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POLYMORPHISM DETECTION IN MTNR1A GENE AND ASSOCIATION WITH LITTER SIZE IN AWASSI SHEEP

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Abstract

In sheep, melatonin has a significant effect on the reproductive system, acting through specific receptors - one of which is melatonin receptor 1A (MTNR1A). It regulates reproductive traits such as seasonality and litter size. The aim of this study was to identify polymorphic variants in exon 2 of the MTNR1A gene and to analyze their association with litter size in Awassi sheep breed in Bulgaria. The two alleles (wild C and mutant T) and the three possible genotypes (homozygous CC, heterozygous CT and homozygous TT) were established in the studied group of ewes. The wild allele C and the heterozygous genotype CT showed a higher frequency (0.61 and 0.51, respectively) than the mutant allele T (0.39) and the homozygous genotypes CC (0.36) and TT (0.13). No statistically significant difference in litter size was observed between the different genotypes of the MTNR1A gene – CC (1.35), CT (1.29) and TT (1.24).

Key words*: Awassi sheep, genetic polymorphism, litter size, MTNR1A gene, PCR-RFLP.*

INTRODUCTION

Sheep are of great importance for the sustenance of humanity and provide important raw materials, whose numerous breeds, raised in extremely different environmental conditions, breeding and selection, show remarkable genetic diversity in productive and reproductive traits (Gootwine, 2020).

Sheep reproduction is an important economic trait (Ibrahim, 2019; Hernández-Montiel et al., 2020), having influence on multiple indicators such as fertility, fecundity, and prolificacy (Cosso et al., 2021), with low (5% to 10%) heritability, which is influenced by both genetic and non-genetic factors (Kianpoor et al., 2018).

Seasonal reproductive activity (controlled by photoperiod) is a common characteristic of sheep from the temperate climates and is one of the main obstacles to year-round lamb production. Seasonal reproduction in animals is determined by internal and external factors, the former being related to the genotype of the animal, while the second are related to the environment in which it lives (Abdoli et al., 2019). Differences in the reproductive characteristics of sheep themselves also depend on management systems and the rearing environment. Controlling environmental factors affecting these traits, as well as improving the genetic potential of mothers, can reduce age at first lambing, increase conception rates, and lead to higher twinning rates (Abdoli et al., 2019; Ajafar et al., 2022). One of the factors influencing these characteristics is melatonin or the "hormone of darkness", so called due to the fact that in all vertebrates, melatonin levels in the blood are higher at night than during the day and change depending on the season (Cazaméa-Catalan et al., 2014). It is a major hormonal regulator of the biological clock in vertebrates. Its production is concentrated in the pineal gland and the retina, reflecting the circadian rhythm and positively influencing reproduction during longer nights (Rosa & Bryant, 2003). In sheep, melatonin (MLT) can induce estrous cycles, increase ovulation (Zuniga et al., 2002), the number of lambs born per litter (Scott et al., 2009), luteal function, embryo viability (Abecia et al., 2008). In rams, melatonin affects sperm capacitation (Casao et al., 2012), increases the speed of motile sperm, and their ability to make contact with oocytes (Casao et al., 2010). Melatonin, produced in the testes of rams, preserves developing sperm from oxidative injury, with seasonal changes detected in the composition of seminal plasma (Abuzahra et al., 2025).

The identification of candidate genes and mutations in them is a fundamental approach to elucidating the genetic influence contributing to the diversity in reproductive performance in sheep (Abuzahra et al., 2025; Xu et al., 2018).
Melatonin acts through two specific receptors, called 1A and 1B. The MTNR1A gene, located on chromosome 26 of the genome and consisting of 2 exons (of which the second is significantly larger), is believed to be the main receptor involved in the regulation of seasonal reproductive activity in sheep and is also considered a candidate gene (Dubocovich et al., 2003; Martínez-Royo et al., 2012). Polymorphic variants in the MTNR1A gene have been found to be associated with seasonal breeding in different breeds of sheep (Carcangiu et al., 2009; Ahmad et al., 2015; Fathy et al., 2018). The association between polymorphisms in exon II of the *MTNR1A* gene and reproduction may show variability across different sheep breeds that exhibit nucleotide variations (He et al., 2019).
These are thought to alter reproductive responses to seasonal changes and generally improve reproduction (Luridiana et al., 2020). *RsaI* polymorphisms for the MTNR1A gene are associated with reproductive seasonality, fecundity rate and litter size (Casao et al., 2025). Melatonin secretion and consequently the seasonality of reproduction underlie the variation in different sheep breeds, with some breeds such as Texel and Serres exhibiting long anestrous seasons, while the Merino, Romanov and Chios breeds are characterized by less pronounced seasonality, and the Chinese Small Tail Han and Hu sheep breeds exhibit completely non-seasonal reproductive physiology (Chu et al., 2006; Giantsis et al., 2016).
Giving consideration to all of the above, the present study was undertaken with the aim of investigating the polymorphism in exon II of the MTNR1A gene and analyzing its relationship with litter size in Awassi sheep raised in Bulgaria.

MATERIALS AND METHODS

Animals and sample collection. The study involved 61 ewes of the Awassi breed from a farm in the village of Sredkovets, Shumen region, with the consent of the farm owner to participate in the experiment (Figure 1).
The sheep are inseminated naturally (once a year) during the months of August-October. The breeding rams are purchased from other farms breeding this breed. The herd is controlled by the "Dairy Sheep Breeding Association".

Figure 1. Awassi ewes from a private herd - the village of Sredkovets, Shumen district, Bulgaria (own source)

The selected animals are of different ages, with typical breed characteristics, in good health and have data on the number of lambs born from at least two consecutive lambings.
Blood samples (approximately 3 mL each) from the animals' peripheral blood were collected in vacuum tubes containing the anticoagulant EDTA by a state veterinarian serving the farm in compliance with all humane requirements. These samples were then transported to the laboratory and stored at –20°C until further analysis.
The laboratory experiment was conducted at the Genetics Laboratory, part of the Faculty of Agronomy of the University of Forestry, Sofia, Bulgaria.
Genomic DNA was extracted using a genomic DNA purification kit (Illustra Blood Genomic Prep DNA Purification Kit, GE Healthcare) following the manufacturer's protocol. The quality and quantity of DNA were assessed by spectrophotometric analysis (Biodrop spectrophotometer) and agarose gel electrophoresis (1% agarose gel). The DNA concentration was determined to be approximately 12-15 ng/μL.
PCR reactions were performed in a total volume of 10 μL, containing 40 ng of genomic

DNA, 0.2 μL ddH_2O, 20 pM of each primer, and 5 μL of 2× (1.5 mM $MgCl_2$) MyTaq™ HS Red Mix (Bioline). Specific PCR fragments of exon 2 of the ovine MTNR1A gene (GenBank U14109) were amplified using primers described by Messer et al. (1997), with the following sequences:
F: 5'-TGTGTTTGTGGTGAGCCTGG-3' and
R: 5'-ATGGAGAGGGTTTGCGTTTA-3'.
Amplification of the MTNR1A gene was performed under the following PCR conditions: an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation (94°C for 30 s), annealing (62°C for 45 s), and elongation (72°C for 1 min), with a final elongation step at 72°C for 10 min.

Restriction fragment length polymorphism (RFLP). The resulting PCR products were subsequently digested using the fast *RsaI* enzyme (Jena Bioscience), following the protocol described by Saxena et al. (2015). The digestion reaction was carried out in a total volume of 10 μL, comprising 6 μL of the PCR amplicon, 0.5 μL of the enzyme, 1 μL of 10× buffer, and 2.5 μL of ddH_2O. The reaction mixture was incubated at 37°C for 15-20 min in a thermal block.

Post-restriction fragments were separated via agarose gel electrophoresis using a 50 bp DNA Ladder (Thermo) on a 2.6% agarose gel (Bioline) stained with 10000×RedGel™ Nucleic Acid Stain (Biotium) and 1× TBE buffer (Jena Bioscience). The final results were visualized under UV light.

Statistical Analysis. Sheep fertility was defined as the number of lambs born per ewe based on the ratio of the number of live births, stillbirths and registered abortions to the number of lambs.

The association of the MTNR1A gene polymorphism with litter size in sheep was determined using a one-way analysis of variance ANOVA model.

RESULTS AND DISCUSSIONS

The results of the RsaI enzyme treatment of the 824 bp fragment from exon 2 of the MTNR1A gene obtained after PCR using specific primers revealed three genotypes: CC (represented by two fragments of size 411 bp and 267 bp), CT (three fragments of length 411 bp, 290 bp and 267 bp) and TT (two fragments of size 411 bp and 290 bp) (Figure 2). All three possible genotypes were identified in the studied Awassi ewes i.e. a genetic polymorphism in the MTNR1A gene has been observed. The wild allele C and the heterozygous genotype CT showed a higher frequency (0.61 and 0.51, respectively) compared to the mutant allele T (0.39) and the homozygous genotypes CC (0.36) and TT (0.13).

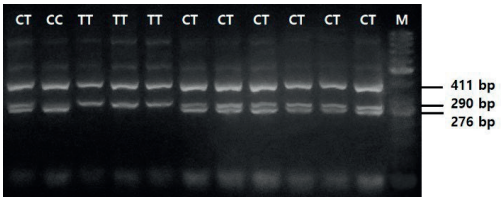

Figure 2. Agarose gel electrophoresis of PCR-RFLP fragments of exon 2 of MTNR1A gene using the enzyme *RsaI* (own source)

A study of ewes from three Bulgarian merino breeds (Caucasian Merino, Ascanian Merino and Karnobat Merino) showed a good genetic diversity with the presence of two alleles and all three possible genotypes (Bozhilova-Sakova & Dimitrova, 2022). The allele frequencies obtained by us for the Awassi breed animals are close to those for Caucasian Merino and Karnobat Merino, but the distribution of genotype frequencies resembles those found in the Ascanian breed with a predominance of the heterozygous genotype CT, in which the most significant diversity is observed.

Our results regarding allele frequencies in animals of the Awassi breed are close to those obtained by Gencheva (2019) for sheep of the autochthonous Bulgarian breeds Sofia (Elin-Pelin), Karakachan and Black-headed Pleven and significantly differ from those obtained for the Copper-red Shumen breed. Regarding the distribution of genotypes, our results for Awassi ewes are similar to those obtained for those of the Sofia (Elin-Pelin) breed and significantly differ from those for the other three breeds.

Studies conducted on other local Bulgarian sheep breeds show a predominance of the T allele in the Stara Zagora breed, while in the local Karnobat breed the C allele predominates more than in our study of the Awassi breed. In this study, the frequencies of the two alleles are

almost equal in the Black-headed Pleven and Sofia (Elin-Pelin) breeds (Hristova et al., 2012).

A similar distribution of genotypes was found by other authors in Awassi sheep in Iraq (Mahdi et al., 2020). Conversely, in the Sarda breed, it was found that the CC genotype (0.53) predominates, with the other two genotypes having an almost equal distribution (Luridiana et al., 2014).

In the studied animals, the observed heterozygosity (0.508) was higher than the expected (0.476), indicating a high level of genetic diversity in this herd of Awassi breed (Table 1).

Table 1. Allele and genotype frequencies, heterozygosity, coefficient of inbreeding, chi-square value and p-value of the investigated animals from Awassi breed

Gene	n	Ae	Allele frequency		Genotype frequency			Heterozygosity		F_{IS}	χ^2	p-value
			C	T	CC	CT	TT	Ho	He			
MTNR1A	61	1.92	0.61	0.39	0.36	0.51	0.13	0.508	0.476	0.067	0.29	0.59

The number of lambs born per ewe is highest in animals carrying the homozygous genotype CC (1.35). The litter size for carriers of the heterozygous genotype CT and homozygous genotype TT shows similar values - 1.29 and 1.24, respectively. In general, it can be said that in Bulgarian conditions, the animals from the studied herd have higher fertility than that typical of the Awassi breed (Alkass et al., 2021; Galal et al., 2008; Gootwine, 2011; Salman et al., 2024; Talafha & Ababneh, 2011; Üstüner & Oğan, 2013).

The results regarding litter size for the established genotypes of Awassi ewes and the performed association analysis are shown in Table 2.

Table 2. Effect of the genotype of MTNR1A gene on the litter size

Traits	Genotype CC (n=22)			Genotype CT (n=31)			Genotype TT (n=8)			P-value
	LS records	$\bar{X}$	CV%	LS records	$\bar{X}$	CV%	LS records	$\bar{X}$	CV%	
Litter size	43	1.35	23.26	66	1.29	20.82	17	1.24	19.12	0.65

The association analysis conducted for the relationship of the MTNR1A gene polymorphism did not confirm significant differences (p-value>0.05) in litter size between the established genotypes. Similar to what we observed, Cosso et al. (2021) did not find such a relationship in a study on the influence of polymorphisms of the MTNR1A gene on the reproductive performance of Awassi sheep bred in Lebanon.

Starič, et al. (2020) also reported that litter size in three Slovenian sheep breeds was not affected by the polymorphism of the MTNR1A gene. However, in other studies, such a relationship has been found in sheep of the same and other breeds of sheep. For example, Mahdi et al. (2020) found a significant effect of the MTNR1 polymorphism on litter size in Awassi sheep, with the highest effect in CC genotype carriers (1.184) and the lowest effect in TT genotype sheep (1.137). In a study of single nucleotide polymorphisms (SNPs) within the gene sequence of the MTNR1A gene in Thin-tailed Indonesian ewes Abuzahra et al., (2025) identified 19 novel SNPs, with 10 being non-synonymous variations, in the MTNR1A gene. One non-synonymous SNP (rs1087815963) showed a significant association with litter size. The study conducted by us to identify a polymorphism in the MTNR1A gene and establish its relationship with litter size in Awassi sheep is the first in Bulgaria. The results obtained cannot be a definitive statement as they refer to a limited number of sheep from one flock. They can serve as a basis for future studies related to both the genetic diversity and the phenotypic expression of reproductive traits in sheep of the

same and other breeds raised in Bulgaria. This would help to clarify the relationship between the traits of the reproductive ability of sheep and this gene.

CONCLUSIONS

The results of the PCR-RFLP analysis showed the presence of a polymorphism in exon II of the gene encoding the melatonin receptor in the genome of the studied Awassi sheep.

Two alleles (wild C and mutant T) were identified in the MTNR1A gene with a predominance of the C allele. All three possible genotypes were observed - CC, CT and TT, with slightly over half of the studied animals carrying the heterozygous CT genotype.

The present study is the first on the polymorphism in exon 2 of the MTNR1A gene in Awassi sheep in Bulgaria and the results obtained cannot be a definitive statement as they refer to a limited number of sheep from only one flock. It is necessary to expand the study with a larger number of sheep from other flocks of the breed, raised in Bulgaria.

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