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Reproductive response to male joining with ewes with different allelic variants of the *MTNR1A* gene

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ABSTRACT

The aims were to evaluate the reproductive response to ram placement with Sarda ewes with different allelic variants at position g.15099485 A > G of the *MTNR1 A* gene. Ram placements occurred between the early and late spring and there was analysis of whether this polymorphism is associated with other nucleotide substitutions. In each of the eight farms where the study was conducted (named F1-F8), 150 ewes (50 with A/A, A/G and G/G genotypes) were selected. In each group of 150 ewes, eight males were joined with ewes on the following dates: 25 March (T1) for F1-F2, 15 April (T2) for F3-F4, 5 May (T3) for F5-F6, and 1 June (T4) for F7-F8. The lambing dates and number of new born lambs were recorded until 220 days after joining rams with ewes. The ewes with G/G or A/G genotypes had a greater fertility rate compared with those with A/A in T1, T2 ($P < 0.01$), and in T3 and T4 ($P < 0.05$). The duration of time in days from the time of ram joining with ewes to lambing was less in the ewes with G/G and A/G compared with those with A/A ($P < 0.01$). The g.15099485A > G variation was always associated with that at position g.15099391G > A. Results indicate there is a positive effect of the g.15099485A > G variant on reproduction when males were joined with ewes in March or April. The association that was ascertained in this study between the g.15099391G > A and g.15099485A > G polymorphisms for the *MTNR1 A* gene, could provide new insights to clarify the mechanism of action of melatonin.

1. Introduction

Melatonin is a pleiotropic signalling molecule that regulates several physiological functions including synchronisation of biological rhythms, such as seasonal reproduction (Tamura et al., 2014). In ewes at temperate latitudes, the seasonal reproductive functions are characterised by periods when there are ovulations and no ovulation occurring, caused by annual variations in day length (Chemineau et al., 2003). Photoperiodic cues are transduced into neuroendocrine signals by melatonin (Karsch et al., 1984). In sheep, this hormone is secreted during the night by the pineal gland and functions in the premammillary hypothalamus to control the release of the GnRH (Malpaux et al., 1998; Carcangiu et al., 2014). Melatonin exerts its action through receptors distributed in different parts of the body (Migaud et al., 2005); among these, MTNR1 A appears to be involved in the regulation of reproductive seasonality (Weaver et al., 1996; Dubocovich et al., 2003). This receptor subtype, encoded by the *MTNR1A* gene, is a G-coupled protein receptor, mostly found in the pars tuberalis area of the pituitary and pre-hypothalamic region (Chabot et al., 1998; Dardente, 2007; Dupré et al., 2008). Polymorphic sites were detected within exon II of the *MTNR1A* gene in sheep, mouflon, goats and buffalo

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(Carcangiu et al., 2009a, 2009b; 2010; Luridiana et al., 2012). In different sheep breeds, the allelic variants at positions g.15099491C > T and g.15099485 A > G of the *MTNR1A* gene exon II (according to the latest genome version Oar4.0, GenBank accession number NW_014639035.1) have been associated with the seasonal reproductive traits (Pelletier et al., 2000; Chu et al., 2006; Luridiana et al., 2015a; Giantsis et al., 2016). In the Aragonesa breed, however, only the g.15099491C > T polymorphism was associated with a greater percentage of oestrous cyclic ewes between January and August (Martínez-Royo et al., 2012). In the Ile de France ewes, the g.15099485A > G polymorphism was not associated with a difference in the onset, cessation, or length of the breeding season among the animals of the two homozygous genotypes (maintained in a controlled photoperiodic setting without contact with males; Hernandez et al., 2005). These results indicate that the relationship between the *MTNR1 A* gene polymorphism and the reproductive season can change with the breed or be affected by management systems, (e.g., presence or absence of males) or other environmental conditions. Thus, the relationship between polymorphisms of this gene and reproductive resumption could also be masked or affected by the observation period in different studies. Indeed, the effect of presence of a male(s) on the reproduction of ewes is affected by breed and season (Chanvallon et al., 2011), and also within the same breed the presence of males effects reproduction in different ways (Fabre-Nys et al., 2015). The utilisation of the management practice of joining males with ewes is an approach that meets modern criteria for ‘clean, green and ethical’ production methods, but its use is limited by the great amount of variability in the reproductive response of ewes (Brown et al., 2014). This could be due to the extent that photoperiodic cues have on the duration of anoestrus of sheep, which in turn could depend on the genotype at the *MTNR1A* locus. The goal of the present research was to clarify the role of this polymorphism on the variability of the response to the joining of males with ewes in the transition from the period of anoestrus and the initiation of oestrous cyclic functions.

Thus, the first aim of the present study was to evaluate, from early to late spring, the reproductive response to rams of Sarda ewes with the different allelic variants - g.15099485A > G of the *MTNR1A* gene. A further aim was to analyse whether this polymorphism is related to other variations in the nucleotide sequence of the *MTNR1A* gene exon II.

2. Material and methods

2.1. Animals and experimental design

All the animals in this research had veterinary care by the National Health Veterinary Service in accordance with the Animal Welfare Act. Blood samples collected by veterinarians of the National Health Service for routine health assessments.

Sarda sheep (more than 3.0 million ewes in Sardinia) are the main dairy breed in Italy, and the milk production in dairy enterprises occurs in a pattern consistent with the pasture growth pattern. The ewes of this breed have a short anoestrous period, generally from late winter to mid-spring. For the present research, ewes were on eight farms located in North Sardinia (on the 40 °N) that were located in a similar climatic zone and at a similar altitude utilising a similar nutritional and management regimens. On each farm, there were approximately 800 Sarda sheep and the animals had been maintained in conditions with a natural photoperiod from birth. During the day, the animals grazed on leguminous and gramineous grasses, and received 300 g per animal daily of concentrated commercial food (crude protein 20.4% and 12.5 MJ ME/kg DM) at the time of milking. At each farm, the genotype at position g.15099485 A > G of the *MTNR1A* gene exon II was identified in all the animals aged 3–5 years (approximately 400 ewes in each farm). There were 150 lactating ewes selected at each farm ($n = 50$ with A/A, A/G and G/G genotypes) that lambed in 2015, between October 20 and December 1, for a total of 1200 ewes for the study. The ewes utilized in the study had at least two previous parities, thus, were at least 3 years of age. Nulliparous ewes and those with a single parity that were in their second gestational period were excluded, as in the Sarda breed, the first lambing generally occurs between January and April with the greatest number of lambs being born between February and March. Sarda sheep have an ample milk yield and consequently their reproductive functions are less than that of ewes of many other breeds for at least 2 months after lambing leading to a delay in the time of lambing in second parity ewes (Luridiana et al., 2015b). This is the reason only multiparous ewes that were pregnant with lambs to be delivered at least at their third parturition were selected for the present study.

The number in the ear tag of each animal was recorded, and the ewes were individually marked with numbered collars to avoid recognition errors. At each farm, (identified as F1-F8) the 150 ewes utilised for the present study were separated from the rest of the flock. In every group of 150 ewes, eight adult (3 to 6 years old) Sarda males of proven fertility (male/female ratio 1/20) were joined

Table 1
Time period of male joining with ewes on eight farms and number of ewes at each farm that was included in the study (1200 ewes).

Time period	Data of male introduction	Farms	n
T1	25 March	F1	150
	25 March	F2	150
T2	15 April	F3	150
	15 April	F4	150
T3	5 May	F5	150
	5 May	F6	150
T4	1 June	F7	150
	1 June	F8	150

with ewes during the 2016 time period (Table 1). The ewes were previously isolated from rams for 90 days (sound, sight and smell, minimum distance > 1500 m). The rams were separated from females after 70 days of joining of ewes and rams. Gestation was diagnosed by trans-abdominal ultrasonography examination using Esaote Piemedical Tringa linear equipment (Esaote Europe B.V., Maastricht, the Netherlands) with a 5.0 to 7.5 MHz multiple frequency linear probe. Pregnancy diagnosis was performed in all ewes, every week, from 45 days after male joining with ewes to 45 days after male removal. The lambing dates and the numbers of new born lambs were recorded until 220 days after joining of rams with the ewes.

2.2. Blood sampling and DNA analysis

To identify the individual allelic variants, DNA analysis was performed using the whole blood from each ewe. Blood samples (10 ml) were collected from the jugular vein, using vacuum tubes with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant (BD Vacutainer Systems, Belliver Industrial Estate, Plymouth, UK). The DNA was extracted from whole blood using a genomic DNA extraction kit (NucleoSpin® Blood, Macherey-Nagel, Germany). An amount of 150 ng of genomic DNA was subjected to polymerase chain reaction (PCR) using specific primers (Sigma Genosys Ltd., Pampisford, Cambs, UK) according to Messer et al. (1997). The primers corresponded to positions 285 to 304 (sense primer 5' – TGT GTT TGT GGT GAG CCT GG – 3') and 1108 to 1089 (antisense primer: 5' – ATG GAG AGG GTT TGC GTT TA – 3') of the sequence reported by Reppert et al. (1994) (GenBank accession number U14109). The PCR reaction was performed for all the samples using the method reported by Carcangiu et al. (2009b). All the PCR products were subjected to restriction enzyme analysis using the MnlI endonuclease (New England Biolabs, Beverly, MA, USA), which recognises an A to a G substitution at position 612 of the U14109 *MTNR1 A* exon II nucleotide sequence, corresponding to position g.15099485 A > G in genome version Oar4.0 (GenBank accession number NW_014639035.1). The digestion reaction was performed using the methods described by Carcangiu et al. (2009b).

2.3. Sequencing

One hundred PCR products for each genotype were sequenced to determine whether the g.15099485A > G variant was associated with other nucleotide substitutions. A total of 300 amplified products was sequenced using Applied Biosystems 3730 DNA Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The sequences were aligned and compared with the ovine sequence GenBank U14109 and NW_014639035.1, to confirm the correspondence of the known nucleotide changes and underscore other possible substitutions. The homology searches were performed using BLAST (National Centre for Biotechnology Information: https://blast.ncbi.nlm.nih. gov/Blast.cgi). To align the sequences, the CLUSTALW tool was used (http://www.genome.jp/tools-bin/clustalw).

2.4. Statistical analysis

Allele and genotype frequencies were determined by direct counting of the observed genotypes. The chi-squared test was used to determine Hardy-Weinberg equilibrium of the mutation (Genepop 4.2). The R statistical software (Version 3.2.2) was used to perform the statistical analysis.

The farm effect was not included in the model as the farms were all located within 20 km each other, therefore, there were similar climatic condictions and animals had the same veterinary, feed and reproductive farm management. The data for the climatic variables (humidity, environmental temperature), and for the animals (number of lambing and the lambing period, BCS and age) were statistically evaluated and there were no differences detected.

A general linear model (GLM) procedure was performed to analyse the effect of treatment period and genotype on the litter size and on the distance in days from ram introduction to lambing, based on the following model:

$$Y_{ilmn} = \mu + {}_{i} + S_i + G_l + P_m + (G_l P_m) + e_{ilmn}$$

where Y_{ilmn} is the variable measured (pregnancy rate or duration of time in days from joining rams with ewes to lambing), μ is the overall mean, S_i is the random effect of the sire, G_l is the fixed effect of the genotype, P_m is the fixed effect of period, $(G_l P_m)$ is the interaction between G_l and P_m, and e_{ilmn} is the error effect. To compare percentages of ewes lambing with each genotype and each time period, a chi-squared test was used. A P value < 0.05 was considered statistically significant.

3. Results

The allelic and genotypic frequency was not different of ewes among the eight farms. The means of allele and genotype frequencies are shown in Table 2. The most frequent allele was G (0.68) at position g.15099485 of the latest genome version Oar4.0 (GenBank accession number NW_014639035.1) and, consequently, G/G was the most frequent genotype (53%). The population was not in Hardy-Weinberg equilibrium due to the small number of heterozygotes ($P < 0.05$). The DNA sequencing confirmed the presence of the polymorphic site in all the samples. The single nucleotide polymorphism (SNP) position reported in this paper refers to the Oar4.0 genome version (GenBank accession number NW_014639035.1).

The alignment of the sequences with that in GenBank indicated there was a total number of eight SNPs: six of which were silent (g.15099644 T > G, g.15099491C > T, g.15099485 A > G, g.15099314 G > A, g.15099296 G > A, g.15099206 T > C), while

Table 2
Genotype and allele frequencies of the *MTNR1 A* gene allelic variant in ewes on the eight farms ($n = 1200$ ewes).

Allelic variant	g.15099485 A > G		
Genotypes	A/A	A/G	G/G
Genotype frequency	0.14	0.33	0.53
Alleles	A	G	
Allele frequency	0.32	0.68	

with the others there was an amino acid change (g.15099391 G > A causing p.Val220Ile, and g.15099204C > A causing a p.Ala282Asp substitution in the amino acid sequence; Table 3). The g.15099485 A > G variation was always associated with g.15099391 G > A. The number of ewes diagnosed as pregnant differed from the number of ewes that lambed by approximately 3%. In T1, the fertility (percentage of ewes lambed) of ewes between the two farms (F1 and F2) was similar. This trend was similar to those of the other farms during the other periods when frequency of pregnancy was assessed. Considering the g.15099485 A > G SNP, the pregnancy rate in the ewes with G/G or A/G genotypes was greater compared to the ewes with A/A in T1, T2 ($P < 0.01$) and for ewes in T3 and T4 ($P < 0.05$; Table 4). Some ewes with G/G or A/G genotypes lambed between 150 and 160 days after rams were joined with ewes in all the four periods (i.e., for the G/G genotype two lambs in T1 and six in T4 meaning approximately 2% and 6% of the ewes lambed, respectively; Fig. 1). None of the ewes with the A/A genotype lambed before 160 days after ewes being joined with rams for all the periods. For the ewes with a G/G and A/A genotype, t there was a difference in lambing rate ($P < 0.05$) between the T1-T2 and T3-T4 periods, whereas for the ewes with the A/G genotype there was a similar lambing rate in the four periods. The average duration of time in days from joining the rams with ewes to lambing was shorter in the animals with the G/G or A/G than i ewes with an A/A genotype ($P < 0.01$). The litter size (number of lambs born per lambing) at the eight farms was affected neither by genotypes nor by the time periods of joining with rams (T1-T4). The total average litter size was similar for ewes with all the three genotypes: 1.20, 1.18 and 1.15 for the G/G, A/G and A/A genotype, respectively.

The lambing trend is depicted in Fig. 1 for the ewes with different genotypes and that conceived during the different periods (from T1 to T4). For all four periods, the ewes with the G/G or A/G genotypes compared with those with the A/A genotype had a greater lambing percentage between 160 and 180 days after ewe joining with rams ($P < 0.01$). There was the greatest number of ewes lambing with the G/G genotype that were joined with rams during the T1 and T2 periods between 160 and 170 days after joining of rams with the ewes. For the ewes with the G/A genotype, there was the greatest number of ewes lambing between 170 and 180 days after joining of rams with ewes. For ewes with the A/A genotype that were joined with rams during T1 and T2, there was the greatest numbers of ewes lambing between 190 and 200 days after joining of ewes with males. For ewes with the G/G and G/A genotypes joined with rams during T3 and T4, the greatest number of ewes lambing was between 160 and 170 days and for the ewes with the A/A genotype between 180 and 190 days after joining ewes with males.

4. Discussion

The genotypic and allelic frequencies of the analysed locus were similar to those previously reported for the same breed (Carcangiu et al., 2009b; Luridiana et al., 2015a). At position g.15099485 A > G of the *MTNR1A* gene exon II sequence, Sarda sheep had a relatively greater frequency of the mutant allele G, similar to that of some other European sheep breeds (Messer et al., 1997; Mateescu et al., 2009). By contrast, in *Ovis gmelini musimon* (a wild sheep) the A allele was most frequently present (Carcangiu et al., 2010). Comparing the frequency of occurrence of the G allele in domestic breeds, in the present study there was a lesser rate than in the Aragonesa and in other sub-temperate and sub-tropical Indian sheep breeds (Magra, Marwari, Chokla, Malpura, Patanwadi, Sandyno and Niligiri; Martínez-Royo et al., 2012; Saxena et al., 2014; 2015a,2015b).

Table 3
Nucleotide and amino acid changes within the *MTNR1 A* gene exon II in Sarda ewes ($n = 300$ sequenced ewes).

SNP position[a]	Nucleotide change[b]	Codon change[c]	Amino acid change[d]
g.15099644	T > G	AC**T**/AC**G**	None: Thr135Thr
g.15099491	C > T	TA**C**/TA**T**	None: Tyr186Tyr
g.15099485	A > G	CC**A**/CC**G**	None: Pro188Pro
g.15099391	G > A	**G**TC/**A**TC	Val220Ile
g.15099314	G > A	CT**G**/CT**A**	None: Leu245Leu
g.15099296	G > A	AG**G**/AG**A**	None: Arg251 Arg
g.15099206	T > C	CC**C**/CC**T**	None: Pro281Pro
g.15099204	C > A	G**C**C/G**A**C	Ala282Asp

[a] According to the latest genome version Oar4.0 (GenBank acc. number NW_014639035.1).

[b] Sequence is in a reverse orientation on the Oar4.0 genome version, so that nucleotide substitution appears in the reverse form compared to the present study.

[c] Nucleotide changes within codons are in bold.

[d] According to NCBI Reference Sequence: NP_001009725.1.

Table 4
Lambing rate and duration of time in days from ram joining with ewes to lambing in the four periods (T1 to T4) ewes were with rams based on genotypes at position g.15099485 (n = 1200 ewes).

	Lambing rate				Duration in days from ram joining with ewes to lambing			
Genotypes	G/G	A/G	A/A	P	G/G	A/G	A/A	P
T1	88%^{b}	78%	60%^{a}	< 0.01	177.6 ± 14.8	179.5 ± 14.2	193.3 ± 14.3	< 0.01
T2	92%^{b}	80%	65%^{a}	< 0.01	176.1 ± 14.5	178.9 ± 14.0	192.1 ± 15.1	< 0.01
T3	85%^{a}	81%	70%^{b}	< 0.01	175.7 ± 14,2	178.8 ± 14.3	189.0 ± 15.6	< 0.01
T4	85%^{a}	81%	74%^{b}	< 0.05	174.8 ± 13.5	178.6 ± 14.6	189.7 ± 15.9	< 0.01

Date of ram joining with ewes - T1: 25 March; T2: 15 April; T3: 5vMay: T4: 1 June.
Different lowercase letters in columns differ $P < 0.05$; P value refers to the significance within the row.

The results from the present study indicate that the reproductive response to joining with males of adult Sarda ewes is affected by the polymorphism at g.15099485A > G. Indeed, the ewes with the G/G or A/G genotype had a greater lambing rate and a lesser duration in days between the time of ram joining with ewes compared to those with the A/A genotype, in all the periods where assessments occurred. Results of the present study are consistent with the findings of previous studies in different sheep breeds, in which the G/G genotype at the same locus was associated with breeding and conceiving out of-season and with a shorter interval between first and second lambing (Chu et al., 2006; Mateescu et al., 2009; Carcangiu et al., 2012). Furthermore, Sarda ewes with the G/G genotype had a shorter period of anoestrus in spring after melatonin administration (Mura et al., 2017). The present results indicate that in the Sarda breed, the presence of even one G allele affects the reproductive response to male joining with ewes and these results are consistent with those previously reported for Sarda and other sheep breeds (Mateescu et al., 2009; Mura et al., 2014).

In other European sheep breeds the same allelic variant assessed in the present study had no effect on reproductive performance (Hernandez et al., 2005; Martínez-Royo et al., 2012). This difference was attributed to the breed or to the effects of environmental factors. In almost all the studies, the fertility of the sheep was recorded after the joining of males with ewes, while, in the study by Hernandez et al. (2005), reproductive activity was evaluated only by recording progesterone concentrations and associating these with whether ewes were or were not pregnant. The lack of the male effect, therefore, could have affected the results in this previous study.

In the present study, the greater lambing rate in the ewes with the G/G or A/G genotype confirmed the hypothesis about a lesser sensitivity to photoperiod of the ewes with these two genotypes. Presumably, this lesser sensitivity resulted in shorter period of anoestrus, which led to a greater response to joining of rams with the ewes, compared to ewes with the A/A genotype, which could have longer periods of anoestrus. The different hypothalamic sensitivity to photoperiodic cues could be the basis for the different reproductive responses among the three genotypes. The difference in fertility rates of the ewes with the A/A genotype between ewes joined with rams in the T1-T2 and T3-T4 periods could be due to the gradual transition from anoestrus to the breeding season, with a consequent greater hypothalamic sensitivity to the oestradiol (E_2)-positive feedback signal, as suggested by Fabre-Nys et al. (2015) or a decreased negative feedback to oestradiol as has previously been reported to occur (Karsch et al., 1984).

Also, from the lambing trend, it can be hypothesized that the animals with the different genotypes have a different duration of anestrous. This is supported by ewes with the G/G and A/G animals lambing before the ewes with the A/A genotype, and this confirms the earlier response to the joining with the males because of shorter periods of anoestrus.

This effect, however, is not easy to explain because this allelic variant is not associated with an amino acid change affecting the trans domains, and therefore it should not involve the receptor functionality. The eight mutations detected in the *MTNR1 A* gene exon II (g.15099644 T > G, g.15099491C > T, g.15099485 A > G, g.15099391 G > A g.15099314 G > A, g.15099296 G > A, g.15099206 T > C, and g.15099204C > A) were identical to those reported as a result of previous studies, both in Sarda (Carcangiu et al., 2009b) and in other sheep breeds (Pelletier et al., 2000; Reppert et al., 1994). Furthermore, in these previous studies (Reppert et al., 1994; Pelletier et al., 2000) of the same gene tract there were two other mutations detected, at position g.426C > T and g.555 G > A of the melatonin receptor sequence with GenBank accession number U14109 that corresponded to position g.15099671C > T and g.15099575 G > A of the latest genome version Oar4.0: NW_014639035.1, respectively. Neither of these allelic variants lead to amino acid changes. Furthermore, Saxena et al. (2014) reported another mutation in the Indian Chokla sheep breed, at position g.931 G > C of the U14109 sequence, corresponding to the g.15099166 of the NW_014639035.1 sequence, which also determines an amino acid substitution (p.Ala295Pro). These differences are, due to the different evolutionary pathways of the breeds, based on the focus of selection for different products (milk, meat or wool). In the present study, and consistent with the report of Saxena et al. (2015b), the variation g.15099485A > G was always associated with g.15099391G > A, which determines an amino acid change at position p.Val220Ile (according to the RefSeq record GenBank accession number NP_001009725.1). These data are very important because of the relationship between these variations and may explain the effect on reproductive seasonality of the polymorphism g.15099485A > G. Although the nucleotide substitution g.15099391G > A leads to an amino acid change in the protein sequence, it is not part of the transmembrane domain of the *MTRN1 A* gene and this is consistent with the findings of Barrett et al. (2003), consequently there should not be a change in the receptor's functionality. Nevertheless, the position in the protein of this amino acid change is close to the histidine at positions 211 and 195 of the amino acid sequence (NP_001009725.1), which are involved in melatonin signal transduction (Conway et al., 1997; Kokkola et al., 1998). This amino acid change could lead to a modification of the steric conformation of the amino acid chain with a consequent signal alteration (Trecherel et al., 2010). The

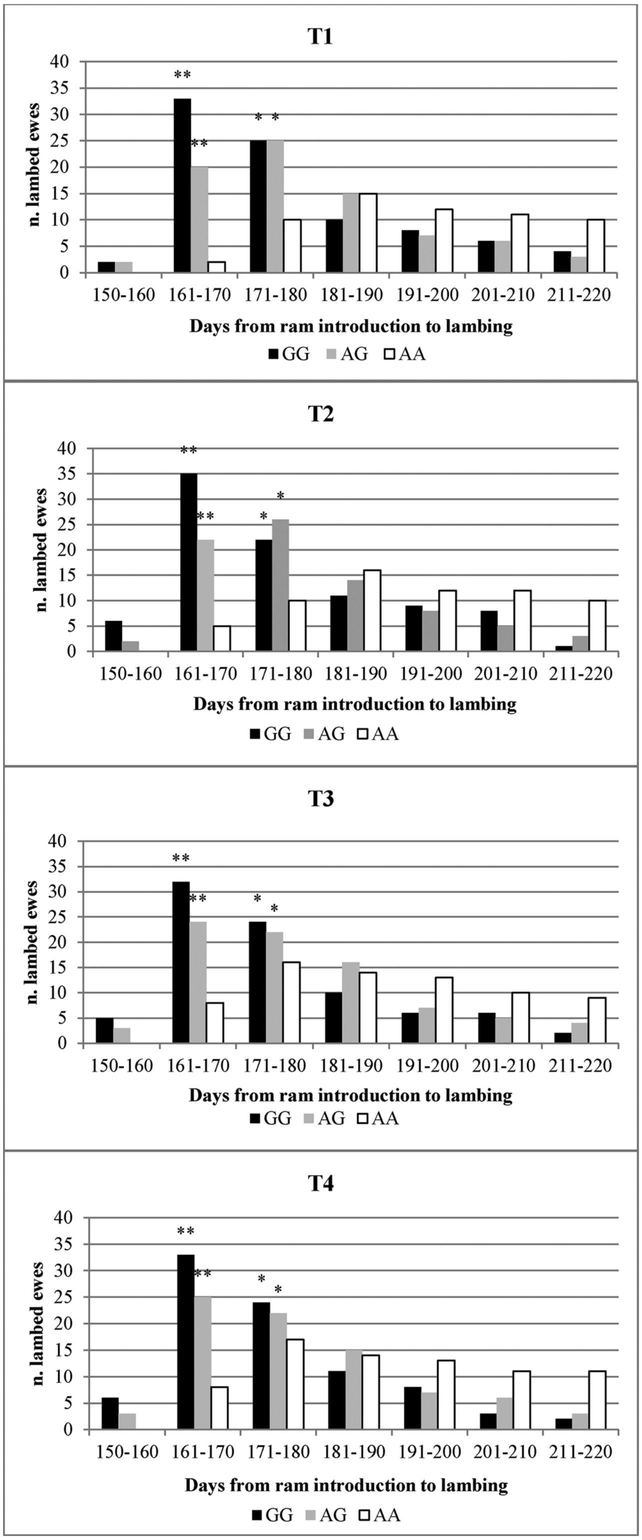

Fig. 1. Depiction of the number of ewes that lambed in 10-day intervals from 150 to 220 days after the joining of males with ewes in the four time periods, according to genotype at the g.15099485 A > G position; T1 = male joining with ewes on March 25; T2 = male joining with ewes on April 15; T3 = male joining with ewes on May 5; T4 = male joining with ewes on June 1; **$P < 0.01$; *$P < 0.05$.

p.Val220Ile amino acid substitution affected the inhibition of adenylate cyclase, thus suggesting a possible modification in the transmission of the melatonin signal in this variant (Trecherel et al., 2010) and, consequently, the differences observed in the reproductive function among ewes with the G/G, A/G and A/A genotypes (Pelletier et al., 2000). Studies on the second messengers involved in the melatonin receptor activation, however, are needed to clarify this hypothesis (Kokkola et al., 1998; Brydon et al., 1999). In a study by Calvo et al. (2018), it was hypothesised that the SNP at position g.15099004C > T could be the causative mutation for the effects on reproductive seasonality traits, as there is an arginine to cysteine substitution in the amino acid sequence at position 349. In the present study with the Sarda breed, this variation was not detected, but this finding indicates there are new and interesting possibilities for further investigations.

Furthermore, data from the present study indicate that in the animals with the G/G or A/G genotype, the placement of males with ewes induces a greater stimulus on the resumption of reproductive activity if the placement occurred at T1 and T2 compared to T3 and T4. This was surprising, as it was expected that the reproductive response in ewes with the G/G and A/G genotype would be increased from March to June. Instead, the effect is greater when the number of ewes that were anoestrus was greatest (March and April), rather than when the time that the initiation of the oestrous cyclic functions was approaching at the end of the anoestrous period when induction of oestrous cyclic functions is usually easier to induce (May and June). It is speculated that the effect of the G allele is greater when day length is shorter, confirming that ewes with one or two G alleles are less sensitive to the photoperiod inhibition of reproductive functions.

The lambing data in the present study was variable when there was assessment of the different genotypes and periods. In fact, the ewes with the G/G or A/G genotype had the peak of lambing earlier than the ewes with the A/A genotype for all the periods when assessments occurred. It is clear that the trend in lambing rate is associated with the trend in mating, so that the ewes with the G/G and A/G genotype had a greater response to the joining of males with ewes. In several studies, there has been an investigation of the origin of the variability in the reproductive response to joining of males with ewes.

Chanvallon et al. (2011) reported that there was a greater response of joining males with ewes at the end of the anoestrus season. Instead, in the present study, the ewes with the G/G and A/G genotype were not affected by the photoperiodic suppression to as great an extent in all periods when there were assessment and the greater response of ewes to the joining of males could be due to a set of positive factors, such as greater oestradiol secretion along with greater amounts of steroid acute regulatory protein (StAR) in granulosa cells Fabre-Nys et al. (2015). Further studies are necessary to clarify if the G allele may be associated with a greater secretion of E_2 and increased amounts of StAR compared to that of ewes with the A allele.

5. Conclusion

In conclusion, the present results indicate that both the *MTNR1 A* gene allelic variant and the period of joining rams with ewes affected the reproductive response to males in the Sarda breed of sheep. In the present study, there was a positive effect of the g.15099485A > G variant on the time of cessation of anoestrus when males were joined with ewes in March or April. Furthermore, the association between the SNPs, g.15099485A > G and g.15099391G > A, that was determined to exist in the present study could provide new insights to clarify the mechanism of action of melatonin and the role of its receptors on reproductive seasonality. In addition, studying the effects of the different genotypes on the granulosal and luteal cells may provide useful information on the action of the *MTNR1 A* gene at the ovary and on fertility in sheep.

Conflict of interest

None of the authors have any conflicts of interest to declare.

Author contribution

I certify on behalf of all coauthors that this article has not been presented in any other place for publication. All coauthors have contributed equally to the research (conception, design of study, acquisition and interpretation of data) as well as to article preparation. All coauthors have approved the final draft of this article.

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