SHORT COMMUNICATION

Detection of polymorphisms in the ovine leptin (*LEP*) gene: Association of a single nucleotide polymorphism with muscle growth and meat quality traits

D. Boucher[1], M. F. Palin[2], F. Castonguay[1,2], C. Gariépy[3], and F. Pothier[1,4]

[1]*Département des sciences animales, Université Laval, Quebec City, Quebec, Canada;* [2]*Agriculture and Agri-Food Canada, Dairy and Swine Research and Development Centre, Lennoxville, Quebec, Canada;* [3]*Agriculture and Agri-Food Canada, Food Research and Development Centre, Saint-Hyacinthe, Quebec, Canada.* *Received 9 June 2005, accepted 3 November 2005.*

Boucher, D., Palin, M. F., Castonguay, F., Gariépy, C. and Pothier, F. 2006. **Detection of polymorphisms in the ovine leptin (*LEP*) gene: Association of a single nucleotide polymorphism with muscle growth and meat quality traits**. Can. J. Anim. Sci. **86**: 31–35. Leptin, the expression product of the obese gene produced primarily in the adipose tissue, is related to feed intake, growth and lipid metabolism. In beef and swine, polymorphisms of the leptin gene (*LEP*) are associated with body composition and fat deposition traits. The objective of this study was to identify polymorphisms in the ovine *LEP* gene and to test for their possible association with growth (average daily gain), carcass composition (% bone, fat and lean, loin eye area, fat and muscle depth), longissimus (L) structural and metabolic characteristics [fiber types, citrate synthase (CS) and lactate deshydrogenase (LDH) activity] and meat quality traits (color, pH, shear forces and chemical composition) in sheep. A population set of Dorset (n = 70) and Suffolk (n = 69) lambs was screened for polymorphisms of the ovine *LEP* gene. Three single nucleotide polymorphisms (SNP) were identified (A103G, C154T and C617G). In the Suffolk breed, the A103G SNP is associated with reduced muscle thickness (–1.36 mm) and loin eye area (–137.58 mm^2) and with increased shear forces (+1.091 kg), pH (+0.168) and cross-sectional area (CSA) of the slow-twitch oxidative (SO) fibers (+818 μm^2) of the L muscle. In the Dorset breed, the A103G variant is also associated with an increased CS activity in the L muscle (+3.19 IU g^{-1}) and in subcutaneous fat (+0.36 IU g^{-1}).

Key words: Carcass, leptin gene, muscle histoenzymology, meat quality, polymorphism, sheep

Boucher, D., Palin, M. F., Castonguay, F., Gariépy, C. et Pothier, F. 2006. **Détection de polymorphismes du gène de la leptine (*LEP*) chez l'ovin : Association entre un SNP et certains caractères de croissance musculaire et de qualité de la viande.** Can. J. Anim. Sci. **86**: 31–35. La leptine est une hormone provenant de l'expression du gène de l'obésité qui est principalement produite par le tissu adipeux. La leptine est reliée à la prise alimentaire, la croissance et le métabolisme lipidique. L'objectif de cette étude était d'identifier des variations à l'intérieur du gène de la leptine (*LEP)* chez une population d'agneaux de races Dorset (n = 70) et Suffolk (n = 69) ainsi que leur association avec certains caractères de croissance (gain moyen quotidien), de caractéristiques structurales et métaboliques du muscle long dorsal (L) (type de fibres, activité de la citrate synthase (CS) et de la lactate déshydrogénase (LDH)) ainsi que de qualité de la viande (pH, couleur, forces de cisaillement et composition chimique) et de carcasse (surface de l'œil de longe, épaisseur de gras et de maigre et % os, gras et maigre). Trois SNP ont été identifiés (A103G, C154T, C617G). Chez les Suffolk, le polymorphisme A103G est associé avec une diminution de l'épaisseur de muscle (–1.36 mm) et de la surface de l'œil de longe (–137.58 mm^2) ainsi qu'avec une augmentation de la force de cisaillement (+1.091 kg), du pH (+0.168) et de la taille moyenne des fibres oxydatives (+818 μm^2) du muscle L. Chez les agneaux de race Dorset, la mutation A103G est associée avec une activité accrue de l'enzyme citrate synthase mesurée dans le muscle L (+3.19 UI g^{-1}) et le tissu adipeux sous-cutané (+0.36 UI g^{-1}).

Mots clés: Carcasse, gène de la leptine, histoenzymologie, mouton, polymorphismes, qualité de la viande

Leptin, the expression product of the *obese* gene, is a hormone of the cytokine family that acts as a satiety signal regulating body weight and energy metabolism (Zhang et al. 1994). The adipose tissue is the main source of leptin before its release into the bloodstream (Houseknecht et al. 1998). In sheep, leptin blood levels are associated with feeding levels (Marie et al. 2001), diet nutritional value (Blache et al. 2000) and body fat mass (Delavaud et al. 2000). Previous studies have shown associations of *LEP* gene polymorphisms and leptin blood levels with carcass and meat quality traits in beef cattle (Buchanan et al. 2002; Kononoff et al. 2005) and swine (Jiang and Gibson 1999; Berg et al. 2003).

[4]*To whom correspondence should be addressed: (e-mail: francois.pothier@crbr.ulaval.ca).*

Abbreviations: **CS**, citrate synthase; **CSA**, cross-sectional area; **FG**, fast twitch glycolytic; **FOG**, fast twitch oxidative-glycolytic; **L**, longissimus; **LDH**, lactate deshydrogenase; **LEA**, loin eye area; ***LEP***, leptin gene; **PCR**, polymerase chain reaction; **SNP**, single nucleotide polymorphisms; **SO**, slow-twitch oxidative

The objective of this study was to identify polymorphisms in the ovine *LEP* gene and their possible association with growth, carcass composition and meat quality traits in sheep.

A population set of randomly selected purebred Dorset (n = 70) and Suffolk (n = 69) lambs was screened for polymorphisms of the *LEP* gene. The choice of these two breeds was based on their opposite phenotypes for fat deposition (% total fat, $P < 0.0001$; % sub-cutaneous and intramuscular fat, $P < 0.0001$) and muscle accretion (% total muscle, $P < 0.0004$) (unpublished data). At the time of weaning (around 55 d old), an equal number of male and female lambs was selected in each breed. All lambs were then raised under the same controlled-environmental conditions. Animals were fed a 18% protein concentrate ad libitum up to 35 kg BW and a 15% protein concentrate to finishing weight. High-quality hay and water was also provided ad libitum. Lambs were allocated to a given slaughter weight interval (36–39 kg, 41–44 kg, 46–49 kg and 51–54 kg) according to the slaughter scheme from another project studying the impact of slaughter weight on carcass composition and meat quality in heavy lambs. The average slaughtering age and weight of lambs for each target weight interval in both breeds is reported in Table 1. Lambs were slaughtered according to the recommended code of practice approved by the local Animal Care Committee following the guidelines of the Canadian Council on Animal Care (1993). Phenotypic data were collected on a weekly basis during the animal growth: average daily gain (kg d^{-1}), ultrasound measurement of fat and L muscle thickness between the 12th and 13th ribs (mm) (Ultrascan50, Alliance Médicale Inc., Montreal, QC); Animals were sacrificed according to their targeted slaughtering weight after a 12-h fasting period. A 10-mL blood sample was collected from each lamb before slaughter. Within 1 h postmortem, a 10-g sample was taken from the right L thoracis muscle and half of it was frozen in liquid nitrogen for further analyses of CS and LDH activities as indicators of the oxidative and glycolytic metabolism of the muscle. The other part was frozen in isopentane cooled in liquid nitrogen for histochemistry. Carcasses were chilled for 48 h at 1°C and were classified according to Canada Livestock and Poultry Carcass Grading Regulations (Agriculture and Agri-Food Canada 1992). The entire L muscle (right) was removed for further quality analyses. Other traits recorded on the carcass were: loin eye area (LEA) (mm^2), fat score at GR site (110 mm from the midline over the 12th rib) (mm), saleable meat yield (%) (Jones et al. 1994), total fat (%) and muscle (%) ratios from half-carcass dissection (left). CS and LDH activity (IU g^{-1}) measurements were performed according to Srere (1969) and Bass et al. (1969), respectively. CS activity was also measured in subcutaneous fat according to Srere . Cryostat prepared frozen transverse serial sections (14 µm) were stained for myosin ATPase following an alkine pre incubation (pH 10.4) (Guth and Samaha 1970) for the determination of fiber twitch. Their oxidative capacity was assessed with succinate deshydrogenase stain (Nachlas et al. 1957). Five serial bundles were analyzed per muscle with an image analysis system (Jandel Scientific Software, San Rafael, CA). Fiber cross-sectional area was also measured. Fibers were classified as slow twitch oxidative (SO), fast twitch oxidative-glycolytic (FOG)

Table 1. Actual mean slaughter weights and ages for each targeted slaughter weight interval according to breed and gender

	Females								Males								P			
	Dorset				Suffolk				Dorset				Suffolk							
Recorded traits at slaughter	36–39 (n = 8)	41–44 (n = 10)	46–49 (n = 9)	51–54 (n = 9)	36–39 (n = 9)	41–44 (n = 9)	46–49 (n = 9)	51–54 (n = 9)	36–39 (n = 9)	41–44 (n = 8)	46–49 (n = 9)	51–54 (n = 8)	36–39 (n = 9)	41–44 (n = 7)	46–49 (n = 9)	51–54 (n = 8)	Breed (B)	Gender (G)	B × G
Age[z] (d)	122,7	140,9	156,4	169,8	98,8	115,0	129,3	145,4	102,9	118,2	132,4	146,1	94,3	103,5	111,9	126,7	0.0002	<0.0001	0.003
Weight[y] (kg)	37,5	43,2	48,1	52,5	37,1	43,7	49,2	53,5	37,8	43,7	48,7	53,6	37,5	42,4	48,3	53,5	0.64	0.99	0.04

Values for age and weight at slaughter are least square means
[z]SEM = 4.0 d.
[y]SEM = 0.5 kg.

and fast twitch glycolytic (FG). Meat quality measurements were ultimate pH, *L* a* b** color indices (CR 300 colorimeter, Minolta, Stone Ridge, NY) and shear forces (kg) (TA.XT2i texturometer, Texture Technologie, New York, NY) measured across 5 cm × 1 cm × 1 cm cores prepared along the fiber axis of L sample cooked to 68°C (internal temperature). Fat (AOAC 991.36) (Association of Official Analytical Chemists 1995) and protein content (AOAC 992.15) (AOAC 1995) were also determined on freeze-dried samples. Leptin levels were also measured in serum from blood samples using a multi-species RIA kit (Linco, St. Louis, MO) as previously reported (Delavaud et al. 2000). Triplicate measurements were performed using an automated gamma counter (1277 Gammamaster, LKB Wallac, Boston, MA) with a 2-min exposition period. Assay buffer differed from the kit and its composition has been taken from Mao et al. (1999). Sensitivity of this test was 0.33 ng mL^{-1} and intra-assay CV was 7.0%. Parallelism of the assay was also verified by adding standard to unknown sample.

Genomic DNA was purified from whole blood samples using MasterPure™ DNA Purification Kit (Epicentre, Madison, WI). Two fragments of the ovine *LEP* gene were amplified using polymerase chain reaction (PCR). Primers were designed based on available ovine genomic sequences (Genbank AF310264 and AY831682) and partial coding sequences (GenBank U84247 and AY911719). The amplicons were 260 bp (fragment 1) and 926 bp (fragment 2) in length. Fragment 1 covered exon 2 and part of intron 2 whereas fragment 2 included the genomic sequence from exon 3 and part of the 3′ UTR of the gene. The design of primers for fragment 1 was possible after amplification and sequencing by our team of intron 2, which lies between the two coding exons of the ovine leptin gene. The sequences for these primers were: forward (1) 5′- CGCAAGGTCCAGGATGACACC-3′; reverse (2) 5′- GTCTGGGAGGGAGGAGAGTGA-3′ for fragment 1; forward (3) 5′- CTCTTGATGTCCCCTTCCTC-3′ reverse (4) 5′-TGGTCCTTCGAGATCCATTC-3′ for fragment 2. The hybridization positions for these primers are as follow: 1: bp 10–30 (U84247); 2: bp 167–188 (AY831682); 3: bp 179–198 (AY911719); 4: bp 57–76 (AF310264).

The amplification reactions were performed in a total volume of 50 µL containing 200 ng of genomic DNA, 200 µM of each dNTP, 2.6 units of Expand HF PCR enzyme mix (Roche Molecular Biochemicals, Mannheim, Germany), 1× Expand HF buffer (1.5 mM $MgCl_2$) and 0.2 µM of each primer. Cycling conditions were 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 60°C (fragment 1) or 54°C (fragment 2) , 1 min at 72°C followed by a final extension of 7 min at 72°C. PCR products were purified using ABI EtOH–EDTA precipitation protocol according to the manufacturer instructions and then sequenced using an Applied Biosystems 3730/XL DNA Analyzer (Foster City, CA). The sequencing reaction was performed using Big Dye v3.1 (Applied Biosystems) followed by EtOH-EDTA precipitation to remove any excess of dying agent.

A total of three polymorphisms were identified in the ovine *LEP* gene including two SNP in intron 2 (A103G and C154T in fragment 1) and one in the 3' UTR of the ovine *LEP* gene (C617G in fragment 2). The allele frequencies of these mutations are shown in Table 2. To our knowledge, this is the first description of allele variations in the ovine *LEP* gene. Genotyping was monitored by direct sequencing in duplicate of each individual. The possibility of an association between breeds and the presence of a given polymorphism was assessed by Fisher's Exact Test (two-tailed). The A103G polymorphism was found in lambs of both breeds with no significant difference in their allele frequencies ($P = 0.16$). Only one Dorset lamb was homozygous (GG) for this mutation. The C617G and C154T mutations were specific to the Dorset breed. Haplotypes consisting of the A103G, C154T and C617G alleles were also found in the Dorset breed only (Table 2).

Table 2. Allele frequencies and breed effects of three *LEP* gene polymorphisms and haplotypes in a population of Dorset and Suffolk lambs

Polymorphisms	Breed	Genotypes (WT/mutants)	Allele frequency	
			WT	Mutant[z]
A103G[y]	Dorset	53/17	0.87	0.13
	Suffolk	60/9	0.93	0.07
C617G	Dorset	51/19	0.86	0.14
	Suffolk	69/0	1.0	0
C154T	Dorset	58/12	0.91	0.09
	Suffolk	69/0	1.0	0
Haplotypes				
A103G + C617G	Dorset	57/13	0.91	0.09
A103G + C154T	Dorset	62/8	0.94	0.06
C617G + C154T	Dorset	59/11	0.92	0.08
A103G + C617G + C154T	Dorset	63/7	0.95	0.05

[z]Frequency of the G allele for A103G, G allele for C617G, T allele for C154T.
[y]Fisher's Exact Test probability for Breed effect of A103G SNP: $P = 0.16$

An ANOVA, using "weight at slaughter" as a covariate, was performed to determine possible association between genotyping results and growth, carcass and meat quality traits (SAS Institute, Inc. 2002). Such associations were only found for the A103G SNP and are shown in Table 3. Because purebred animals were used in this study, data from Suffolk and Dorset lambs were analyzed separately. In the Suffolk breed, the presence of the A103G SNP is associated with a decrease in LEA (–137.58 mm^2; $P < 0.05$) and ultrasound L thickness (–1.36 mm; $P < 0.05$). An increase in L shear forces (+1.091 kg; $P < 0.05$), pH (+0.168; $P < 0.05$) and CSA of the SO fibers (+818 μm^2; $P < 0.05$) were also found significant in the Suffolk breed. Dorset lambs carriers of the A103G variant showed an increase of CS activity in both L muscle (+3.19 IU g^{-1}; $P < 0.05$) and subcutaneous fat (+0.36 IU g^{-1}; $P < 0.05$) compared with animals having two wild-type alleles. The covariate "weight at slaughter" was not related to L pH ($P = 0.6$) and to CS activity of L ($P = 0.3$) and subcutaneous fat ($P = 0.6$). However, the use of this covariate explained part of the variation measured for LEA ($P < 0.01$), muscle thickness ($P < 0.01$) and L shear forces ($P < 0.01$). There was no difference observed between carriers of the wild-type (AA) and mutant alleles (AG or GG) of the A103G SNP for any carcass classification, meat composition and fibers proportion traits recorded (data not shown).

Significant gender effects were obtained for some of the phenotypic traits presented therein. Despite these effects, we decid-

Table 3. Effect of ovine *LEP* gene A103G SNP on growth, carcass composition and meat quality traits measured on Dorset and Suffolk lambs

	Dorset				Suffolk			
	AA	AG	SEM	P^z	AA	AG	SEM	P^z
Growth								
Avg daily gain (kg d^{-1})	0.378	0.395	0.017	0.40	0.479	0.490	0.029	0.75
Ultrasound fat depth (mm)	8.8	8.5	0.4	0.64	7.6	7.5	0.5	0.75
Ultrasound muscle depth (mm)	31.1	31.4	0.6	0.70	30.3	28.9	0.6	0.05
Carcass composition								
Loin eye area (mm^2)	1612.8	1575.4	36.9	0.38	1622.4	1484.8	57.6	0.03
Total dissected fat (%)	29.2	29.4	1.3	0.88	24.5	23.4	1.8	0.57
Total dissected lean (%)	55.5	55.2	0.8	0.78	57.1	56.4	1.0	0.55
Meat quality								
pH	5.52	5.53	0.04	0.90	5.53	5.70	0.07	0.03
Color								
*a**	19.3	18.6	0.6	0.29	18.2	17.9	1.2	0.82
*b**	9.8	9.0	0.5	0.20	9.1	8.3	1.1	0.47
*L**	39.4	39.0	0.8	0.63	40.1	37.9	1.8	0.22
Shear forces (kg)	3.5	3.3	0.3	0.60	3.6	4.7	0.5	0.05
Enzymatic activity								
CS (IU g^{-1}) – L	16.2	19.0	1.1	0.03	16.4	14.4	1.4	0.20
CS (IU g^{-1}) – fat	1.44	1.80	0.13	0.02	1.59	1.45	0.16	0.44
LDH (IU g^{-1})	1479.2	1451.2	54.9	0.66	1390.8	1325.2	121.0	0.62
Fibers cross-sectional area (μm^2)								
FG	2259.7	2392.9	176.7	0.50	2226.1	2619.1	266.3	0.17
FOG	1884.1	1822.0	132.2	0.67	1668.6	1805.3	173.3	0.46
SO	2406.7	2419.7	233.9	0.96	2205.0	3023.2	324.9	0.02

Values are least square means.
[z]Using Proc Mixed with weight at slaughter as a fixed effect.

ed to group all carriers of the A103G SNP only according to breed. Moreover, an equal proportion of male and female carriers of the G allele was obtained for both Suffolk (males, n = 5; females, n = 4) and Dorset (males, n = 9; females, n = 8) breeds. We are aware of the limitation introduced by pooling gender in our analysis in regard to some traits. Owing to the low allele frequency of the G allele, a larger population set will be needed to allow gender-specific analysis.

Results obtained in this study suggest a negative effect of the A103G variant of the *LEP* gene on muscle growth for Suffolk lambs. The leptin and leptin receptor genes are expressed in skeletal muscle of fetal sheep, and ovine fetal leptin concentrations show an inverse association with fetal body weight (Buchbinder et al. 2001). It was previously reported that leptin, as well as insulin, affects muscle cell proliferation in chick embryo (Lamosova and Zeman 2001). Moreover, in vitro experiments revealed that leptin mimics or interferes with insulin at the level of glycogen uptake and synthesis (Berti et al. 1997). Although our results and above cited studies strongly suggest an effect of leptin on skeletal muscle cells, further investigations will be needed to assess the implication of leptin on sheep muscle growth.

We also report an effect of the A103G variant on L pH, shear forces and cross-sectional area of the SO fibers for Suffolk lambs. As it was not possible to follow the cooking procedures with thermocouples in each sample, it could be speculated that the higher shear forces measured in Suffolk might be associated in part with their smaller L size. Correlation values between shear forces and LEA (r = 0.52, $P < 0.001$) in this breed support this possibility but indicate that 73% of shear force variations are independent of muscle size. Higher pH could also be involved in shear forces variation. This higher pH, however, is not explained by change in oxidative enzyme activities (CS) and fiber type distribution in the Suffolk breed. However, the large increase in CSA of the SO fibers associated with the A103G variant in this breed might reflect a change in oxidative capacity as well as other muscle properties. It is known that changes in enzyme activities can occur in asynchrony with transitions in fiber type and these changes in beef were reported to be dependant upon muscle or developmental stages (Brandsetter et al. 1998). Such observations may account for the different expression of the variant in the two lamb breeds in this study. This warrants further research.

Leptin blood levels have been shown to positively affect CS activity in rat adipose tissue (Ceddia et al. 2000) and muscle (Zou et al. 2004). In humans, greater CS activity was found in muscles submitted to exercise-induced oxidative stress (Carter et al. 2001). The higher CS activity found in L muscle of A103G heterozygous Dorset lambs may indeed reflect a greater fatigue resistance, yet no changes in fiber type were observed. At the meat level, this could translate into reduced color stability during storage. Higher CS activity in adipose tissue may reflect a change in lipid oxidation potential although this hypothesis has not been investigated in this study.

Circulating leptin concentrations were not affected by the A103G SNP of the *LEP* gene in either breed. Thus, the reported effects of this mutation would not be related to a variation of leptin concentration in the bloodstream.

It has been reported that intronic mutations, such as the A103G variant, can affect gene regulation and transcription levels. As such, Tokuhiro et al. (2003) have characterized a SNP in intron 1 of the human *SLC22A4* gene that affects affinity with a transcriptional regulator of the hematopoietic system (RUNX1). In the same way, a SNP in intron 1 of the human *LTA* gene, associated to myocardial infarction, has been shown to increase its transcription level (Ozaki et al. 2002). Intronic mutations can also results in splicing abnormalities, which often change the structure of mature protein (Faustino and Cooper 2003).

In this study, we characterized the first association between a single nucleotide polymorphism in the ovine *LEP* gene and skeletal muscle growth and meat quality traits. Further work will be needed on a larger sample of sheep before we can conclude on the implications of the A103G for the sheep breeding industry.

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