Genotype at the MTNR1A locus and response to melatonin treatment in Sarda lambs

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ABSTRACT - With the aim to evaluate the effect of melatonin treatment and melatonin receptor 1A (MTNR1A) genotype on advance of puberty, 423 Sarda lambs were chosen. On June 26th, they were divided into three groups, each of 141 animals (groups 0, 1, and 2), on the basis of live weight. On June 30th, animals in group 1 received a single implant (18 mg melatonin), while group 2 received two implants. Group 0 was untreated. Thirty-five days after treatment (August 4th), rams were introduced and after 40 days they were removed. From January 1st to February 10th lambing dates were recorded. Genomic DNA was extracted and subjected to PCR for the amplification of exon II and then digested with enzymes MnlI and RsaI and placed into +/+, +/–, or –/– group for MnlI and C/C, C/T, or T/T group for RsaI. Samples were cloned and sequenced. Data obtained were subjected to χ^2 test in order to evaluate the difference in fertility among groups and the link between genotype and reproductive activity. Genotype +/+ and C/C showed the highest incidence. Treated groups showed a higher number of lambing at 10th February compared to control group ($P<0.04$). Melatonin treatment results more efficient in +/+ genotype.

Key words: MTNR1A locus, Sarda lambs, Melatonin implants.

Introduction – One of the major problems in the management of sheep breeding is the reduction of unproductive time that passes between birth and first lambing. The reaching of puberty in ewes is influenced by many factors among which age, body weight, and photoperiod are the most significant (Dyrmundsson, 1981). Photoperiod is a factor that considerably influences puberty in sheep (Kennaway *et al.*, 1985). The endocrine signal responsible for mediating the effects of photoperiod on the hypothalamo-pituitary axis is the circadian rhythm of melatonin secretion (Sunderland *et al.*, 1995). This hormone, synthesized by pineal gland, relays information about night/day alternation to the tissues that express specific receptors (Boutin *et al.*, 2005), so regulating circadian rhythms and the variations in reproductive activity in seasonally responsive animals such as sheep (Barrett *et al.*, 1997). Indeed, in sheep the high melatonin levels, that are typical of decreasing photoperiods, stimulate GnRH secretion so favouring reproductive activity (Carcangiu *et al.*, 2009). Thus, lambs born in November-December, at temperate latitudes such as Sardinia, start their reproductive activity mainly during the subsequent autumn mating period and as a consequence lambing occur in late winter-early spring, with a delay in the onset of reproductive activity (Carcangiu *et al.*, 2005). Indeed, exogenous administrations of this hormone near to the summer solstice, mimic short photoperiods and encourage the onset of puberty, when body weight and age are adequate (Haresign et al., 1990). Reproductive effects of melatonin are mediated by receptors located in the hypophyseal pars tuberalis (Reppert *et al.*, 1994). In mammals, two types of receptors with high affinity for melatonin have been identified but only one is involved in the regulation of reproductive activity (Reppert *et al.*, 1994). The second exon of the gene encoding for MTNR1A receptor presents polymorphisms using endonucleases MnlI and RsaI (Messer *et al.*, 1997). Thanks to the relationship between gene allelic isoforms and reproductive performances in

sheep, the genotype at MTNR1A locus can become a marker able to study sexual activity in sheep (Notter and Cockett, 2005). The goals of the present study are firstly to evaluate the effect of one or two melatonin implants on the onset of puberty in Sarda lambs; secondly to assess if genotype at MTNR1A locus can influence the response to melatonin treatment.

Material and methods – In total, 423 lambs born in November coming from three different farms located in the centre of Sardinia (39° 36' N) were chosen. All the animals were weaned at 35 days, kept under natural photoperiod, and separated from the flock. Feeding was based on extensive pasture of natural grass land with a supplement of commercial pellets (300g/head/day); hay and water were *ad libitum*. In each farm, 141 animals were chosen and on June 26th were weighed and assigned to one of three groups (0, 1, and 2), each of 141 animals on the basis of their body weight. In the same days, in order to allow genotypic analyses, a 10 ml blood sample from jugular vein was taken from each head using tube with EDTA as an anticoagulant (Believer Industrial Estate, Plymouth, UK). Only the animals with a minimum of 26 kg of body weight were included. On June 30th, lambs in group 1 received a single slow release subcutaneous implant containing melatonin 18 mg (MELOVINE®, CEVA VETEM, Agrate Brianza, MI), in the left retroauricolar region using an apposite implanter, while lambs in Group 2 received two implants (total dose melatonin 36 mg). Group 0 received no treatment. Thirty-five days after treatment, on August 4th, rams of proved fertility were introduced with a ratio of 1/20, and removed after 40 days. From January 1st to February 10th lambing date, number and sex of born lambs were recorded. Genomic DNA was extracted from whole blood by DNA extraction kit (Purgene, Gentra, Minneapolis, Minnesota, USA), and amplification of the second exon of ovine MTNR1A locus was performed as described by Messer *et al.* (1997). PCR products were subjected to MnlI and RsaI restriction enzymes (New England Biolabs, Beverly, MA, USA) and then placed into +/+, +/-, or -/- group for MnlI and C/C, C/T, or T/T group for RsaI enzyme. Before sequencing, the resulting fragment from PCR of all samples was cloned as described by Carcangiu *et al.* (2009). Then samples were sequenced from both directions and the obtained sequences were aligned with the U14109 sequence of GenBank. Data were subjected to χ^2 test in order to evaluate the difference in fertility among groups and the link between genotype and reproductive activity (MINITAB®).

Results and conclusions – Melatonin treatment produces an advance of the puberty in Sarda lambs. At day 190th from ram introduction, about 20 more animals lambed in treated groups compared to control, and experimental data evidenced that a single implant is adequate for obtain this effect (Table 1). These data are in accord with what recorded by Nowak and Rodway (1985) which found, in treated animals, an advance of puberty of three weeks. However, in our observation the puberty advance was not so marked. This could be due to the fact that treated and untreated groups were kept together and so the difference between groups was attenuated, which might be the result of a carry-over effect from treated to control ewes (Abecia *et al.*, 2006). PCR products consist in a fragment of 824 bp corresponding to the main part of the exon II. Sequencing evidenced the same substitutions and polymorphisms already recorded in Sarda and other sheep breed (Carcangiu *et al.*, 2009). Digestion with MnlI enzyme evidenced one polymorphic site in position 612 of the reference sequence, corresponding to the presence of a G (allele +) or a A (allele -). Genotypic frequency was 62% for +/+ (92 in group 0, 80 in group 1, and 90 in group 2), 19% for +/- (28 in group 0, 34 in group 1, and 34 in group 2), and 19% for -/- (21 in group 0, 27 in group 1, and 17 in group 2). Digestion with RsaI enzyme evidenced one polymorphic site in position 606 of the reference sequence corresponding to the presence of a C (allele C) or a T (allele T). Genotypic frequency was 53% for C/C (73 in group 0, 68 in group 1, and 85 in group 2), 30% for C/T (43 in group 0, 48 in group 1, and 35 in group 2), and 17% for T/T (25 in group 0, 25 in group 1, and 21 in group 2) (Table 2). From our data it results that genotypes +/+ and C/C are the most representative compared to the majority of other breeds (Notter and Cockett, 2005). No relationship was evidenced about polymorphism in RsaI site and response to melatonin treatment. Subjects with +/+ genotype showed a better response to melatonin treatment compared with the animals +/+ of the control group ($p<0.04$). So lambs with this genetic character resulted more

easily stimulated by melatonin treatment, and this permitted an earlier onset of sexual activity. In conclusion our data evidenced the effect of melatonin treatment on advance of puberty and focus an important role of +/+ genotype in this response.

Table 1. Lambing distribution in the three groups and on the basis of the MnlI genotype.

	Group 0			Group 1			Group 2		
Total lambing at 10th February	54a			72b			73b		
Genotype MnlI	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-
Ewes lambed at 10th February	43	8	3	50	11	11	57	11	5
Ewes Not lambed	49	20	18	30	23	16	33	23	12

a, b = p-value= 0.038; chi-squared for genotype +/+ p=0.04, for +/- and -/- n.s.

Table 2. Total genotypic frequency percentage and genotypic distribution on the basis of restriction enzymes in the three groups of Sarda lambs.

		MnlI			RsaI		
		+/+	+/-	-/-	C/C	C/T	T/T
Total genotypic frequency	(%)	62%	19%	19%	53%	30%	17%
Group 0	(n)	92	28	21	73	43	25
Group 1	(n)	80	34	27	68	48	25
Group 2	(n)	90	34	17	85	35	21

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