Revised

Polymorphisms of the melatonin receptor 1A (*MTNR1A*) gene influence the age at first mating in autumn-born ram-lambs and sexual activity of adult rams in spring

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ABSTRACT

The aim of this study was to determine whether polymorphisms of the melatonin receptor 1A (*MTNR1A*) gene influence the age at first mating in autumn-born ram-lambs and influence the out-of-season sexual activity of adult rams. In experiment 1, 24 Rasa Aragonesa ram-lambs born in September were genotyped for their RsaI and MnlI allelic variants of the *MTNR1A* gene, and the date of their first mounting with ejaculation after a period of semen collection training was documented. In experiment 2, the reproductive behavior, testicle size, and plasma testosterone concentrations of 18 adult rams (6 rams for each RsaI genotype) were recorded at the beginning (March) and end (May) of the seasonal anestrus. The number of days of training to achieve the first mating with ejaculation in T/T (C/C: 85.17± 12.08 C/T: 86.60±18.87; T/T; 26.50±24.50 d; $P<0.05$), and G/G ram-lambs (G/G: 51.57±14.99; A/G: 95.58±10.95 d; $P<0.05$) was significantly fewer than it was in the other genotypes. Likewise, for the RsaI genotype, 55% of the vulva-sniffing ($P<0.001$), 48% of the approaches ($P<0.01$), 48% of the mountings ($P<0.05$) and 49% total activities ($P<0.001$) were performed by T/T rams in March, and 50% of the sexual events in May ($P<0.001$). For the Mnll variant, G/G rams performed a significantly ($P<0.001$) larger proportion of the vulva-sniffing (41%), approaches (46%) and total activities (40%) in March, and 52% of the vulva-sniffing ($P<0.001$), 43%, of the approaches ($P<0.001$), 46% of the mountings ($P<0.05$), and 47% of the total activities ($P<0.001$) in May. Scrotal circumference, testicular volume, and plasma testosterone concentrations did not differ significantly among genotypes. Results confirmed that the polymorphisms of the *MTNR1A* gene sequence can influence reproductive performance in young and adult rams. Autumn-born ram-lambs that carried the T/T or G/G genotype had an advanced ability to reproduce, and T/T or G/G adult rams exhibited the most intense reproductive behavior. Genotyping might be a useful procedure for identifying the correct and rational use of rams in modern sheep farming.

Keywords: sheep, melatonin, receptor, sexual activity

1. Introduction

The synchronization of reproductive activity and living environment are important requirements for the survival of wild animals. In particular, small ruminants reproduce seasonally and their offspring are born in spring, which is the most favorable time of the year for their growth and survival. Sheep and goats maintained at temperate latitudes under natural conditions are sexually active in autumn, which leads to births in spring [1].

In Mediterranean areas, an advance in lambing at the beginning of autumn increases farm incomes, significantly, by reducing the length of the unproductive period in sheep, although it requires that reproduction occurs in spring, which is the non-breeding season [2]. Under natural variations in day-length at temperate latitudes, female small ruminant exhibit highly repeatable and distinct anovulatory and anestrous periods, and males exhibit significant variability in reproductive behavior and spermatogenetic activity from early autumn to mid-summer [3]. Photoperiod is the main environmental factor that influences those seasonal patterns [4]. Long days inhibit and short days stimulate sexual activity in sheep [5]. The light detected by the retina is translated into a neuroendocrine message by the epiphysis through the secretion of melatonin [6]. Blood concentrations of that hormone are low in day-light and high at night, thus, it is an organic informer of photoperiod [7-8].

In females of many mammals, melatonin influences seasonal reproductive activity through its effects on the *pars tuberalis* (review: [9]). Melatonin acts on specific receptors in various nuclei in the central nervous system including those that regulate reproduction [10]. In mammals, several types of melatonin receptors have been identified, but MT1, only, appears to be involved in the regulation of reproductive activity [11-12]. The MT1 receptor belongs to the G protein-coupled receptor family, and its gene has been cloned [13] and mapped in several animal species [14]. The melatonin receptor 1A (*MTNR1A*) gene exhibits several polymorphic sites, which are associated with seasonal reproductive activity in ewes [15-16] and other mammals [17-19]. Although males exhibit a seasonal pattern in sexual activity, it is unknown whether those polymorphisms influence the reproductive performance of rams. Melatonin production occurs in testis and melatonin receptors have been described in various testicular regions by our group [20]; therefore, we hypothesized whether polymorphisms of the melatonin receptor gene differ in their effects on reproductive seasonality in rams. To test that hypothesis, timing of first mating in autumn-born ram-lambs and the sexual activity of adult Rasa Aragonesa rams in spring, carrying different polymorphisms of the *MTNR1A* gene, have been studied.

2. Material and methods

The experiment was conducted at the experimental farm of the University of Zaragoza, Spain (41°40'N). The Ethics Committee for Animal Experiments at the University of Zaragoza approved the procedures performed in the study. The care and use of animals were in accordance with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

2.1. Experiment 1

Twenty-four Rasa Aragonesa ram-lambs born in early September were genotyped for the RsaI and MnlI allelic variants of the *MTNR1A* gene (Table 1). At the age (±S.D.) of 5±0.3 mo (Feb) (live weight: 33.4±0.2 kg), the rams were initiated into semen collection training, which occurred every 10 d for six months until the end of July. Each session lasted 20 min or until two ejaculates were obtained from the ram, whichever occurred first. Rams were individually exposed to one immobilized female in estrus, which had been induced by intravaginal sponges that contained 40 mg of flurogestone acetate and an i.m. injection of 400 IU of eCG (Syncro-Part, CEVA Salud Animal, Spain) at pessary withdrawal, 14 d later. The same technician was present with the animals throughout the training period. The semen collection materials and procedures followed Evans and Maxwell [21]. Rams were housed in a group and fed to meet their maintenance requirements. The date of the first mounting with ejaculation of each ram was documented, and the time between the onset of the training period (6 February) and the date of first mounting was calculated.

2.2. Experiment 2

Eighteen sexually-experienced 2.5 year-old Rasa Aragonesa rams (live weight: 89.2±5.7 kg) were selected from among 39 animals (Table 2) based on their Rsal polymorphism: genotype C/C (n=6), C/T (n=6), and T/T (n=6). Based on their Mnll allele, rams were classified as G/G (n=9), G/A (n=3), or A/A (n=6). Rams were housed as a single group, which was isolated from another group of rams and ewes, and fed to meet their maintenance requirements. Rams had not undergone any previous serving capacity test, and some sporadic homosexual behavior had been observed.

In late March and late May, individual serving-capacity tests [22-23] were performed. To that end, to induce synchronized estrus, 40 adult Rasa Aragonesa ewes received intravaginal pessaries for 12 d and 300 IU eCG i.m. (Syncro-Part, CEVA Salud Animal, Spain). Forty-eight hours later (20 Mar and 20 May), ewes were used in an individual ram serving-capacity test. For 20 min, individual rams were exposed to five estrous ewes in a 15-m^2 pen and the following information was recorded: the number of acts of flehmen (elevating the head and upper lip feedback in response to taste and odor of ewe urine or ambient odor), ano-genital sniffing (sniff in the genital region of ewe), approaches (rubbing, licking, or superficially nibbling the flank of the ewe with intensity), attempted mounting (stands behind the ewe and moves with the intention to copulate, with front legs in the air, but not placed safely on the ewe), and mounting (intrusion of the penis into vagina of ewe with one or more thrusts and, thereby, ejaculation can occur, which is indicated by the backward elevation of the ram's head). The definitions of sexual events followed Calderón-Leyva et al. [24].

The day before the sexual-capacity test, scrotal circumference (SC) was measured. To estimate testicular volume (TV), testicle width and length were measured and TV was calculated as (0.0396 x (average testis length) x (scrotal circumference)^2 [25]. To measure plasma testosterone concentrations, a blood sample was collected (08:00 am) by jugular venipuncture, placed into a heparinized tube, and centrifuged at 3,500 × g for 30 min. The plasma fraction was stored at −20°C until testosterone concentrations were measured.

2.3. Blood sampling and DNA analysis

To identify the individual allelic variants, DNA analysis was performed using whole blood from each ram. Blood samples (10 ml) were collected from the jugular vein into vacuum tubes that contained ethylenediaminetetra acetic acid (EDTA) as an anticoagulant. The DNA was extracted using a genomic DNA extraction kit (NucleoSpin® Blood, Macherey-Nagel, Germany). Polymerase chain reaction (PCR) was performed on 150 ng of genomic DNA from each ram and specific primers (Sigma Genosys Ltd., Pampisford, Cambs, UK) according to Messer et al. [14]. The primers were 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’) [13]

(GenBank accession number U14109). Thereafter, we referenced to the newest ovine *MTNR1A* gene sequence included in the latest ovine genome version (Oar_rambouillet_v1.0 - GenBank assembly accession number: GCA_002742125.1). The PCR reaction was performed according to Mura et al. [26]. The PCR products were subjected to a double restriction enzyme analysis involving the MnlI and RsaI endonucleases (New England Biolabs, Beverly, MA, USA). The MnlI restriction enzyme recognizes an A to a G substitution at position g.17355452, and RsaI recognizes a C to a T substitution at position g.17355458 of the GCA_002742125.1 genome sequence (corresponding, respectively, to position 612 and 606 of the older MTNR1 A exon II U14109 nucleotide sequence). The digestion reactions were performed according to Carcangiu et al. [16].

2.4. Sequencing

To determine whether the variants identified by endonucleases digestion were associated with other nucleotide substitutions, the PCR products for each genotype were sequenced by an Applied Biosystems 3730 DNA Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). To confirm the correspondences among the known nucleotide changes and identify other possible substitutions, the sequences were aligned and compared with the ovine sequence GenBank U14109 and GCA_002742125.1. The homology searches were performed through 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).

2.5. Hormonal assay

Plasma testosterone concentrations were measured in duplicate by direct radioimmunoassay [27]. Sensitivity was 0.3 ng/ml. Samples were run in a single assay (intra-assay CV = 6%).

2.6. Statistical analysis

In experiment 1, statistically significant differences among genotypes in the timing of first mating, and in the number of days of training until the first mating were identified by a log-rank test for trend and a 2-way ANOVA, respectively.

In experiment 2, statistically significant differences among genotypes in proportions of events performed were identified by X^2 tests. Differences in SC and TV were assessed by ANOVA, with genotype variant as the main effect. To calculate statistical differences among genotypes for SC and TV, an ANOVA was used. To assess the statistical significance of differences in SC and TV between March and May, a Paired T-test for related samples was used.

3. Results

3.1. Experiment 1

At the end of the experiment (July), the proportion of the rams which mated at least once and remained sexually active throughout the experiment was 70.8%. Five of the rams (5 C/C, 0 C/T, 0 T/T of the RsaI genotype; P<0.001) (1 A/A, 2 A/G, and 2 G/G for the MnlI genotype, P>0.05) did not respond to the female stimulus neither ejaculate.

Among the RsaI genotypes, T/T rams (26.50±24.50 d) required significantly (P<0.05) fewer days of training to achieve their first ejaculation with the AV than did the other genotypes (C/C: 85.17± 12.08 d, C/T: 86.60±18.87 d) (Fig. 1). Among the MnlI genotypes, the number of days of training until the first mounting was significantly (P<0.05) less in the G/G rams (51.57±14.99 d) than it was in the A/G genotype. (95.58±10.95 d).

3.2. Experiment 2

In March and May, T/T rams exhibited a significantly higher level of sexual activity than did the other genotypes (Fig. 2); specifically, 55% of the vulva-sniffing (P<0.001), 48% of the approaches (P<0.01), 48% of the mountings (P<0.05), and 49% of total activities (P<0.001) in March, and 50% of all sexual events in May (P<0.001). Among the Mnll genotypes, G/G rams performed a significantly (P<0.001) higher proportion of the vulva-sniffing (41%), approaches (46%), and total activities (40%) in March, and 52% of the vulva-sniffing (P<0.001), 43% of the approaches (P<0.001), 46% of the mountings (P<0.05), and 47% of total activities (P<0.001) in May.

Scrotal circumference, TV, and plasma testosterone concentrations did not differ significantly among genotypes (Table 3). In March and May, some of the genotypes exhibited a significant (P<0.05) increase in SC (C/C, G/G, and A/A) and or TV (C/C, G/G, and T/T). Testosterone concentrations did not differ significantly among genotypes (Table 3).

4. Discussion

Results of this experiment show that T/T and G/G genotypes of the MTNR1A gene were associated with an earlier mating activity of ram-lambs, and a more intensive reproductive behavior of adult rams in spring. The genotypic and allelic frequencies of the MTNR1A gene observed in this study were similar to those reported for the same breed [28], with small differences probably due to the smaller number of animals included in the present study. The frequency of the mutant allele G at position g.17355452 G>A of the MTNR1A gene exon II sequence in our study was similar to those found in other sheep breeds [16,29-30]. Our results, moreover, showed that at the position g.17355458 C>T, the frequency of the T allele was higher than it is in the Sarda breed, but very similar to those in some Indian and Egyptian sheep breeds [31-33].

In our study, the two MTNR1A gene loci appeared to influence the reproductive behavior of Rasa Aragonesa rams. In particular, the T/T genotype at position g.17355458 C> T had a positive effect on the sexual performance of young and adult rams. Young rams that carried the T/T genotype were advanced in their ability to reproduce, and adult males exhibited higher reproductive behavior in March and May than did the other genotypes. Published data on those phenomena in rams are unavailable; however, a correlation between the T/T genotype and a high proportion of cyclic sheep between January and August has been observed in Rasa Aragonesa and in some Slovenian ewes [34-35]. In one study, the C/C genotype had a more advanced reproductive recovery in spring than did the C/T and the T/T genotype in Sarda ewes [16].

In Rasa Aragonesa, the polymorphism at position g.17355452 G>A appeared to be involved in the reproductive performance of young and adult rams. Specifically, ram-lambs that carried the G/G genotype were advanced considerably in the age at first mounting, and adult G/G rams performed significantly disproportionally more of the reproductive behaviors (vulva-sniffing, approaches, mountings, and total activities) than did the other genotypes. Although there is no published information on the effects of this genotype in rams, in ewes of several sheep breeds, the G/G genotype appears to have the best reproductive recovery, ovulatory cyclicity throughout the year, and reproductive response to treatment with melatonin or synthetic progestins [17,36-38]. It is uncertain why those two SNPs influence reproductive behavior because they do not involve amino acid substitutions; however, variation g.17355452G>A is linked to g.17355358C>T substitution, which produces an amino acid change, and might affect the melatonin transmission system, as reported also by other authors [39]. Sensitivity to photoperiod and thus to melatonin, might be affected by genotype, which would make G/G ewes the most responsive to the onset of reproductive activity. Consequently, it can be hypothesized that also in males the different genotypes could influence the reproductive performance as found in the present research. In addition, melatonin receptors have been identified in various testicular areas [20], which underscores the importance of melatonin in testicular function. Indeed, because of its antioxidant properties, melatonin can protect spermatozoa from apoptosis [40-41]. Furthermore, the in vitro reduction of nitric oxide levels in ram spermatozoa by melatonin treatment, modulates capacitation by cAMP [42]. Possibly, in our study, changes in melatonin message transmission caused by the polymorphism have improved ram reproductive activity and sexual behavior.

For the g.17355452G>A and the g.17355458C>T polymorphisms, blood testosterone concentrations in adult rams did not differ significantly among genotypes; however, in May, rams that carried the T/T or G/G variants had the highest testosterone circulating levels. Possibly, the animals that carried those variants were preparing for reproductive recovery earlier than were the animals that had the other genetic variants. Data about SC and TV in g.17355458C>T locus for different genotypes are difficult to explain. In fact, from March to May, rams carrying the T/T genotype significantly increased their TV, while C/C rams increased both SC and TV. However, the values registered in May were similar for both the above-mentioned genotypes, which suggests a similar preparation of the reproductive system for sexual recovery. This little difference in the observed parameters would require a longer observation period to achieve a more accurate evaluation. Regarding the g.17355452G>A locus, G/G rams exhibited a significant increase in SC and TV from March to May, while A/A rams only increased their SC. The values exhibited by G/G rams were higher for both parameters, confirming their better sexual behavior and reproductive recovery. The role of the polymorphisms of the *MTNR1A* gene might be better clarified by extending observations in a greater number of animals for longer periods, including semen quality analysis.

5. Conclusions

This study confirmed that the polymorphisms at the *MTNR1A* gene sequence influenced the reproductive performance of young and adult Rasa Aragonesa rams. The T/T and G/G genotypes were associated with an advance in the ability of autumn-born ram-lambs to reproduce, and an improvement in the reproductive behavior of adult rams. Genotyping might be a useful procedure for identifying the correct and rational use of rams in modern sheep farming.

Declaration of competing interest

The authors have no conflicts of interest to declare

CRediT authorship contribution statement

J.A. Abecia: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition, Writing -review&editing. M.C. Mura: Conceptualization, Methodology, Writing - Original Draft, Supervision, Writing -review&editing. M. Carvajal-Serna: Formal analysis, Investigation, Visualization. L. Pulinas: Conceptualization, Methodology, Investigation, Writing - Original Draft. A. Macías: Resources, Investigation. A. Casao: Formal analysis, Investigation, Visualization. R. Pérez-Pe: Formal analysis, Investigation. V. Carcangiu: Conceptualization, Methodology, Writing - Original Draft, Supervision, Funding acquisition, Writing -review&editing.

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References

[1] Chemineau P, Malpaux B, Thiery JC, Viguie C, Morello H, Zarazaga L, Pelletier J. The control of seasonality: a challenge to small ruminant breeding. In: Enne G, Greppi GF, Lauria A, editors. Reproduction and Animal Breeding. Advances and Strategy, Amsterdam: Elsevier; 1995, p. 225-250.

[2] Luridiana S, Mura MC, Daga C, Farci F, Di Stefano MV, Zidda F, Carcangiu V. Melatonin treatment in spring and reproductive recovery in sheep with different body condition score and age. Anim Reprod Sci 2015;160:68-73. http://dx.doi.org/10.1016/j.anireprosci.2015.07.004.

[3] Chemineau P, Pelletier J, Guérin Y, Colas G, Ravault JP, Touré G, Almeida G, Thimonier J, Ortavant R. Photoperiodic and melatonin treatments for the control

of seasonal reproduction in sheep and goats. Reprod Nutr Dev 1988;28:409-422. https://doi.org/10.1051/rnd:19880307.

[4] Hansen PJ. Photoperiodic regulation of reproduction in mammals breeding during long days versus mammals breeding during short days. Anim Reprod Sci 1985;4:301-315. https://doi.org/10.1016/0378-4320(85)90059-4.

[5] Karsch FJ, Bittman EL, Goodman RL, Legan SJ, Robinson JE. Neuroendocrine basis of seasonal reproduction. Recent Prog Horm Res 1984;40:185-225. https://doi.org./10.1016/b978-0-12-571140-1.50010-4.

[6] Goldman BD. Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J Biol Rhythms 2001;16:283–301. https://doi.org/10.1177/074873001129001980.

[7] Lincoln GA, Johnston JD, Andersson H, Wagner G, Hazlerigg DG. Photorefractoriness in mammals: dissociating a seasonal timer from the circadian-based photoperiod response. Endocrinology 2005;146:3782-3790. https://doi.org/10.1210/en.2005-0132.

[8] Carcangiu V, Mura MC, Parmeggiani A, Piccione G, Bini PP, Cosso G, Luridiana S. Daily rhythm of blood melatonin concentrations in sheep of different ages. Biol Rhythm Res 2013;44:908-915. https://dx.doi.org/10.1080/09291016.2013.781317.

[9] Dardente H. Melatonin-dependent timing of seasonal reproduction by the pars tuberalis: pivotal roles for long daylengths and thyroid hormones. J Neuroendocrinol. 2012;24:249-266. https://doi:10.1111/j.1365-2826.2011.02250.x

[10] Sliwowska J, Billings HJ, Goodman RL, Coolen LM, Lehman MN. The premammillary hypothalamic area of the ewe: anatomical characterization of a melatonin target area mediating seasonal reproduction. Biol Reprod 2004 ;70 :1768-1775. https://doi.org/10.1095/biolreprod.103.024182.

[11] Weaver DR, Liu C, Reppert SM. Nature's knock-out: the Mel1b receptor is not necessary for reproductive and circadian responses to melatonin in Siberian hamsters. Mol Endocrinol 1996;10:1478–1487. https:/doi.org/10.1210/mend.10.11.8923472.

[12] Dubocovich ML, Yun K, Al-Ghoul WM, Benloucif S, Masana MI. Selective MT2 Melatonin receptor antagonists block melatonin-mediated phase advances of

circadian rhythms. FASEB J 1998;12:1211-1220. https://doi.org/10.1096/fasebj.12.12.1211.

[13] Reppert SM, Weaver DR, Ebisawa T. Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron 1994;13:1177–1185. https://doi.org/10.1016/0896-6273(94)90055-8.

[14] Messer LA, Wang L, Tuggle CK, Yerle M, Chardon P, Pomp D, Womack JE, Barendse W, Crawford AM, Notter DR, Rothschild MF. Mapping of the melatonin receptor 1a (*MTNR1A*) gene in pigs, sheep and cattle. Mamm Genome 1997;8:368–370. https://doi.org/10.1007/s003359900444.

[15] Pelletier J, Bodin L, Hanocq E, Malpaux B, Teyssier J, Thimonier J, Chemineau P. Association between expression of reproductive seasonality and alleles of the gene Mel1a receptor in the ewe. Biol Reprod 2000;62:1096–1101. https://doi.org/10.1095/biolreprod62.4.1096.

[16] Carcangiu V, Mura MC, Vacca GM, Pazzola M, Dettori ML, Luridiana S, Bini PP. Polymorphism of the melatonin receptor MT1 gene and its relationship with seasonal reproductive activity in the Sarda sheep breed. Anim Reprod Sci 2009;116:65–72. https://doi.org/10.1016/j.anireprosci.2009.01.005.

[17] Chu MX, Cheng DX, Liu WZ, Fang L, Ye SC. Association between melatonin receptor 1A gene and expression of reproductive seasonality in sheep. Asian-Austral J Anim Sci 2006;19:1079-1084. https://doi.org/10.5713/ajas.2006.1079.

[18] Carcangiu V, Mura MC, Vacca GM, Dettori ML, Pazzola M, Daga C, Luridiana S. Characterization of the melatonin receptor gene MT1 in mouflon (Ovis Gmelini Musimon) and its relationship with reproductive activity. Mol Reprod Dev 2010;77:196. https://doi.org/10.1002/mrd.21125.

[19] Luridiana S, Mura MC, Pazzola M, Paludo M, Cosso G, Dettori ML, Bua S, Vacca GM, Carcangiu V. Association between melatonin receptor 1A (MTNR1A) gene polymorphism and the reproductive performance of Mediterranean Italian buffaloes Reprod Fertil Dev 2012;24:983-987. https://doi.org/10.1071/RD11297.

[20] Casao A, Gallego M, Abecia JA, Forcada F, Pérez-Pé R, Muiño-Blanco T, Cebrián-Pérez JA. Identification and immunolocalisation of melatonin MT(1) and MT(2) receptors in Rasa Aragonesa ram spermatozoa. Reprod Fertil Dev 2012;24:953-961. https://doi.org/10.1071/RD11242.

[21] Evans G, Maxwell WMC.. Handling and examination semen. In: Salamon S (editor). Salamon's Artificial Insemination of Sheep and Goats. Butterworths, Sydney; 1987, p. 93–106.

[22] Kilgour RJ, Whale RG. The relation between mating activity of rams in pens and subsequent flock mating performance. Aust J Exp Agric 1980;20:5–8.

[23] Damián JP, Beracochea F, Hötzel MJ, Banchero G, Ungerfeld R. Reproductive and sexual behaviour development of dam or artificially reared male lambs. Physiol Behav 2015;147:47–53.

[24] Calderón-Leyva G, Meza-Herrera CA, Martinez-Rodriguez R, Angel-García O, Rivas-Muñoz R, Delgado-Bermejo JV, Véliz-Deras FG. Influence of sexual behavior of Dorper rams treated with glutamate and/or testosterone on reproductive performance of anovulatory ewes. Theriogenology 2018;106: 79–86.

[25] Godfrey RW, Collins JR, Gray ML. Evaluation of sexual behavior of hair sheep rams in a tropical environment. J Anim Sci 1988;76:714-717.

[26] Mura MC, Luridiana S, Pulinas L, Di Stefano MV, Carcangiu V. Reproductive response to male joining with ewes with different allelic variants of the *MTNR1A* gene. Anim Reprod Sci 2019;200:67-74. https://doi.org/10.1016/j.anireprosci.2018.11.012.

[27] Hochereau-de Reviers MT, Copin M, Seck M, Monet-Kuntz M, Cornu C, Fontaine C, Perreau C, Elsen JM, Boomarov O. Stimulation of testosterone production by PMSG injection in the ovine male: Effect of breed and age and application to males carrying or not carrying the F Booroola gene. Anim Reprod Sci, 1990;23;21–32.

[28] Calvo JH, Serrano M, Martinez-Royo A, Lahoz B, Sarto P, Ibañez-Deler A, Folch J, Alabart JL. SNP rs403212791 in exon 2 of the MTNR1A gene is associated with reproductive seasonality in the Rasa Aragonesa sheep breed. Theriogenology 2018;113:63–72. https://doi.org/10.1016/j.theriogenology.2018.02.013.

[29] Notter DR, Cockett NE. Opportunities for detection and use of QTL influencing seasonal reproduction in sheep: a review. Genet Sel Evol 2005;37:39–53. https://doi.org/10.1186/1297-9686-37-S1-S39.

[30] Saxena VK, Jha BK, Meena AS, Naqvi SM. Characterization of MTNR1A gene in terms of genetic variability in a panel of subtemperate and subtropical Indian

sheep breeds. J Genet 2015;94:715–721. https://doi.org/10.1007/s12041-015-0587-9.

[31] Mura MC, Luridiana S, Bodano S, Daga C, Cosso G, Diaz ML, Bini PP, Carcangiu V. Influence of melatonin receptor 1A gene polymorphisms on seasonal reproduction in Sarda ewes with different body condition scores and ages. Anim Reprod Sci 2014;149:173–177. https://doi.org/10.1016/j.anireprosci.2014.07.022.

[32] Saxena, V.K., Jha, B.K., Meena, A.S., Narula, H.K., Kumar, D., Naqvi, S.M.K., 2015. Assessment of genetic variability in the coding sequence of melatonin receptor gene (MTNR1A) in tropical arid sheep breeds of India. Reprod Domest Anim 50, 517–521. https://doi.org/10.1111/rda.12503.

[33] Fathy HA, Gouda EM, Gafer JA, Galal MK, Nowier AM. Genetic polymorphism in melatonin receptor 1A and arylalkylamine N-acetyltransferase and its impact on seasonal reproduction in Egyptian sheep breeds. Arch Anim Breed 2018;61:505–516. https://doi.org/10.5194/aab-61-505-2018.

[34] Martínez-Royo A, Lahoz B, Alabart JL, Folch J, Calvo JH. Characterisation of the Melatonin Receptor 1A (MTNR1A) gene in the Rasa Aragonesa sheep breed: Association with reproductive seasonality. Anim Reprod Sci 2012;133:169–175. https://doi.org/10.1016/j.anireprosci.2012.06.018.

[35] Starič J, Farci F, Luridiana S, Mura MC, Pulinas L, Cosso G, Carcangiu V. Reproductive performance in three Slovenian sheep breeds with different alleles for the MTNR1A gene. Anim Reprod Sci 2020;216 :106352. https://doi.org/10.1016/j.anireprosci.2020.106352.

[36] Luridiana S, Mura MC, Daga C, Diaz ML, Bini PP, Cosso G, Carcangiu V. The relationship between melatonin receptor 1A gene (*MTNR1A*) polymorphism and reproductive performance in Sarda breed sheep. Livest Sci 2015;171:78–83. https://doi.org/10.1016/j.livsci.2014.11.004.

[37] Luridiana S, Mura MC, Daga C, Cosso G, Bodano S, Farci F, Zidda F, Carcangiu V. Influences of melatonin treatment, melatonin receptor 1A (MTNR1A) and kisspeptin (KiSS-1) gene polymorphisms on first conception in Sarda ewe lambs. Reprod Fertil Dev 2016;28:750–756. https://doi.org/10.1071/RD14120.

[38] Carcangiu V, Luridiana S, Vacca GM, Daga C, Mura MC. A polymorphism at the melatonin receptor 1A (MTNR1A) gene in Sarda ewes affects fertility after AI in the spring. Reprod Fert Dev 2011;23, 376-380. https://doi.org/10.1071/RD10014.

[39] Trecherel E, Batailler M, Chesneau D, Delagrange P, Malpaux B, Chemineau P, Migaud M. Functional characterization of polymorphic variants for ovine MT1 melatonin receptors: Possible implication for seasonal reproduction in sheep. Anim Reprod Sci 2010;122:328–334. https://doi.org/10.1016/j.anireprosci.2010.10.007.

[40] Espino J, Ortiz A, Bejarano I, Lozano GM, Monllor F, Garcia JF, Rodriguez AB, Pariente JA. Melatonin protects human spermatozoa from apoptosis via melatonin receptor- and extracellular signal-regulated kinase-mediated pathways. Fertil Steril 2011;95:2290–2296. https://doi.org/10.1016/j.fertnstert.2011.03.063.

[41] Jang HY, Kim YH, Kim BW, Park IC, Cheong HT, Kim JT, Park CK, Kong HS, Lee HK, Yang BK. Ameliorative effects of melatonin against hydrogen peroxide-induced oxidative stress on boar sperm characteristics and subsequent in vitro embryo development. Reprod Domest Anim 2010;45:943–950. https://doi.org./10.1111/j.1439-0531.2009.01466.x.

[42] Gimeno-Martos S, Casao A, Yeste M, Cebrián-Pérez JA, Muiño-Blanco T, Pérez-Pé R. Melatonin reduces cAMP-stimulated capacitation of ram spermatozoa. Reprod Fertil Dev 2019;31:420-431. https://doi: 10.1071/RD18087.

Table 1

Genotypes of the rams in a study of the effects of polymorphisms of the melatonin receptor 1A gene on the timing of first mating in autumn-born Rasa Aragonesa ram-lambs (*n* = 24).

		RsaI			
MnlI		**C/C**	**C/T**	**T/T**	
	n	6	1	2	**9**
G/G	%	25.00	4.17	8.33	**37.50**
	n	10	4	0	**14**
G/A	%	41.67	16.67	0.00	**58.33**
	n	1	0	0	**1**
A/A	%	4.17	0.00	0.00	**4.17**
	n	**17**	**5**	**2**	**24**
	%	**70.83**	**20.83**	**8.33**	**100.00**
		Allele frequency			
		C = 0.81	T = 0.19	G = 0.67	A = 0.33

Table 2

Genotypes of the initial group of 39 Rasa Aragonesa rams from which 24 individuals were used in a study of the effects of polymorphisms of the melatonin receptor 1A gene on sexual activity in adult rams in spring.

			Rsal		
Mnll		**C/C**	**C/T**	**T/T**	
	n	19	0	8	**27**
G/G	%	48.70	0.00	20.51	**69.21**
	n	5	1	0	**6**
G/A	%	12.82	2.56	0.00	**15.38**
	n	0	5	1	**6**
A/A	%	0.00	12.82	2.56	**15.38**
		24	**6**	**9**	**39**
		61.52	**15.38**	**23.07**	**100.00**
				Allele frequency	
		C = 0.69	T = 0.31	G = 0.77	A = 0.23

Table 3

Mean (±S.E.M.) scrotal circumference (cm), testicular volume (cm^3), and plasma testosterone concentration (ng/ml) of Rasa Aragonesa rams and the polymorphisms of the melatonin receptor 1A gene (a,b denotes statistical differences between months $P<0.05$).

	Scrotal circumference (cm)		Testicular volume (cm^3)		Testosterone (ng/ml)	
RsaI	March	May	March	May	March	May
C/C (6)	30.8±0.8[a]	34.6±0.7[b]	345.4±24.3[a]	508.7±32.3[b]	7.0±1.6	8.3±2.5
C/T (6)	32.3±1.5	33.8±0.9	393.8±33.8	446.0±39.6	8.1±2.5	9.4±2.9
T/T (6)	31.6±0.8	34.7±0.7	373.3±22.7[a]	504.3±27.1[b]	8.2±1.7	10.8±2.5
MnlI	March	May	March	May	March	May
G/G (9)	31.3±0.7[a]	34.5±0.6[b]	360.4±21.1[a]	500.4±26.4[b]	7.8±1.4	9.6±2.0
A/G (3)	34.22.5	33.8±0.4	418.8±49.4	445.7±41.6	7.2±2.3	9.7±2.8
A/A (6)	30.7±0.7[a]	34.4±0.9[b]	362.6±25.5	484.9±42.3	8.9±2.9	8.6±4.5

Fig. 1. Distribution (%) of the first mating by rams with an estrus-synchronized ewe, ejaculating into an artificial vagina, and the polymorphism of the melatonin receptor 1A gene that they carried (▲C/C; ■ C/T; ● T/T; ∆ G/G; □ A/G; ○ A/A).

Fig.2. Proportion (%) of flehmen, anogenital sniffing, approaches, mounting attempts, and mountings in a 20-min individual serving capacity test (one ram-lamb with three estrous ewes) by Rasa Aragonesa rams and the polymorphisms of the melatonin receptor 1A gene.