hormone that is produced and secreted by white adipose tissue mainly *(Chilliarda et al., 2001).* Other tissues, which are capable of producing leptin, are skeletal muscle, placenta, mammary glands and the pituitary. The ob-gene is a highly conserved gene across different species and is located on chromosome 4 in the bovine genome *(Stone et al., 1995)*. Its DNA sequence consists out of 15000 base pairs containing 3 exon regions of which exon two and three contain the protein-coding region *(Zieba et al., 2005)*. In the last years, it is well studied and it is proven to be involved in the regulation of feed intake, energy balance and milk production *(Ramsay and Cranwell., 1999)*. Also, leptin is known to play an important role in fertility, reproduction and certain immune system functions *(Dubey et al., 2007)*. Figure 1 shows an overview of the main body functions of leptin.

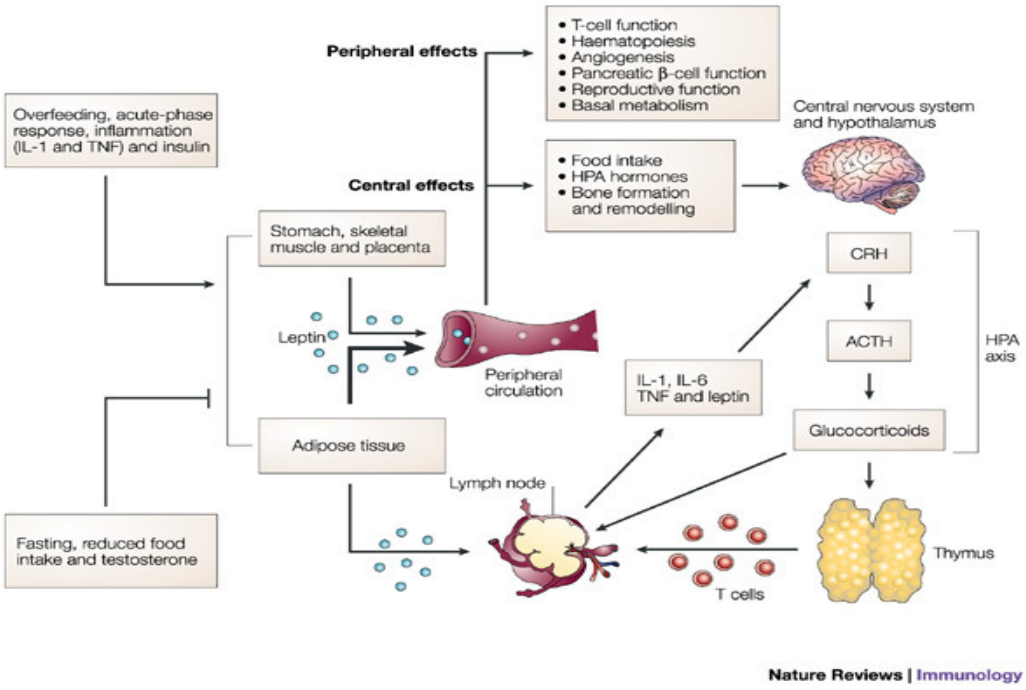

figure 1: central and peripheral effects of leptin in the body

Source: http://contagions.wordpress.com

The protein exerts its function by transmission of a lipostatic signal from the adipocytes to a leptin receptor in the hypothalamus. This signal results mainly in a suppression of appetite *(Dubey et al., 2007)*. Other studies suggest that leptin can directly modulate energy metabolism in peripheral tissues, increase lipolysis and decrease lipogenesis, and may antagonize insulin activities in adipose tissue *(Buchanan et al., 2002, Daix et al., 2008)*. In humans and rodents, expression of the ob-gene and circulating leptin concentrations are highly correlated with body fat content *(Daix et al., 2008).* Also, circulating leptin levels increase during fattening in rodents, chickens, pigs and sheep *(Buchanan et al., 2002)*. This made the ob-gene a strong candidate for evaluation of genetic polymorphisms that may influence fat content in the beef cattle carcass *(Buchanan et al., 2002).* Therefore, the objective of

this study was to determine if there is a correlation between specific polymorphisms in the obese gene and intramuscular fat content in the longissimus muscle of Simmental bulls.

MATERIAL AND METHODS

Animals

51 Healthy, 2-year-old, non-castrated pure breed Simmental bulls from the experiment station of Chinese National Beef Cattle Industrial Technology System at Zhangye, Gansu province, were used in this study. These animals were housed in traditional cowsheds and were fed grass silage, shredded corn and had access to water twice a day. Blood samples were collected from the jugular vein in 5 ml vacutainer tubes containing 0.5 mL of 2.7% ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Tubes were stored in 4°C overnight. Genomic DNA was extracted from 2 ml whole blood using the Relaxgene DNA extraction kit from Tiangen Biotech Co. Ltd. (Beijing, China) following the manufactures protocol.

Measurement of marbling percentage

From all animals, marbling percentage was measured using a Honda HS-2100 ultrasound device with a linear transducer and a frequency of 7,5 MHz, as recommended by Schröder and Staufenbiel *(2006)*. Skin contact with the transducer was ensured using motor oil. Images of Longissimus thoracis et lumborum muscle (L.M.) were made. In this study, the probe was placed between the 12^{th} and 13^{th} rib, 2 cm under the dorsal spinal condyl. The probe was placed parallel with the rib lining. For ultrasound scanning, all L.M. images were examined at the same position and following the same procedure. All images were saved on the ultrasound device in order to analyse them later and to maintain all data. Marbling was analysed using the measurement function of the ultrasound device (BCF Technology software).

Leptin Gene analysis

Nine primer pairs, covering the three exons in leptin gene, were designed from the published sequence of the *Bos Taurus* leptin gene (NC_007302.5 on NCBI GeneBank) using the program Primer Premier 5 (Premier Biosoft International, Palo Alto USA, *Lalitha, 2000*) (Table 1). Primer pairs for genotyping were synthesized by Invitrogen Biotechnology co., Ltd. (Shanghai).

Table 1: sequence and position of oligonucleotide primers.

Exon	Fragment	Primer sequence	Tm[1]	Topt[2]	GC%	Location	Size (bp)
Exon 1	Lep1 F:	5'-GCTTCGGCGGCTATAA-3'	40.8	55.0	56,2	2457-2999	543
	Lep1 R:	5'-CTTCGCATTCACAAGTCAG-3'	43.8		47,4		
Exon 2	Lep2 F:	5'-GCAAATCTTGTTGTTATCCG-3'	42.5	53.5	40,0	14488-14798	311
	Lep2 R:	5'-TTCTACCTCGTCTCCCAGTC-3'	48.7		55,0		
Exon 3	Lep3a F:	5'-GTGGGCACAAGAAGTAAGG-3'	46.0	49.5	52,6	16378-17083	706
	Lep3a R:	5'-GTGGGCGTGGAATCTGTA-3'	45.2		55,6		
	Lep3b F:	5'-GGCAGGAAACCAAAGATA-3'	40.6	53.5	44,4	17044-17821	798
	Lep3b R:	5'-TCACCACTACCCCGACA -3'	44.3		58,8		
	Lep3c F:	5'-AACCCATTTTGAGTGACTTG-3'	42.5	55.0	40,0	17434-18054	620
	Lep3c R:	5'-GAAAATCCTTCCCCAATG-3'	40.6		44,4		
	Lep3d F:	5'-GACCTTCTTTGGGATTTGC-3'	43.8	55.0	47,4	17985-18441	457
	Lep3d R:	5'-TTGCGTGCTGGTGAGTG-3'	40.8		56,2		
	Lep3e F:	5'-CCACCAACCTGCCATT-3'	40.8	52.4	56,2	18221-18801	581
	Lep3e R:	5'-TCTTCCAGCAAACACTCC-3'	42.9		50,0		
	Lep3f F:	5'-TCAGCAGGTGGGAAATG-3'	41.9	55.0	52,9	18490-19141	651
	Lep3f R:	5'-GCCAGGTTTTATGAGCAA-3'	40.6		44,4		
	Lep3g F:	5'-GATGGGAGGGAAGGGTT-3'	44.3	55.0	58,8	18949-19437	488
	Lep3g R:	5'-AGTGCTCTGGGCTTTGG-3'	44.3		58,8		

[1] Optimal individual annealing temperature in degrees Celcius, determined by Invitrogen Biotechnology co., Ltd at 50mM Na+.

[2] Optimal primer pair annealing temperature in degrees Celcius, determined like described in this paper.

Polymerase chain amplification (PCR) was carried out in a Biorad DNA Engine Dyad Peltier thermal cycler. First, the optimal primer temperature was determined for each primer pair using 1.0 µl DNA template derived and mixed from 5 randomly selected individuals, 3.4 µl purified water, 5.0µl Taq mastermix (CW Biotech) and 0.5µl from both the forward and reverse primer. The machine was programmed for the following conditions: denaturation at 94°C for 4.5 minutes, 30 cycles in temperature ranging from 45°C to 55°C for 40 seconds and 72°C for 1.5 minutes with a final extension step at 72°C of 10 minutes. To determine optimal primer temperature, DNA fragments were analyzed using gel electrophoresis on a 1.5% agarose gel (Agarose G-10, Triacetate EDTA (TAE) and fluorescent dye (S Green Nucleic Acid)). Then, DNA from 20 different samples was solved in purified water to create a concentration of 500ng/µl each. DNA was mixed and amplified using the 9 different primer pairs. The machine was programmed for the following conditions: denaturation at 94°C for 4.5

minutes, 30 cycles in the optimal annealing temperature determined before for 30 seconds and 72°C for 1.5 minutes with a final extension step at 72°C of 10 minutes. After amplification, PCR products were sent to Life company (Shanghai) to be sequenced, using the Applied Biosystems 3730 DNA analyzer (Applied Biosystems) (*Shendure and Ji, 2008*). Sequenced products were analyzed for single nucleotide polymorphism's (SNP's) using BLAST software (*NCBI website*). This way, sequencing results were compared with the known bovine genome. SNP genotyping from all 51 animals was completed by individual sample sequencing using only the fragments in which the SNP's were found: primers Lep2, Lep3a, Lep3d and Lep3g.

Statistical analysis

All analyses were performed using the statistical software SPSS 22 for Windows. The analysis of variance test (ANOVA) was used to statistically analyze the effect of genotype on all individual SNP's on intra muscular fat content and body weight. Also, the combination of genotypes at the 5 SNP's taken together (so called haplotypes), were analyzed in relation to IMF content and body weight.

H0 = There is no significant difference in the means of IMF content value for L.M. between animals with different ob-gene polymorphisms,

H1 = There is significant difference in the means of IMF content value for L.M. between animals with different ob-gene polymorphisms,

Additionally, Pearson's correlation test was used to determine the possible presence of a correlation between IMF content and body weight.

RESULTS

All PCR amplified products were consistent with the target fragment and of good quality and were, therefore, suitable for DNA sequencing. Doing this, five SNP's were found in the leptin gene. There was a C-T substitution at location 14697 of leptin gene exon 2, as shown in figure 1. This SNP was also found in a study by Buchanan and Tian (*Buchanan et al., 2002; Tian et al., 2013)*.

The mutation is located at the 25th nucleotide triplet and the C-T substitution results in a change in amino acid from Arganine (R) to Cysteine (C). Therefore, this SNP will be called E2-R25C in the rest of this paper.

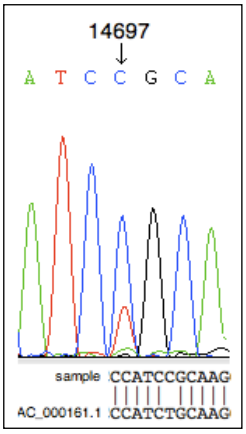

Figure 1: a T-C substitution was found at location 14697 in exon 2. The colored peaks are the sequencing results, showing variation between samples at location 14697. The line of letters underneath show blast-results. The upper line depicts the DNA sequence of our sample, the lower line shows the sequence from the same piece of Bos Taurus DNA, from the NCBI database (number AC_000161.1). This blast result confirms the variation that we already found in the colored peaks.

In the non-coding region following exon 3 (the 3' untranslated region), the following SNP's were found: A-G substitution at location 18095, C-T substitution at location 18268, T-C substitution at 19130 and a G-T substitution at 19263, as shown in figure 2. These polymorphisms are located at silent codon positions, which means they are not located in the coding region of the gene, but in the regulating region. Therefore, they do not affect the encoded amino acid.

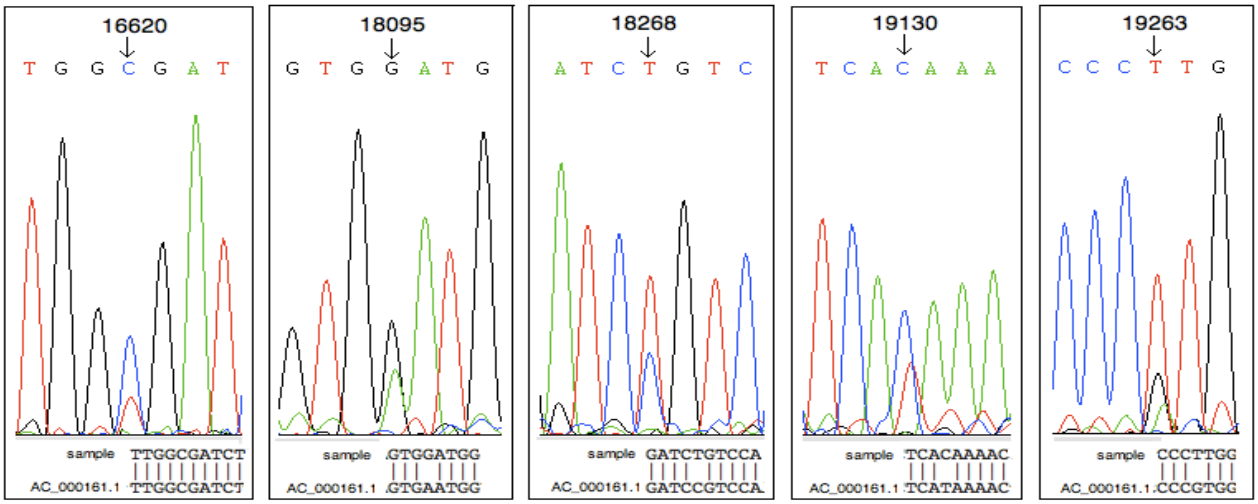

Figure 2: subsequent SNP's found in the 3' Untranslated region (non-coding region following exon 3): an A-G substitution at location 18095, C-T substitution at location 18268, T-C substitution at 19130 and a G-T substitution at 19263. Since these SNP's are located in the non-coding region of the ob-gene, mutations do not result in amino acid substitutions.

Genotype distribution

Genotypes on all five SNP-locations were determined from each individual animal. The frequencies of the different alleles are shown in table 2.

Population	*N*	*SNP location (exon-basepair)*	*Allel frequency*		*Genotype frequency*		
Simmental	51	E2-14679	T(0.69)	C(0.31)	CC(0.47)	CT(0.43)	TT(0.10)
	51	E3-18095	A(0.26)	G(0.74)	GG(0.49)	GA(0.49)	AA(0.02)
	51	E3-18268	T(0.74)	C(0.26)	TT(0.49)	CT(0.49)	CC(0.02)
	51	E3-19130	T(0.27)	C(0.73)	CC(0.45)	CT(0.55)	TT(0)
	51	E3-19263	T(0.76)	G(0.24)	TT(0.53)	GT(0.47)	GG(0)

Table 2: Different SNP's and associated allele and genotype frequencies.

No significant correlations between single nucleotide polymorphisms and the percentage of intra muscular fat content were detected for SNP locations: E2-14679 (P = 0.226), E3-18095 (P = 0.908), E3-18268 (P = 0.908), E3-19130 (P = 0.341) and for E3-19263 (P = 0.419) using the Chi Square test. Some of the genotype frequencies were not detected once: the TT-genotype of snp E3-19130 and the GG-genotype of snp E3-19263. Other genotypes were detected at very low frequencies, like snp E3-18095 and E3-19263, respectively genotype AA and CC, each detected once.

Linkage disequilibrium was noticed for the SNP's on locations 18095, 18268, 19130 and 19263. Linkage disequilibrium describes the condition when specific alleles of two or more different genes tend to appear together more frequently than random chance predicts. 43 Out of the 51 studied animals (84%) showed one of the two observed fixed sequences of these 4 alleles; either all alleles were homozygous (GG, TT, CC, TT) or all alleles were heterozygous (GA, CT, CT, GT). Only 8 out of 51 animals deviated from this fixed combination.

9 Different haplotypes were determined for the alleles on locations 14697, 18095, 18268, 19130 and 19236. Two different chromosomes make up the genotype of animals; hence one animal's leptin gene consists of two haplotypes. Three haplotypes, CGTCT, TGTCT and CACTG make up the leptin gene for 86% off all animals (43 out of 51 animals). Table 3 shows the different haplotypes, the number of times this haplotype was determined, and the frequency of these haplotypes in the population studied

Haplotypes	*Frequency*	Mean marbling	Std. Deviation	*Sig.*
CGTCT	0,33	3.9582	0.81296	0.488
TGTCT	0,25	3.7960	0.76513	0.359
CACTG	0,22	3.7982	0.77571	0.425
TACTT	0,02	4.6800	0.77782	0.165
TGTTT	0,02	3.2700	0.45255	0.260
CACCG	0,01	5.0700	0.0	0.144
CACTT	0,01	4.7800	0.0	0.274

CGTTT	0,01	5.1400	0.0	0.121
CACCT	0,01	4.7800	0.0	0.274

Table 3: Different haplotypes and associated frequencies in the population studied; mean marbling of all the individuals containing at least one time the haplotype; Sig.: significance of difference between individuals containing the haplotype and the one without.

From these different haplotypes, we can predict that some of the more rare haplotypes have originated by crossing over from another haplotype; for example TGTTT can originate from TGTCT when crossing over takes place before and after nucleotide 19130.

Two different statistical analyses were carried out: associations of both genotype and haplotypes with intra muscular fat content were evaluated. Descriptive characteristics of quantitative traits were obtained using Boxplots: data on intra muscular fat content and body weight were plotted against individual genotype (figure 3).

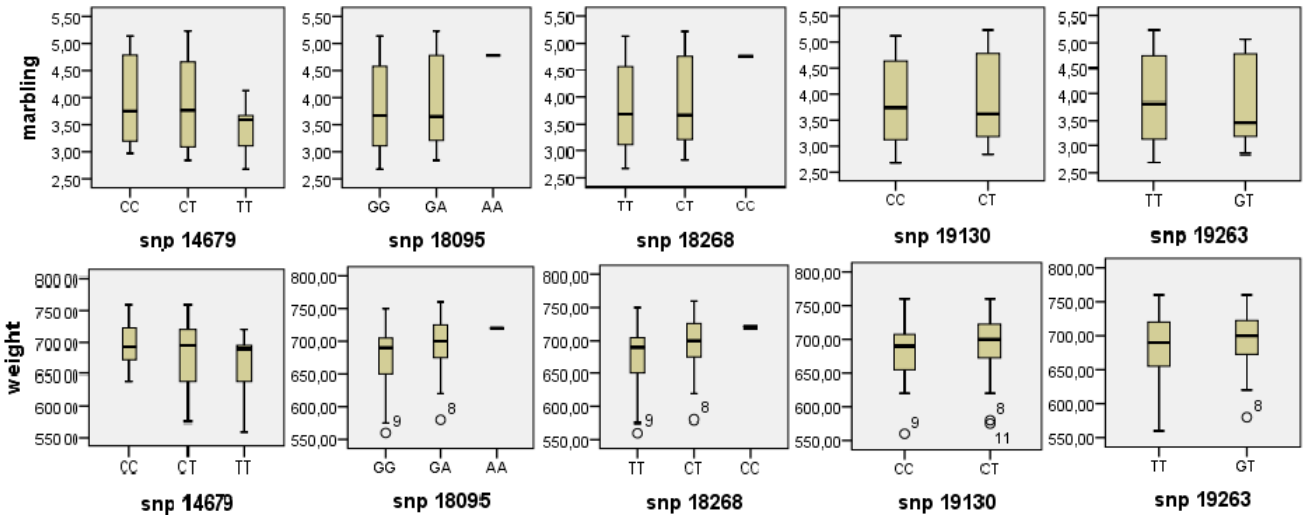

Figure 3: Data on individual SNP's plotted against BFT and bodyweight, shown in boxplots. No obvious trends were observed this way.

No obvious trends were observed this way. Results of one-way ANOVA analysis of the Simmental population for intra muscular fat content (3.9024±0.8033) (IMF Mean ± Standard Deviation) are shown in table 4. No significant differences in intra muscular fat content and individual leptin SNP genotypes were observed, nor was there any tendency towards significance. Also, there was no significant effect between different genotypes. Also, intra muscular fat content and body weight were similar for the different haplotypes. Therefore, It seems unlikely that variations in intra muscular fat content and body weight are caused by differences in genotype on the 5 SNP´s we found.

These results indicate that there is no association whatsoever between the genotypes and traits studied.

Loci	*Genotype*	*IMF LM±se*	*N*	*P*
E2-14679	CC	3.9975±0.17	24	0.371
	CT	3.9045±0.18	22	
	TT	3.4360±0.25	5	
E3-18095	GG	3.8500±0.16	25	0.528
	GA	3.9196±016	25	
	AA	4.7800±0	1	
E3-18268	TT	3.8500±0.16	25	0.528
	CT	3.9196±016	25	
	CC	4.7800±0	1	
E3-19130	CC	3.8974±0.17	23	0.371
	CT	3.9064±0.15	28	
E3-19263	TT	3.9425±0.15	28	0.698
	GT	3.8538±0.17	23	

Table 4: ANOVAs between genotypes of different SNP's and IMF values (%) for LM

DISCUSSION

In this study the hypothesis, that there is difference in the means of IMF values for *L.M. muscle* between animals with different ob-gene polymorphisms has not been confirmed. Neither was there any significant association between body weight and different genotypes. Not even tendencies towards significant association between genotypes and observed carcass traits were observed. This may suggest that these SNP's, found in exon 2 and 3 of the leptin gene, are not affecting the IMF and, therefore, may not be of great value by means of marker identification.

Several SNP's have been identified in the leptin gene since the interest in the leptin gene began. Some of those SNP's are and some are not associated with different carcass traits. One of the SNP's, which has been studied intensively, is the R25C mutation of exon 2 *(Anton et al., 2011, Buchanan et al., 2002, Nkrumah et al., 2003, Schenkel et al., 2005, Tian et al., 2013).* In our study SNP R25C was detected as well. The mutation is located at the 25th nucleotide triplet and the C-T substitution results in a change in amino acid from Arganine (R) to Cysteine (C). Although this substitution did not lead to any significant results with regard to IMF content in our study, there are studies which describe different outcomes. Cerón-Muñoz et al. (2008) for example, although they examined the amount of back fat thickness and weight, instead of IMF, significant effects have been reported by them. They found that animals with the TT genotype had higher adjusted means for BFT and weight compared to TC and CC animals for different breeds. Similar results for IMF have been described by Buchanan et al. (2002) and Anton et al. (2011). Buchanan et al. found that animals homozygous for T expressed higher levels of leptin mRNA, which might result in higher levels of serum leptin. Geary et al. (2003) proved that serum concentrations of leptin were significantly positively associated ($P<0.01$) with marbling score, BFT and quality grade (*Geary et al., 2003*). Anton et al. (2011) measured intramuscular fat content of the *musculus longissimus dorsi* (LD) and *musculus semitendinosus* (ST) in Hungarian Angus beef cattle. They found a significantly higher marbling percentage in TT-animals compared with CC and TC animals for LD samples as well. However, at leptin gene level there was no significant effect of the genotype on the fat content of LD according to our results. In contrast, Tian et al., found that the C-bearing genotypes of R25C mutation showed significantly higher dressed weight and higher IMF (*Tian et al., 2013*). As in the current study: Xin Li et al., (2013), Schenkel et al., (2005), Crews et al., (2004), Nkrumah et al., (2003), and Buchanan et al., (2002) found no association between marbling score and different genotypes of the leptin gene. However, some of these papers describe significant associations between different genotypes and other carcass traits. Schenkel et al., (2005) for example, confirms an association between the E2FB leptin exon 2 SNP and lean meat yield and fatness (*Schenkel et al., 2005*). Nevertheless, the increased fatness does not translate into higher IMF *(Schenkel et al., 2005).* Due to all the different outcomes of different studies, it can be noticed that conflicting evidence exists towards the relation between SNP's in the leptin gene and different carcass traits, whereof especially marbling.

One of the reasons why we did not find any association between leptin polymorphism and BFT in Bos taurus Simmental bulls could be the limited sample size (n=51). In our study some of the SNP-genotypes occurred only once. In addition, the mean IMF content was 3.9024 with a Standard Deviation of ±0.8033 resulting in small differences between different genotypes. However, Barendse et al. (2005) conducted a study, in which 3129 individual animals were sampled: they also found no association between leptin gene polymorphism and marbling, BFT en total fat. Crews et al. (2004) compared the R25C genotypes with carcass traits of 433 Charolais steers and found no association with BFT and carcass marbling as well (*Crews et al., 2005; Schenkel et al., 2005*). Another explanation for these different outcomes could be environmental factors such as feeding regimes or climate, which can affect significant differences in IMF (*L. Pannier et al. 2009*). Also, age might be of importance. In 1994 Gregory et al., proved that continental breeds like Simmental and Charolais are characterized by their late maturity compared to British breeds like Angus and Herford (*Gregory et al., 1994*). Therefore, it could be that the differences in IMF of different genotypes will come to expression at a later age. Beside these factors, our method of IMF determination might be inadequate due to a lack of experience. However, in this study we determent the IMF content with the method of

Schröder and Staufenbiel (2006). They call it an objective and very precise method of intra muscular fat measurement and has been used by researchers before (*Schröder and Staufenbiel, 2006)*. In regard to genes, which contribute towards IMF content, the leptin gene is not the only one. Andersson & Georges (2004), underline the hypothesis that IMF content is regulated by multiple genes or several genetic loci, each specific gene responsible for a small amount of overall variation (*Andersson & Georges, 2004*). This suggests that not only the leptin gene affects the amount of IMF.

In this study, several SNP's in the leptin gene were detected. However, none of the SNP's was significantly associated with IMF, also called marbling. Other authors, whom have studied the same SNP's, report conflicting results. Therefore, more research is necessary to obtain a full understanding of the role of these SNP's in the (intramuscular) fat content of Simmental bull.

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