The relationship between melatonin receptor 1A gene (MTNR1A) polymorphism and reproductive performance in Sarda breed sheep

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A B S T R A C T

In several species the circadian changes in melatonin secretion acts as the organic informer of the photoperiodic trend. Some physiological functions, such as reproduction, are influenced by seasonal melatonin secretion. In small ruminants polymorphisms at the MTNR1A gene are associated with non-seasonal breeding. The aim of this research was to evaluate if the MTNR1A gene polymorphism influences first conception, second and third lambing in Sarda breed sheep. Two-hundred ewe lambs, born between 10th November and 10th December 2008, were randomly chosen from four selected farms and genotyped for the A612G polymorphism at the MTNR1A gene. To rule out a possible confounding effect of the low frequency of the A/A genotype in Sarda sheep, 800 ewe lambs were genotyped and 85 individuals were selected for each genotype (A/A, A/G and G/G). Fertile rams were given access to the selected ewe lambs from 30th July to 30th October 2009 during the first year, and from 1st May to 30th October 2010 and 2011 on the second and third year, respectively. Mating and lambing dates were recorded for each ewe during the three years. In the first year ewe lambs showed no differences among genotypes in their fertility rates, litter size and mean number of days from ram introduction to parturition. However, in the second and third year G/G and A/G genotypes showed, a shorter interval (expressed in days) from ram introduction to lambing, compared to A/A genotype (Po0.05). During

the second year fertility rate for the G/G, A/G and A/A genotypes, were 91.7%, 90.2% and 89.9%, respectively; whereas in the third year fertility rates were 91.5% for G/G, 91.8% for A/G and 93.0% for A/A genotypes. We speculate that the similarity in fertility rates among different genotypes may be due to a carry-over effect because ewes carrying different genotypes were not separated. Results indicate that the MTNR1A gene polymorphism does not influence the onset of reproductive activity in Sarda ewe lambs, but this single nucleotide polymorphism (SNP) reveals its influence in the earlier reproductive resumption in the adult ewes. The interval from rams' introduction to lambing may be longer if animals with different genotypes were kept separated.

Introduction

Small ruminants living in temperate latitudes exhibit marked seasonal variation in their reproductive activity (Chemineau et al., 2003). Although domestication has mitigated this reproductive trend, a clear distinction between oestrous and anoestrous period is commonly seen in sheep. Generally, ewes show their sexual activity between late summer and early winter, and the photo- period is the principal driver of these physiological changes (Karsch et al., 1984). However, photoperiod in mammals does not generate circadian rhythms, but it acts by melatonin, as the principal timing system of an endogenous time-keeping system, called “biological clock” (Bartness et al., 1993; Weaver, 1999). Indeed, circannual rhythms have been found in several animal species as they have a pivotal role in behavioural and physiological functions, such as migration, hibernation and pelage moult (Goldman, 2001; Lincoln and Hazlerigg, 2010). Melatonin secretion by pineal gland occurs mainly during night hours, thus this hormone can be considered the organic informer of the photoperiodic trend (Lincoln et al., 2005). The circadian effects of melatonin are mediated by mela- tonin receptors in the hypothalamic suprachiasmatic nucleus, which is the site of the circadian clock (Weaver et al., 1996), whereas the reproductive effects of melatonin occur in the premammillary hypothalamus (Migaud et al., 2005). Melatonin exerts its reproductive and circadian effects through the binding with high-affinity, G-protein-coupled receptors (Dubocovich and Takahashi, 1987; Reppert et al., 1994). Among these receptors, the MT1 seems to be the only one involved in the regulation of the reproductive activity (Dubocovich, 1988; Weaver et al., 1996). Melatonin receptor 1A (MTNR1A) gene has been mapped in the human chromosome 4q35.1; in

the proximal portion of the mouse chromosome 8 (Slaugenhaupt et al., 1995); in the porcine chromosome 17q1.2; in the ovine chromosome 26; and bovine chromosome 27 (Messer et al., 1997). The discovery of two polymorphic RFLP sites within the ovine MTNR1A gene allowed the evaluation of the influence of this gene on seasonal reproduction (Messer et al., 1997). Several authors (Chu et al., 2006; Notter et al., 2003; Pelletier et al., 2000) reported that the G/G genotype of the exon II of MTNR1A gene was associated with an autumnal lambing performance in ewes. Consequently, the same exon exhibits a relationship with reproductive seasonality in goats, buffaloes and mouflons (Carcangiu et al., 2009a, 2010, 2011b). In Sarda breed sheep, Carcangiu et al. (2009b) found that the MTNR1A gene polymorphism influences reproductive resumption in spring. Therefore, the aim of this research was to investigate the influence of MTNR1A gene poly- morphism on the first conception and on the second and third lambing in Sarda breed sheep.

Material and methods

Animals and management

The productive cycle in Sarda sheep is very strictly linked to the Mediterranean climate: characterised by mild winter, rainfalls occurring in autumn and spring and a very dry summer. Such climate affects the growth of herbage, which is usually available in autumn and mostly in the spring season. In addition, temperatures during high summer also lead to a significant reduction in milk yield. The typical Sarda dairy sheep productive cycle is distinguished by one out-of-season lambing, which makes for optimal exploitation of the herbage growth cycle. Adult ewes' lambing occurs in autumn when the grass starts to grow right after the summer drought. On the other hand, yearling ewes (on average 20–25% of the total flock) usually lamb between January and March. The lambing period of ewe lambs is determined not only by their period of birth, but also by the achievement of the suitable body development. The ewe lambs reach about 80% of their adult weight in July, so that they can only reproduce thereafter.

The research was conducted in four farms located in central Sardinia (391360 N) where each farm raised approximately 800 ewes. First, 200 ewe lambs from each of the four farms were genotyped at the MTNR1A gene. Then, to rule out the possibility that the low frequency of the A/A genotype in the Sarda sheep population (about 14%) could affect the results, 85 ewe lambs for each genotype (A/A, A/G, G/G) were included in the

present study. The research lasted three years, covering a time span between 2009 and 2011. In the first year the reproductive activity of 255 ewe lambs was recorded. Forty-three ewes in the second year and 24 in the third were respectively excluded from the study because they were culled or did not lamb. The 255 Sarda ewe lambs, born in 2008 between 10th November and 10th December, were selected during the first year. These lambs were weaned at 35 days of age and then kept under natural photoperiod, separated from males and the rest of the flock. The same animals, with the exception of those excluded, were monitored for the next two years (2010 and 2011). Ewe lambs grazed all day on grass-legume and gramineous pastures. In addition they received, daily as a supplement, 300 g per head of concentrate commercial food when they were housed in the sheepfold for the night. For adult and lactating ewes the feeding consisted of natural extensive pasture supplemented daily with 400 g per head of commercial pellets in two doses, which were administered during the morning and the evening milking. Hay and water were ad libitum.

Sampling, DNA preparation and primers sequences

On 1st March 2009 blood was collected from the jugular vein of 800 ewe lambs, using 10 ml vacuum tubes with EDTA as an anticoagulant (BD Vacutainer Systems, Belliver Indus- trial Estate, Plymouth, UK). Ovine genomic DNA was extracted from whole blood using a genomic DNA extraction kit (NucleoSpinsBlood, Macherey-Nagel, Germany) and then kept at 20 1C until further use. An aliquot of 100–150 ng of genomic DNA was subjected to a polymerase chain reaction (PCR) using specific primers synthesised by Sigma Genosys Ltd (Pampisford, Cambs, UK). The above mentioned primers were of similar brand as those used by Messer et al. (1997). These specified primers allow amplification of the main part of the exon II of the ovine MTNR1A gene (GenBank U14109).

DNA amplification and genotyping

The polymerase chain reaction (PCR) was carried out according to the method reported by Carcangiu et al. (2009a, 2009b) on Mastercyclers Gradient thermocycler (Eppendorf AG, Hamburg, Germany). All samples of the PCR amplification reaction were subjected to MnlI restriction enzyme analysis (New England Biolabs, Beverly, MA, USA) according to the method reported by Carcangiu et al. (2011a). MnlI enzyme cuts the sequence at the succession of bases 5'-CCTC(n)7^-3' and 3'-GGAG(n)6^-5'. MnlI endo- nuclease recognises an A to a G substitution at position 612 of the MTNR1A exon II nucleotide sequence. Products of digestion

were resolved by electrophoresis on a 4% agarose gel (GellyPhor, Euroclone, UK) in parallel with a 50 bp DNA marker (Invitrogen, Carlsbad, CA, USA).

Experimental design

In the first year, on 30th July 2009 fertile and experienced Sarda rams (1:25 male to female ratio), were introduced to the flock and removed on 30th October 2009. Males were introduced on these dates because ewe lambs were over 7 months old and at this age Sarda lambs reach the optimal body development to start reproductive activity, corresponding to the 60% of the adult weight (Mura et al., 2009, 2010). The rams were marked on their keel to colour the back of the serviced females. The number of ewe lambs with ram keel marks was counted and recorded each day, and keel colour was changed every ten days. Gestations were diagnosed by transabdominal ultrasonography examination using an Esaote Pie Medical Tringa linear instrument (Esaote Europe B.V., Maastricht, The Netherlands) provided with a 5.0–7.5 MHz multiple frequency linear probe. From December 27th 2009 to March 31st 2010 lambing dates and number of newborns were recorded. In the second and third year (2010 and 2011) from 1st May the males were introduced in the flocks until October 1st Rams were introduced on different dates in the second and third year compared to the first year because of the age of the ewes. During the second and third year ewe lambs became adult ewes, hence their ability to resume the reproductive activity during long photoperiods was studied. Rams were marked on their keel, marked ewes were counted daily and keel colour was changed every ten days. Gestations were diagnosed by transabdominal ultrasonography examination as described above. Lambing dates and number of newborn lambs were recorded from 25th September to 5th March.

Statistical analysis

Allelic frequencies were determined by direct counting of the observed genotypes. The $\chi 2$-test was used to determine Hardy–Weinberg equilibrium of the mutation (Gene- pop 4.2). Statistical analysis was performed by R statistical software (Version 3.0.0). Analysis of variance was used to analyse the association between MTNR1A genotypes and reproductive activity (obtained according to the number of days' interval between ram introduction and lambing). The following linear model was utilised:

$$Y_{ikj} = \mu + G_k + F_j + G_kF_j + E_{ikj}$$

where Y_{ikj} was the trait measured on each animal (days between ram introduction and lambing); μ is the overall mean, G_k is the fixed effect of the genotype (3 levels); F_j is the fixed effect of farm (4 levels); G_kF_j is the effect of interaction between genotype and farm; and E_{ikj} is the error effect. Data were analysed within year and orthogonal contrasts were constructed to evaluate the effect of genotype (A/A vs. A/G and G/G, and A/G vs. G/G) on the number of days from ram introduction to lambing. A logistic regression was calculated to determine the effects of genotypes and farm on fertility rate. P o0.05 was considered statistically significant.

Results

The PCR results consisted of a single band of 824 bp in length, corresponding to the main part of the exon II of the MTNR1A gene. Restriction enzyme analyses, using MnlI, confirmed the existence of the polymorphism A612G. The allelic and genotypic frequencies are shown in Table 1. The most frequent allele was G in position 612 and consequently the most frequent genotype was G/G. The population resulted not in Hardy–Weinberg equilibrium due to the low number of heterozygotes. Returns to oestrus and embryo resorption were within physiological limits of the specie (approximately 3%) in all three year of the study. There was no significant effect of farm on fertility rate. In the first year ewe lambs in each genotype showed the same fertility rates and the interval in days from rams introduction to lambing (Table 2). Litter size was also similar among ewe lambs with different genotypes: 1.20 in G/G, 1.18 in A/G and 1.15 in A/A genotype (Table 2). Indeed, in the second and third year animals carrying G/G and A/G genotypes had a shorter interval in days from ram introduction to lambing, compared to ewes with A/A genotype (P o0.05) whereas no difference was detected in the interval from rams' intro- duction to parturition between ewes carrying the A/G and the G/G genotype (Tables 3 and 4). The estimates for each contrast, and probability 4|t| for days from rams introduction to lambing in each year are presented in Table 5. In the second year fertility rates for the G/G, A/G and A/A genotypes were 91.7%, 90.2% and 89.9%, respectively, whereas in the third year fertility rates were 91.5% 91.8% and 93.0%, respectively. The litter size rate was similar among the three genotypes during the second and third year of the study (Tables 2 and 3, respectively).

Discussion

The genotypic and allelic frequencies of the analysed polymorphism were similar to previous studies (Carcangiu et al., 2009b; Mura et al., 2010). Sarda sheep exhibit a high frequency of the mutant allele G at position 612 of the MTNR1A gene, similarly to other European sheep breeds (Martinez-Royo et al., 2012; Messer et al., 1997; Pelletier et al., 2000). Notter and Cockett (2005) postulated that the G allele was mutant because the ewes carrying it exhibited a reduced reproductive seasonality. Moreover, wild sheep (Ovis Gmelini Musimon) had a higher A allele frequency coupled with a reproductive activity mostly influenced by photoperiod confirming that this allele might be considered ancestral (Carcangiu et al., 2010). Nevertheless the different genetic selections may be linked to the production attitude of the single breed (milk, meat and/or wool), leading to the genetic variability of this polymorphism. In the present study, the MTNR1A gene polymorphism showed no influence on the onset of reproductive activity in Sarda ewe lambs, since days between the introduction of rams and lambing were similar among the three genotypes. More- over, the rams' introduction did not determine a prompt beginning of the reproductive activity in ewe lambs, even though the daylight hours were decreasing. The ewe lambs mating peak was only observed in late September when the

daylight hours were substantially reduced causing a strong stimulus for the onset of reproductive activity. Thus, the inhibitory effect of photoperiod on the reproductive activity of ewe lambs remained still very strong for the entire month of August. These results confirm previous findings in Sarda sheep (Mura et al., 2010), but differ from data recorded in Dorset sheep (Mateescu et al., 2009). In Dorset sheep breed the animals with a G allele (either in homozygosis or in heterozygosis) lambed earlier than those with the A/A genotype. The different relationship between polymorph- ism and first lambing that was found in the two above cited studies is certainly due to difference in breed. Dorset and Sarda have different productive attitudes: Dorset is a dual purpose breed (meat and wool) whereas Sarda is a dairy sheep. In addition, Dorset is a prolific breed while Sarda usually has low litter sizes (Carcangiu et al., 2011a). Moreover, the two studies have been conducted in differ- ent seasons and this variable could have influenced the results. In this research same age ewe lambs were studied while in the study on the Dorset breed, animals were of different ages with the males being introduced in different seasons, as the STAR system requires (Mateescu et al., 2009). All these factors are likely to have had a high impact on the reproductive response. In the second and third years of this study the time of lambing showed that the MTNR1A gene polymorphism, influenced the reproductive resumption in spring. Ewes carrying G/G or A/G genotype lambed approximately 10 days later compared to ewes with A/A

genotype in both years. This result differs from that found in Merinos d'Arles, Ile de France and Aragonesa breeds (Hernandez et al., 2005; Martìnez-Royo et al., 2010; Teyssier et al., 2011). Another SNP in the MTNR1A exon II, C606T, has been detected and associated with the reproductive activity in sheep, but results are contradictory among different authors. Martinez-Royo et al. (2012) found that in the Aragonesa breed the T allele at position 606 was associated with an early onset of reproductive activity in the spring. These results are in disagreement with those by other authors in several other sheep breeds, in which the C allele exhibited a better reproductive efficiency in spring (Chu et al., 2006; Carcangiu et al., 2009b; Mateescu et al., 2009; Notter et al., 2003). The results by Teyssier et al. (2011) on the SNP at position 612 in the Merinos d'Arles breed, do not agree with Pelletier etal.(2000). Teyssieretal.(2011) concluded that the A612G SNP cannot be used as a single genetic selection marker for out of breeding season in the Merinos d'Arles sheep, while Pelletier et al.(2000) hypothesised that the relationship between the seasonal ovarian inactivity and the homozygous A/A has a genetic linkage. This difference could be due to the lower frequency of the A/A genotype as compared to the other two genotypes (Teyssieretal.,2011). The same number of animals for each genotype was included in this study to rule out possible confounding effect on the results of the low frequency of the A allele in the Sarda sheep. The discrepancy between results obtained in different ovine breeds could to be in line with the report by Hernandez et al. (2005), and could be due to various elements such as difference in breed and number of animals used, distribution of the genotypes and body condition of the ewes. The relationship between the polymorphism at position 612andtheseasonalreproductiveactivityisdifficultto explain, since this SNP does not result in an aminoacid change, hence it does not modify the functionality of the receptor. Pelletier et al. (2000), showed a different binding capacity of the receptor to melatonin in the various genotypes, but their finding has not been entirely confirmed by Trecherel et al. (2010) who report a difference in cAMP signalling in melatonin receptors between ewes carrying A/A versus G/G genotype suggesting a modification in the melatonin signal interpretation in the A/A genotype animals. Therefore, it can be hypothesised that this polymorphism could be linked to other mutations in other sites of the nucleotide sequence which lead to a different receptor functionality. Mutation at position 612 resulted indubitably in an association with another SNP, G702A, producing an aminoacid change (a valine in a isoleucine, V220I) (Pelletieretal.,2000). This change leads to an altered signal transmission, without modifying either the receptor's ability to bind to melatonin or its density (Trechereletal.,2010). The aminoacid change occurs within

the Vdomain, close to the histidine at position 211and 195, which are involved in the melatonin signal transduction pathways (Conwayetal.,1997; Kokkolaetal., 1998). Thus, it can be hypothesised that the change from valine to isoleucine could result in a different steric conformation, leading to an altered signal transduction. The outcome of this study evidenced that the animals carrying G/G or A/G genotype, during the second and third year resumed their reproductive activity earlier compared to the ewes with A/A genotype. The animals carrying different genotypes were kept together, so we cannot exclude that a carry-over effect may have influenced the reproductive activity (Muraetal.,2010). Data from lambings and matings show that ewes carrying G/G or A/G genotype respond faster to the male effect compared to A/A animals. Therefore, it is reasonable to hypothesise that the difference in days from rams introduction to lambing could be greater if genotypes were separated.

Conclusion

In conclusion, our data evidenced an association between MTNR1A gene polymorphism and the reproductive resumption in spring, in the adult Sarda sheep breed. Moreover the present study confirmed that this polymorphism does not influence the onset of reproductive activity in Sarda ewe lambs. From an economic standpoint, the advance of reproductive activity in out of breeding season leads to an extension of the lactation period. These findings lead to conclude that this SNP could be used in the sheep selection plans.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of this paper.

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Table 1

Genotype and allele frequency of the MTNR1A gene polymorphisms in Sarda sheep (n ¼ 800).

SNP position A612G

Genotype	A/A	A/G	G/G
Genotype frequency	0.14	0.33	0.53
Allele	A	G	
Allele frequency	0.32	0.68	

Table 2

Results of first year in the studied Sarda ewe lambs: mean time in days from ram introduction to lambing, age at first lambing, percentage of fertility and litter size according to genotype.

Genotype	first year			sem
	A/A	A/G	G/G	
n. ewes lambs	85	85	85	
DRIL	198.4	192.5	191.1	1.9
Age at first lambing (days)	440.8	436.5	433.4	2.7
Fertility (%)	89.4	90.6	89.4	
Litter size	1.15	1.18	1.20	0.01

DRIL = mean time in Days from Ram Introduction to Lambing. Sem = standard error of the means

Table 3

Results of second year in the studied Sarda ewes: mean time in days from ram introduction to lambing, percentage of fertility and litter size according to genotype.

Genotype	second year			sem
	A/A	A/G	G/G	
n. ewes lambs	69	71	72	
DRIL	197.3	190.3	188.1	1.9
Fertility (%)	89.9	90.2	91.7	
Litter size	1.16	1.19	1.21	0.01

DRIL = Mean time in Days from Ram Introduction to Lambing. Sem = standard error of the means.

Means within a row with different superscripts are significantly different (P o 0.05).

Table 4

Results of second year in the studied Sarda ewes: mean time in days from ram introduction to lambing, percentage of fertility and litter size according to genotype.

Genotype	third year			sem
	A/A	A/G	G/G	
n. ewes lambs	57	60	59	
DRIL	192.9	184.5	183.2	2.8
Fertility (%)	93.0	91.8	91.5	
Litter size	1.19	1.22	1.20	0.01

DRIL = Mean time in Days from Ram Introduction to Lambing. Sem = standard error of the means.

Means within a row with different superscripts are significantly different (P o 0.05).

Table 5

Effect estimates and probability >|t| for contrasts evaluating the effect of genotype on the number of days from ram introduction to lambing in each year.

	Days from ram introduction to lambing					
	first year		second year		third year	
	Effect estimates	P value	Effect estimates	P value	Effect estimates	P value
A/A vs A/G and G/G	7.1	n.s	15.2	<0.05	14.2	<0.05
A/G vs G/G	1.4	n.s	3.3	n.s	1.3	n.s