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SNP rs403212791 in exon 2 of the *MTNR1A* gene is associated with reproductive seasonality in the Rasa aragonesa sheep breed

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2 **SNP rs403212791 in exon 2 of the *MTNR1A* gene is associated with**  
3 **reproductive seasonality in the Rasa aragonesa sheep breed.**

4

5 **Running title:** *MTNR1A* in Reproductive Seasonality

6

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9

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## 27 **Abstract**

28 The aim of this study was to characterize and identify causative SNPs in the  
29 *MTNR1A* gene responsible for the reproductive seasonality traits in the Rasa  
30 aragonesa sheep breed. A total of 290 ewes (155, 84 and 51 mature, young  
31 and ewe lambs, respectively) from one flock were controlled from January to  
32 August. The following three reproductive seasonality traits were considered: the  
33 total days of anoestrus (TDA) and the progesterone cycling months (P4CM);  
34 both ovarian function seasonality traits based on blood progesterone levels; and  
35 the oestrus cycling months (OCM) based on oestrous detection, which indicate  
36 behavioural signs of oestrous. We have sequenced the total coding region plus  
37 733 and 251 bp from the promoter and 3'-UTR regions, respectively, from the  
38 gene in 268 ewes. We found 9 and 4 SNPs associated with seasonality traits in  
39 the promoter (for TDA and P4CM) and exon 2 (for the three traits), respectively.  
40 The SNPs located in the gene promoter modify the putative binding sites for  
41 various trans-acting factors. In exon 2, two synonymous SNPs affect RFLP  
42 sites, rs406779174/Rsal (for the three traits) and rs430181568/MnII (for OCM),  
43 and they have been related with seasonal reproductive activity in previous  
44 association studies with other breeds. SNP rs400830807, which is located in  
45 the 3'-UTR, was associated with the three traits, but this did not modify the  
46 putative target sites for ovine miRNAs according to *in silico* predictions. Finally,  
47 the SNP rs403212791 (NW\_014639035.1: g.15099004G>A), which is also  
48 associated with the three seasonality phenotypes, was the most significant SNP  
49 detected in this study and was a non-synonymous polymorphism, leading a

50 change from an Arginine to a Cysteine (R336C). Haplotype analyses confirmed  
51 the association results and showed that the effects found for the seasonality  
52 traits were caused by the SNPs located in exon 2. We have demonstrated that  
53 the T allele in the SNP rs403212791 in the *MNTR1A* gene is associated with a  
54 lower TDA and higher P4CM and OCM values in the Rasa Aragonesa breed.

55

56 **Keywords:** sheep, oestrous behaviour, ovulatory activity, *MTNR1A*, SNP

57

## 58 1. Introduction

59 Rasa Aragonesa is an autochthonous Mediterranean sheep breed from  
60 northeastern Spain that is mainly reared in extensive or semi-extensive farming  
61 systems and oriented for meat production. Improvements in efficiency on farms  
62 are made possible by exploiting changes in genetics, nutrition and the  
63 management of an ewe flock. In this sense, the Cooperative Oviaragon-Grupo  
64 Pastores carries out a selection programme for prolificacy in Rasa Aragonesa  
65 sheep that began in 1994, currently includes 490,337 ewes because the  
66 number of lambs born per ewe plays a key role in the efficiency and viability of  
67 these farms [1]. The annual lambing rate can also be increased by getting a  
68 higher proportion of ewes to breed out-of-season. Sheep breeds from the  
69 Mediterranean area have a marked seasonality for breeding activities, showing  
70 seasonal oestrous behaviours and ovulation patterns. The maximal  
71 reproductive activity is associated with short days, with the highest percentage  
72 of ewes exhibiting ovulatory activities from August to March. This reproductive  
73 seasonality causes a seasonal fluctuation in lamb market prices, with the lowest

74 prices being when the lamb supply is the highest (late spring to early fall).  
75 Several methods to control the reproduction of sheep have been used such as  
76 environmental manipulation (light control), the sudden introduction of rams,  
77 which induces oestrus in ewes (ram effect), or other methods based on the  
78 administration of exogenous hormones. However, the increasing demand for  
79 hormone-free products and the evolution of European rules and directives  
80 towards a reduction in or even complete cessation of the use of exogenous  
81 hormones has led to the search for alternative methods, such as light control,  
82 the ram effect or the use of genetic markers. Spring ovulatory activities have  
83 heritability and repeatability values of 0.20 and 0.30, respectively [2], but they  
84 are only measured in females, are exhibited relatively late in an ewe's life and  
85 are only present in some management systems. Thus, the use of genetic  
86 markers would be a powerful tool in selection programmes. Only two candidate  
87 genes related to reproductive seasonality traits have been successfully  
88 identified, including *melatonin receptor subtype 1A (MTNR1A)* [3-13], and the  
89 *arylalkylamine N-acetyltransferase (AANAT)* [14]. Arylalkylamine N-  
90 acetyltransferase is involved in the biosynthesis of melatonin and controls daily  
91 changes in melatonin production. Melatonin acts through high-affinity G-protein  
92 coupled receptors, one of which is melatonin receptor 1A encoded by the  
93 *MTNR1A* gene. *MTNR1A* has been characterized in several breeds as a  
94 candidate gene and appears to play a key role in the control of photoperiod-  
95 induced seasonality mediated by circadian melatonin concentrations [15-16].  
96 However, *MTNR1A* genotypes did not show an association with reproductive  
97 seasonality in Ile de France and Cornell flocks (composed of East Friesian  
98 Cross, Dorset, Finnsheep × Dorset, and Finnsheep ewes) [17-18], suggesting

99 that the effects of *MTNR1A* polymorphisms may depend on the genetic  
100 background and/or environmental conditions. Furthermore, previous studies  
101 have associated two polymorphic RFLP sites (606/RsaI and 612/MnII) within the  
102 *MTNR1A* coding sequence with reproductive activity. However, since these  
103 SNPs are synonymous, they are not causative mutations, indicating that other  
104 polymorphisms in linkage disequilibrium or regulatory sequences in the  
105 *MTNR1A* gene could be influencing the ability to breed out of season. Genetic  
106 mapping of quantitative trait loci (QTL) and a genome-wide association study  
107 (GWAS) for aseasonal reproduction in sheep using microsatellites and SNPs,  
108 respectively, revealed several chromosomes and SNPs that could be implicated  
109 in this trait [19-20].

110 Therefore, the main objective of this study was to identify causative SNPs  
111 responsible for reproductive seasonality traits in the Rasa aragonesa sheep  
112 breed. Polymorphisms were detected and characterized from the entire  
113 *MTNR1A* gene coding region and promoter. Then, an association study was  
114 performed between all the polymorphisms detected and the three reproductive  
115 seasonality traits.

116

## 117 **2. Material and Methods**

### 118 *2.1 Ethics statement*

119 All experimental procedures were performed in accordance with the guidelines  
120 of the European Union (2003/65/CE) and Spanish regulations (RD 1201/2005,  
121 BOE 252/34367-91) for the use and care of animals in research. No hormonal  
122 treatments were applied to ewes during the study.

## 123 2.2 *Animal resources*

124 Phenotypic seasonality data were obtained from a Rasa aragonesa sheep flock  
125 managed at an experimental farm ("Pardina de Ayés") owned by Oviaragón  
126 S.C.L., located in the Pre-Pyrenees (Ayés, Sabiñánigo, Huesca; North-Eastern  
127 Spain, 42° 29' 48.55" N 0° 23' 37.54", 790 m above sea level) and described in  
128 Martínez-Royo et al. [20]. The experimental period lasted from January to  
129 August in 2012. The flock was composed of 290 ewes in the following three age  
130 groups: mature (5.2-7.2 y, n=155;  $5.5 \pm 0.5$ ; mean  $\pm$  SD), young (all: 1.9 y,  
131 n=84) and, ewe lambs (all: 0.94 y; n=51) at the beginning of the experiment.  
132 Their individual live weight (LW) and body condition score (BCS) on a 1 to 5  
133 scale [21] were assessed every three weeks. Mean LW and BCS values were  
134 similar in mature and young ewes age groups. Pooled overall means and  
135 standard deviations for the entire experimental period were  $52.5 \pm 7.7$  kg and  
136  $2.9 \pm 0.3$  for LW and BCS, respectively, for mature and young ewes. However,  
137 ewe lambs had an LW and BCS of  $40.6 \pm 3.8$  kg and  $2.8 \pm 0.1$ , respectively.  
138 Management of the ewes is described in Martínez-Royo et al.[20]. The ewes  
139 were handled in a single lot and subjected to the same management, nutrition  
140 and environmental conditions.

## 141 2.3 *Measurement of reproductive seasonality traits*

142 Three reproductive seasonality traits were considered and described in  
143 Martínez-Royo et al. [20]. Briefly, the first was the total days of anoestrus (TDA)  
144 based on weekly individual plasma progesterone levels. TDA was the sum of  
145 the days in anoestrus, considering anoestrus as periods with three or more  
146 consecutive progesterone concentrations lower than 0.5 ng/ml. Likewise, ewes  
147 were not considered for this study if they were not cycling in the preceding

148 breeding season (based on three samples one week apart taken in October),  
149 had progesterone levels below the threshold in all samples taken in January,  
150 and had more than 4 consecutive samples higher than or equal to the threshold  
151 (possible pathological ewes).

152 The second reproductive seasonality trait was the progesterone cycling months  
153 (P4CM), which was defined for each ewe as the rate of cycling months based  
154 on progesterone determination. When progesterone levels were higher than or  
155 equal to 0.5 ng/ml in at least one blood sample in the month, an ewe was  
156 considered cyclic.

157 Finally, the third reproductive seasonality trait considered was the oestrus  
158 cycling months (OCM), which is defined as the rate of months cycling based on  
159 daily oestrous records for each ewe. Eight vasectomised rams fitted with  
160 harnesses and marking crayons were mixed with the ewes, and daily oestrous  
161 detection was examined [22]. Thus, after natural mating, oestrus was recorded  
162 as a coloured mark on the ewes' rump.

### 163 *2.3 Sampling and genotyping*

164 Genomic DNA was extracted from blood samples from 268 ewes (138, 79 and  
165 51 mature, young and ewe lambs, respectively) from the total ewes in the flock  
166 (n=290) using the FlavorPrep Genomic DNA mini kit (Flavorgen, Ibian,  
167 Zaragoza, Spain). Twenty-two ewes were not considered because of missing  
168 data for some variables. Direct Sanger sequencing of the PCR products from all  
169 the ewes (n=268) was used to search for genotype polymorphisms in the  
170 experimental population. Primers were used to amplify the total coding, 5'-UTR,  
171 partial 3'-UTR, and promoter genomic regions in the *MNTR1A* gene using three  
172 different PCR reactions (Supplementary Table 1). Genomic DNA (50 ng) was



173 amplified in a final PCR volume of 25  $\mu$ l containing 5 pmol of each primer, 200  
174 nM dNTPs, 2.0 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100  
175 and 1 U Taq polymerase (Biotools, Madrid, Spain). The following cycling  
176 conditions were used: an initial denaturation step at 94 °C for 3 min; 35 cycles  
177 of 94 °C for 1 min, the annealing temperature for 1 min, and 72 °C for 1 min and  
178 30 s for fragments 1-3 and 2, respectively; and a final extension step at 72 °C  
179 for 10 min. The annealing temperatures for each fragment are indicated in  
180 Supplementary Table 1. All PCR amplifications of genomic DNA were  
181 performed in a MyCycler thermal cycler (BioRad, Madrid, Spain). The PCR  
182 products were purified using the FlavorPrep Gel/PCR purification mini kit  
183 (Flavorgen, Ibian, Zaragoza, Spain), according to the manufacturer's  
184 instructions. The PCR products were sequenced in both directions by STAB  
185 Vida Lda. (Caparica, Portugal) using an ABI 3730XL sequencer (Applied  
186 Biosystems, CA, USA).

187 The homology searches were performed using BLAST (National Centre for  
188 Biotechnology Information: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). To align the  
189 sequences, the CLUSTAL Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>)  
190 software was used. The Variant Effect Predictor software (VEP:  
191 [http://www.ensembl.org/Ovis\\_aries/Tools/VEP?db=core](http://www.ensembl.org/Ovis_aries/Tools/VEP?db=core)), which predicts the  
192 possible impact of an amino acid substitution on the structure and function of a  
193 protein, was used. The degree of the amino acid change was also assessed  
194 using the BLOSUM 62 scoring matrix ([http://www.ncbi.nlm.nih.](http://www.ncbi.nlm.nih.gov/Class/FieldGuide/BLOSUM62.txt)  
195 [Gov/Class/FieldGuide/BLOSUM62.txt](http://www.ncbi.nlm.nih.gov/Class/FieldGuide/BLOSUM62.txt)), where low values indicate more drastic  
196 changes. Antisense matches for individual miRNAs in the 3'-UTR sequences of  
197 the isolated ovine *MTNR1A* gene were determined using MirTarget

198 (<http://mirdb.org/>) and TargetScan ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/))  
199 programs. The locations of the SNPs and gene clusters were identified based  
200 on the latest sheep genome version for *Ovis aries* (Oar\_v4.0,  
201 <https://www.ncbi.nlm.nih.gov/genome/?term=ovis+aries>).

202

#### 203 *2.4 Linkage disequilibrium (LD)*

204 The gametic LD among SNPs ( $D'$  and  $r^2$ ) within the *MTNR1A* gene was  
205 calculated and visualized using the Haploview v4.2 program [23]. LD blocks  
206 were defined based on the four-gamete rule algorithm [24].

#### 207 *2.5 Statistical analysis*

##### 208 *2.5.1. SNP association studies*

209 The Hardy–Weinberg equilibrium exact test values and observed and expected  
210 heterozygosities and minor allele frequency (MAF) for each SNP were  
211 calculated using the PLINK 1.9 software [25].

212 The associations between the *MTNR1A* gene polymorphisms and reproductive  
213 seasonality traits (TDA, P4CM, and OCM) were determined by fitting a Linear  
214 Mixed Model using the Mixed procedure (MIXED) in the SAS statistical  
215 package. The model included the genotype of the SNPs (S), the age (mature,  
216 young and ewe lambs) (A), and the interaction between the age × genotype of  
217 the SNPs (A × S) as fixed effects; the live weight (LW) and body condition score  
218 (BCS) as covariates; and the animal (A) and residual I as random effects.  
219 Homogeneous variance was considered for the residual ( $e \sim N(0, \sigma^2)$ ). The  
220 equation for the model was as follows:

$$221 \quad Y = \mu + S + A + (A \times S) + LW + BCS + A + e$$

222 To test the differences between genotypes for each breed, the least square  
223 means (LSMs) for each pair-wise comparison were estimated. A Bonferroni  
224 correction was fitted to take into account the multiple tests. All SNPs were  
225 independently analysed with the same statistical model.

226

227 2.5.2. Haplotype association studies

228 SNPs were phased with PLINK1.9 [23] considering the blocks defined by  
229 Haploview and using the expectation–maximization (E–M) algorithm to assign  
230 individual haplotypes. We considered diplotypes with a posterior probability  
231 higher than 0.8.

232 Associations between the haplotypes and reproductive seasonality traits were  
233 performed using a Linear Mixed Model using the Mixed procedure (MIXED) in  
234 SAS. The fitted model was similar to that used for the SNP association studies  
235 but included the haplotype (H) effect and the interaction between the age ×  
236 haplotype (A × H). Haplotypes for each individual were codified as 0, 1 or 2,  
237 indicating the number of copies of each haplotype. The equation for the model  
238 was as follows:

$$239 \quad Y = \mu + H + A + (A \times H) + LW + BCS + A + e$$

240 Only haplotypes with a frequency greater than or equal to 1% were considered.

241 To test the differences between the haplotypes, the LSMs for each pair-wise  
242 comparison were estimated. The Bonferroni correction was applied to take into  
243 account multiple tests.

244

### 245 3. Results and discussion.

246 3.1. Structural characterization and linkage disequilibrium

247

248 All of exon 1, 1120 bp from exon 2 (869 bp from the protein coding region plus  
249 251 bp from the 3'-UTR) and 733 bp from the promoter region were sequenced.  
250 Exons were identified by comparison with ovine sequences (GenBank  
251 sequence NM 001009725 and AF078545). Sequences from the total population  
252 (n=268) revealed 35 polymorphisms, including 13 SNPs in the promoter region,  
253 1 SNP in exon 1 and 21 SNPs in exon 2 (17 and 4 SNPs in the coding and 3'-  
254 UTR regions, respectively) (Table 1). Table 1 shows the location, alias  
255 (nomenclature in this manuscript), dbSNP identifiers, amino acid substitution  
256 effect, and genotypic and allelic frequencies of the SNPs. *MTNR1A* is in reverse  
257 orientation on the genome, and SNPs are ordered according to their position in  
258 the latest genome version (Oar4.0: GenBank acc. number NW\_014639035.1).  
259 All SNPs were in Hardy-Weinberg equilibrium, though exon 1 (snp\_22) showed  
260 a reduced number of heterozygous animals. In the promoter region, we  
261 sequenced the same fragment as in Martinez-Royo et al. [12], which is located  
262 536 bp upstream from the transcription start site (TSS). We did not find any new  
263 SNPs in this region. As described in Martinez-Royo et al. [12], six of these  
264 polymorphisms modify putative binding sites for various trans-acting factors,  
265 including snp\_26 (Brn-2 and Oct-1 consensus sites), snp\_27 (SRY), snp\_31  
266 (SRY), snp\_32 (Nkx-2), snp\_33 (SRY), and snp\_34 (EF2). In exon 1, a  
267 synonymous polymorphism was detected; in contrast, 9 non-synonymous SNPs  
268 were found in exon 2. Four of the SNPs had not been previously described in  
269 other sheep breeds, with two of these being non-synonymous polymorphisms,  
270 one that changes from a Lysine to Threonine (K335T) (snp\_9) and the other a  
271 non-conservative change from a Glycine to Valine (G123V) (snp\_20), and both

272 substitutions were predicted as deleterious (with a SIFT value of 0.04) by the  
273 VEP software. However, we did not find homozygous animals for the predicted  
274 deleterious allele (G and A for snp\_9 and snp\_20, respectively), and only one  
275 heterozygous animal for each SNP was found in two different animals. None of  
276 the other non-synonymous substitutions were predicted as deleterious; all were  
277 considered tolerated with SIFT values ranging from 0.37 (snp\_10) to 0.05  
278 (snp\_12).

279 Fig. 1 shows the linkage disequilibrium plot among *the* SNPs in *MTNR1A*. Four  
280 LD blocks were predicted (Fig. 1). One block included all the promoter SNPs  
281 except snp\_23, snp\_24, snp\_25 and snp\_27 (Block 4). These two last SNPs  
282 (snp\_23 and snp\_24) constituted Block 3. Finally, two different blocks were  
283 predicted in exon 2. Polymorphisms in the promoter and exon 2 showed low  
284 linkage disequilibrium. The maximum value of  $D'$  and  $r^2$  between the promoter  
285 and exon 2 SNPs were 0.82 and 0.14 (Figure 1), respectively, being separated  
286 by approximately 24 Kb.

287

### 288 3.2. SNP association studies

289 The median values and 5th–95th percentiles for the TDA, P4CM and OCM traits  
290 for the population are shown in Supplementary Table 2. Thirty-seven of the  
291 ewes (29, 6 and 2 mature, young and ewe lambs, respectively) did not present  
292 anoestrus during the experiment (TDA=0). Similarly, 87 (60, 17 and 10 mature,  
293 young and ewe lambs, respectively) and 9 (7 and 2 mature and young ewes,  
294 respectively) ewes were cycling throughout the experiment when considering  
295 the P4CM and OCM traits, respectively. TDA and P4CM had a negative  
296 correlation because P4CM was a less strict trait for ovarian function than TDA in

297 the three age groups studied. The phenotypic correlations among the three  
298 traits are shown in Supplementary Table 3.

299

### 300 3.2.1. Promoter region

301 Results from the association studies are shown in Supplementary Table 4.

302 SNPs snp\_9 and snp\_20 were not considered in the association analysis

303 because only one heterozygous animal was found ( $MAF < 0.01$ ). The interaction

304 between the SNP and age affected the TDA and P4CM traits in the promoter

305 region (Table 2 and Supplementary Table 4). The two blocks associated with

306 these SNPs were in complete linkage disequilibrium ( $D' = 1$  and  $r^2 = 1$ ) ([snp\_29,

307 snp\_30 and snp\_34] and [snp\_24, snp\_26, snp\_32 and snp\_33]) (Figure 1).

308 After Bonferroni correction, only the TDA phenotype differed among the SNPs

309 ( $P < 0.05$ ) at ewe lamb age (SNP x age fixed effect), showing significant

310 differences between the homozygous alternative genotypes. However, this

311 association may be spurious as it relies upon an unbalanced distribution of

312 genotypes and a small number of ewe lambs ( $n = 51$ ) for each SNP. In this

313 sense, only 3 homozygous animals for the MAF allele were found for snp\_24,

314 snp\_26, snp\_29, snp\_30, snp\_32, snp\_33 and snp\_34 among the ewe lambs.

315 Considering all ages (SNP fixed effect), the SNPs were significantly associated

316 with the TDA and P4MC traits, showing a balanced distribution of genotypes

317 (Table 2 and Supplementary Table 4), with a homozygous animal frequency for

318 the MAF allele of approximately 17% (Table 1). The present results are in

319 agreement with those described by Martinez-Royo et al. [12] in that SNPs in the

320 *MNTR1A* gene promoter are associated with reproductive seasonality.

321 However, our results are slightly different because only the OCM trait was

322 examined in that study. Martinez-Royo et al. [12] found 5 associated SNPs, two  
323 of which were also associated with TDA and P4CM in the present study,  
324 snp\_28 (called 677 in Martinez-Royo et al. [12]) and snp\_34 (called 422 in  
325 Martinez-Royo et al. [12]). The C (snp\_26), A (snp\_32), A (snp\_33), and A  
326 (snp\_34) alleles were associated with lower TDA and higher P4CM values and  
327 created putative binding sites for various trans-acting factors, such as EF-2,  
328 SRY, Nkx-2 and Brn-2, respectively. These SNPs showed strong linkage  
329 disequilibrium (Fig. 1). These allelic variants may play a role in gene regulation  
330 by increasing the *MTNR1A* gene expression level (mRNA) and thus the final  
331 protein contents. Therefore, these variations may enhance the ability of  
332 *MNTR1A* to mediate the physiological function of melatonin, decreasing TDA  
333 and increasing P4CM.

334

### 335 3.2.2. Coding region

336 The SNP found in exon 1 was synonymous and not associated with  
337 reproductive seasonality traits. Furthermore, this SNP was not in Hardy-  
338 Weinberg equilibrium. For any SNP, the interaction between the SNP and age  
339 was not significantly associated with reproductive seasonality traits, though four  
340 SNP effect associations were found (Table 3 and Supplementary Table 4). Two  
341 of them were at the snp\_17 (612/MnII) and snp\_18 (606/RsaI) polymorphic  
342 RFLP sites that have been previously associated with seasonality traits in Rasa  
343 aragonesa and other breeds [3-13]. These results are in agreement with those  
344 from Martinez-Royo et al. [12], in which snp\_18 was associated with the OCM  
345 trait in the Rasa aragonesa sheep breed. In that study, snp\_17 was not  
346 associated, but here, we found a significant association with the OCM trait.

347 However, these SNPs are synonymous polymorphisms and therefore not  
348 causative mutations. One of the other two significant SNPs is a non-  
349 synonymous polymorphism (snp\_8), which led to a change of an Arginine to a  
350 Cysteine (R336C); the other one is located in the 3'-UTR region (snp\_2). Snp\_8  
351 was the most significant SNP and was predicted as tolerated (with a SIFT value  
352 of 0.22) by the VEP software. The GG genotype had greater value than the AA  
353 genotype, whereas the AG genotype presented intermediate values for the TDA  
354 ( $73.08 \pm 7.25$ ,  $53.57 \pm 7.36$  and  $20.85 \pm 13.13$  for the GG, AG and AA genotypes,  
355 respectively) and P4CM ( $0.80 \pm 0.03$ ,  $0.86 \pm 0.03$  and  $0.94 \pm 0.05$  for the GG, AG  
356 and AA genotypes, respectively) traits. However, only significant differences  
357 were observed between the GG and AA genotypes for the OCM trait  
358 ( $0.49 \pm 0.03$ ,  $0.54 \pm 0.03$  and  $0.64 \pm 0.06$  for the GG, AG and AA genotypes,  
359 respectively). This SNP has been previously described in two populations from  
360 Iran and Morocco (NEXTGEN project, <http://nextgen.epfl.ch/>). Similar results  
361 were also found for snp\_2, as the two SNPs showed high linkage disequilibrium  
362 ( $D'=1$ ,  $r^2=0.94$ ). Snp\_2 was located in the 3'-UTR region; this SNP could modify  
363 putative ovine miRNA target sites. However, this SNP did not modify a miRNA  
364 target sequence according to the MirTarget and Targetscan software  
365 predictions. Snp\_8 could be the causative mutation for the observed effects in  
366 the reproductive seasonality traits because it was the most significant SNP and  
367 produces an amino acid change. According to the BLOSUM 62 scoring matrix,  
368 the substitution generated by snp\_8 (arginine to cysteine) is rated -3, with the  
369 most severe rating (i.e., introduction of a premature stop codon) being -4.  
370 Arginine is a positively charged amino acid, whereas cysteine is polar in nature.  
371 Moreover, cysteine is important for the generation of cysteine knots and



372 disulfide linkages between subunits in some proteins and could potentially affect  
373 the binding of melatonin to its receptor.

374

### 375 3.3. Haplotype association studies

376 Haplotype association studies were performed considering the four LD blocks  
377 predicted with Haploview (Figure 1 and Supplementary Table 5) and a block  
378 containing all the significant SNPs (Block 5; Supplementary Table 5). Totals of  
379 5, 22, 3, 6 and 21 haplotypes were found for blocks 1, 2, 3, 4 and 5,  
380 respectively. Because we only considered diplotypes with a posterior probability  
381 higher than 0.8, we performed the haplotype analysis with 245, 243, 268, 268  
382 and 233 ewes for blocks 1, 2, 3, 4 and 5, respectively.

#### 383 3.3.1. Promoter region.

384 The statistically significant SNP associations were confirmed by *MTNR1A*-  
385 specific haplotype analyses. The haplotype and haplotype x age effects affected  
386 the TDA and P4CM traits (Table 4 and Supplementary Table 5). Haplotypes 3  
387 and 1 for blocks 3 and 4, respectively, showed significant associations with the  
388 TDA and P4CM traits. In this sense, homozygous h3/h3 animals (n= 102) had  
389 higher and lower values for TDA and P4CM, respectively, than animals with one  
390 copy (n= 119) or no h3 copies (n= 47) for block 3. However, only significant  
391 differences were found between having 0 and 2 copies of the haplotype. For  
392 this haplotype, only snp\_24 was associated with TDA and P4CM, as having the  
393 G allele showed higher and lower values for these traits (Table 2), respectively,  
394 but without modifying any trans-acting factor putative binding sites. Thus, the  
395 SNPs in block 3 could not be the responsible for the observed effects. Similar  
396 results were found for haplotype 1 in block 4 because the of the high degree of

397 LD between the two blocks ( $D'=1$ ), as homozygous h1/h1 animals ( $n= 103$ ) had  
398 higher values than animals with one copy ( $n= 117$ ) or no h1 copies ( $n= 48$ ) for  
399 block 4 (Table 4). For this haplotype, the T, C, A, C, G, G and G alleles for  
400 SNPs snp\_26, snp\_28, snp\_29, snp\_30, snp\_32, snp\_33 and snp\_34,  
401 respectively, were also associated with higher and lower TDA and P4CM  
402 values, respectively, in the SNP association studies (Table 2). *In silico* analysis  
403 showed that the T (snp\_26), G (snp\_32), G (snp\_33), and G (snp\_34) alleles  
404 were associated with higher and lower TDA and P4CM, respectively, and could  
405 destroy the putative binding sites for various trans-acting factors (EF-2, SRY,  
406 Nkx-2 and Brn-2, respectively), demonstrating that the SNPs may play a role  
407 in regulating gene expression. However, the LSMs for the haplotype x age  
408 effect were only significant for the TDA trait in the SNP association studies,  
409 while for P4CM, a trend was only observed between having 0 or 2 copies for  
410 haplotypes at an ewe lamb age after Bonferroni correction. In this sense, only  
411 three ewe lambs had 0 copies of the h3 (block 3) and h1 (block 4) haplotypes  
412 for the TDA trait, and even then, these associations were not very confident.

### 413 3.3.2. Coding region.

414 In exon 2, the haplotype affected the TDA, P4CM and OCM traits (Table 5 and  
415 Supplementary Table 5), but the interaction between the haplotype and the age  
416 was not significant. Homozygous h3/h3 animals ( $n= 15$ ) had lower values for  
417 TDA than animals with one copy ( $n= 97$ ) or no h3 copies ( $n= 133$ ) for block 1.  
418 For the P4CM and OCM traits, homozygous h3/h3 animals had higher values  
419 than animals with one copy or no h3 copies. Significant differences were found  
420 between animals carrying 0 copies and 1 or 2 copies of the haplotype for the  
421 three traits. A trend was also observed between 1 and 2 copies ( $P=0.064$ ) for

422 the TDA trait. Regarding block 2, results similar to those found for block 1 were  
423 observed due to the high degree of LD between the two blocks ( $D'=0.97$ ).  
424 Significant differences were found between the three diplotypes for haplotype  
425 h2, showing that homozygous h2/h2 animals ( $n= 13$ ) had lower values than  
426 animals with no h2 copies ( $n= 131$ ). An intermediate TDA value was observed  
427 for the heterozygous diplotype ( $n= 99$ ). For the P4CM and OCM traits,  
428 homozygous h2/h2 animals had higher values than animals with one copy or no  
429 h2 copies, showing the significant differences between having 0 and 2 copies  
430 for the haplotype for both traits.

431 For these haplotypes, the A, A, C and A alleles in snp\_2 (block 1), snp\_8 (block  
432 2), snp\_17/MnII (block 2) and snp\_18/RsaI (block 2), respectively, were  
433 associated with lower TDA and higher P4CM and OCM values in the SNP  
434 associations studies (Table 3). Only snp\_8 produces a putative functional effect  
435 (a change of an Arginine to a Cysteine) (see section 3.2.2). The association  
436 results obtained for snp\_2, snp\_17 and snp\_18 could be due to the high linkage  
437 disequilibrium between these SNPs and snp\_8, as the causative mutation of the  
438 traits studied in this work.

### 439 3.3.3. Block with significant SNPs.

440 To determine whether the phenotype effects observed were caused by snp\_8 in  
441 exon 2 or SNPs in the promoter, we created another haplotype block with all the  
442 significant SNPs associated with the seasonality traits (block 5; Supplementary  
443 Table 5). We found 3 haplotypes associated with TDA and P4CM and two  
444 associated with OCM (Table 6 and Supplementary Table 5). One copy of the h4  
445 (**AACAACCTCTAAA**) and h13 (**AACAGTTCACGGG**) haplotypes showed  
446 lower TDA values than having no copies of this haplotype (alleles significantly

447 associated with lower TDA and higher P4CM and OCM values for each SNP in  
448 each haplotype according to the SNP association results are shown in bold).  
449 These haplotypes had the alleles from exon 2 (**A**, **A**, and **A** for snp\_2, snp\_8,  
450 and snp\_18/Rsal, respectively), but not those from the promoter region  
451 associated with lower TDA values in the SNP association studies (Tables 2 and  
452 3). In the same way, the h12 haplotype (**GGCGATCTCTAAA**), which was  
453 associated with higher TDA values, showed that the alleles from the SNPs in  
454 exon 2 were associated with an increase in this trait (G, G, and G for snp\_2,  
455 snp\_8, and snp\_18/Rsal, respectively). However, SNPs in the promoter region  
456 were associated with lower instead of higher TDA values (the **A**, **T**, **C**, **T**, **C**, **T**,  
457 **A**, **A**, and **A** alleles for snp\_24, snp\_25, snp\_26, snp\_28, snp\_29, snp\_30,  
458 snp\_32, snp\_33 and snp\_34, respectively). Similar results were found for P4CM  
459 (the same significant haplotypes were found in the TDA phenotype). For the  
460 OCM trait, the two haplotypes associated with higher OCM values (h10,  
461 **AACAGCTCACGGG** and h13, **AACAGTTCACGGG**) had the alleles from exon  
462 2 (**A**, **A**, and **A** for snp\_2, snp\_8, and snp\_18/Rsal, respectively), but not those  
463 from the promoter region associated with higher OCM values in the SNP  
464 association studies (Tables 2 and 3). These results indicated that the effect is  
465 due to SNPs in exon 2 and not from those in the promoter region. As discussed  
466 in sections 3.2.2 and 3.3.2, snp\_8 located in exon 2 might be the causative  
467 mutation of the effect found in this study because this SNP leads to a change in  
468 an amino acid. Despite the long distance between the SNPs located in the  
469 promoter and exon 2 (approximately 24 kb) and the low linkage disequilibrium  
470 found between them (the maximum values of  $r^2$  and  $D'$  between snp\_8 and the  
471 significant SNPs in promoter region for snp\_29 snp\_30, and snp\_34 were 0.06

472 and 0.33, respectively), it appears that the effects observed for the SNPs in the  
473 promoter region are due to the linkage disequilibrium with snp\_8.

474

#### 475 **4. Conclusions**

476 We have sequenced the entire coding region plus 733 and 251 bp from the  
477 promoter and 3'-UTR regions, respectively, in the *MTNR1A* gene in 268 Rasa  
478 aragonesa sheep breed ewes, finding 13 SNPs associated with seasonality  
479 traits in the promoter and exon 2 regions. Haplotype analyses confirmed these  
480 results and allowed an assessment of the impact of the SNPs located in the  
481 promoter and exon 2 regions on the effects observed for the seasonality values.  
482 In this sense, alleles associated with lower TDA values (or higher values for  
483 P4CM) in the promoter are in linkage disequilibrium with those in exon 2  
484 responsible for the effects found in this study, and they can be segregated  
485 together. Thus, the observed effects could be due to SNPs in exon 2 but not the  
486 SNPs in the promoter. We have demonstrated that the snp\_8 T allele in the  
487 *MNTR1A* gene is associated with lower and higher TDA and P4CM values,  
488 respectively, both of which are seasonality traits for ovarian function based on  
489 blood progesterone levels; this allele is also associated with higher OCM  
490 values, which indicates behavioural signs of oestrous in the Rasa Aragonesa  
491 breed. This SNP is in linkage disequilibrium with snp\_17 (612/MnII) and snp\_18  
492 (606/RsaI), which have been related with seasonal reproductive activities in  
493 several association studies with other breeds. However, these SNPs are  
494 synonymous polymorphisms and do not appear to be the causative mutations.  
495 In this sense, snp\_8 could be the causative mutation for the observed effects in

496 the reproductive seasonality traits because it produces an amino acid change  
497 from an Arginine to a Cysteine (R336C).

498

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505

#### 506 **References**

- 507 [1] Pardos L, Maza Rubio MT, Fantova E. The diversity of sheep production  
508 systems in Aragón (Spain): characterisation and typification of meat  
509 sheep farms. *Spanish J Agric Res* 2008;6:497. doi:10.5424/sjar/2008064-  
510 344.
- 511 [2] Hanocq E, Bodin L, Thimonier J, Teyssier J, Malpoux B, Chemineau P.  
512 Genetic parameters of spontaneous spring ovulatory activity in Mérinos  
513 d'Arles sheep. *Genet Sel Evol* 1999;31:77–99. doi:10.1186/1297-9686-  
514 31-1-77.
- 515 [3] Pelletier J, Bodin L, Hanocq E, Malpoux B, Teyssier J, Thimonier J, et al.  
516 Association between expression of reproductive seasonality and alleles of  
517 the gene for Mel(1a) receptor in the ewe. *Biol Reprod* 2000;62:1096–101.
- 518 [4] Notter DR, Cockett NE, Hadfield TS. Evaluation of melatonin receptor 1a  
519 as a candidate gene influencing reproduction in an autumn-lambing  
520 sheep flock. *J Anim Sci* 2003;81:912–7.

- 521 [5] Chu M, Ji C, Chen G. Association between PCR-RFLP of melatonin  
522 receptor 1a gene and high prolificacy in Small Tail Han sheep. *Asian*  
523 *Australas J Anim ...* 2003;16:1701–4.
- 524 [6] Faigl V, Kerestes M, Kulcsar M, Reiczigel J, Cseh S, Huszenicza G, et al.  
525 Seasonality of reproduction and MT1 receptor gene polymorphism in  
526 Awassi sheep. *Reprod Domest Anim* 2009;43:11.
- 527 [7] Mura MC, Luridiana S, Vacca GM, Bini PP, Carcangiu V. Effect of  
528 genotype at the MTNR1A locus and melatonin treatment on first  
529 conception in Sarda ewe lambs. *Theriogenology* 2010;74:1579–86.  
530 doi:10.1016/j.theriogenology.2010.06.028.
- 531 [8] Mateescu RG, Lunsford AK, Thonney ML. Association between melatonin  
532 receptor 1A gene polymorphism and reproductive performance in Dorset  
533 ewes. *J Anim Sci* 2009;87:2485–8. doi:10.2527/jas.2008-1688.
- 534 [9] Teyssier J, Migaud M, Debus N, Maton C, Tillard E, Malpoux B, et al.  
535 Expression of seasonality in Merinos d'Arles ewes of different genotypes  
536 at the MT1 melatonin receptor gene. *Animal* 2011;5:329–36.  
537 doi:10.1017/S1751731110001813.
- 538 [10] Carcangiu V, Mura MC, Vacca GM, Pazzola M, Dettori ML, Luridiana S,  
539 et al. Polymorphism of the melatonin receptor MT1 gene and its  
540 relationship with seasonal reproductive activity in the Sarda sheep breed.  
541 *Anim Reprod Sci* 2009;116:65–72. doi:10.1016/j.anireprosci.2009.01.005.
- 542 [11] Carcangiu V, Luridiana S, Vacca GM, Daga C, Mura MC. A polymorphism  
543 at the melatonin receptor 1A (MTNR1A) gene in Sarda ewes affects  
544 fertility after AI in the spring. *Reprod Fertil Dev* 2011;23:376–80.  
545 doi:10.1071/RD10014.

- 546 [12] Martínez-Royo A, Lahoz B, Alabart JL, Folch J, Calvo JH.  
547 Characterisation of the Melatonin Receptor 1A (MTNR1A) gene in the  
548 Rasa Aragonesa sheep breed: association with reproductive seasonality.  
549 Anim Reprod Sci 2012;133:169–75.  
550 doi:10.1016/j.anireprosci.2012.06.018.
- 551 [13] Mura MC, Luridiana S, Bodano S, Daga C, Cosso G, Diaz ML, et al.  
552 Influence of melatonin receptor 1A gene polymorphisms on seasonal  
553 reproduction in Sarda ewes with different body condition scores and ages.  
554 Anim Reprod Sci 2014;149:173–7. doi:10.1016/j.anireprosci.2014.07.022.
- 555 [14] Ding-ping B, Cheng-jiang Y, Yu-lin C. Association between AA-NAT gene  
556 polymorphism and reproductive performance in sheep. Electron J  
557 Biotechnol 2012;15. doi:10.2225/vol15-issue2-fulltext-6.
- 558 [15] Weaver DR, Liu C, Reppert SM. Nature's knockout: the Mel1b receptor is  
559 not necessary for reproductive and circadian responses to melatonin in  
560 Siberian hamsters. Mol Endocrinol 1996;10:1478–87.  
561 doi:10.1210/mend.10.11.8923472.
- 562 [16] Notter DR, Cockett NE. Opportunities for detection and use of QTL  
563 influencing seasonal reproduction in sheep: a review. Genet Sel Evol  
564 2005;37 Suppl 1:S39-53. doi:10.1051/gse:2004031.
- 565 [17] Hernandez X, Bodin L, Chesneau D, Guillaume D, Allain D, Chemineau  
566 P, et al. Relationship between MT1 melatonin receptor gene  
567 polymorphism and seasonal physiological responses in Ile-de-France  
568 ewes. Reprod Nutr Dev 2005;45:151–62.
- 569 [18] Posbergh CJ, Murphy RJ, Thonney ML. Further testing of for out-of-  
570 season reproduction in the Cornell flock and allelic frequencies compared



- 571 with Romney sheep. *J Anim Sci* 2017;95:1939.  
572 doi:10.2527/jas.2016.1345.
- 573 [19] Mateescu RG, Thonney ML. Genetic mapping of quantitative trait loci for  
574 aseasonal reproduction in sheep. *Anim Genet* 2010;41:454–9.  
575 doi:10.1111/j.1365-2052.2010.02023.x.
- 576 [20] Martinez-Royo A, Alabart JL, Sarto P, Serrano M, Lahoz B, Folch J, et al.  
577 Genome-wide association studies for reproductive seasonality traits in  
578 Rasa Aragonesa sheep breed. *Theriogenology* 2017;99:21–9.  
579 doi:10.1016/j.theriogenology.2017.05.011.
- 580 [21] Russel AJF, Doney JM, Gunn RG. Subjective assessment of body fat in  
581 live sheep. *J Agric Sci* 1969;72:451–4. doi:10.1017/S0021859600024874.
- 582 [22] Radford HM, Watson RH, Wood GF. A crayon and associated harness for  
583 the detection op mating under field conditions. *Aust Vet J* 1960;36:57–66.  
584 doi:10.1111/j.1751-0813.1960.tb03764.x.
- 585 [23] Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization  
586 of LD and haplotype maps. *Bioinformatics* 2005;21:263–5.  
587 doi:10.1093/bioinformatics/bth457.
- 588 [24] Wang N, Akey JM, Zhang K, Chakraborty R, Jin L. Distribution of  
589 Recombination Crossovers and the Origin of Haplotype Blocks: The  
590 Interplay of Population History, Recombination, and Mutation. *Am J Hum*  
591 *Genet* 2002;71:1227–34. doi:10.1086/344398.
- 592
- 593 [25] Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR, Bender D,  
594 et al. PLINK: a tool set for whole-genome association and population-  
595 based linkage analyses. *Am J Hum Genet* 2007;81:559–75.

596 doi:10.1086/519795.

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602 **Figure captions**

603

604 **Fig. 1.** Schematic representation of ovine *MTNR1A* and the linkage  
605 disequilibrium plot for the SNPs in *MTNR1A* for the Rasa aragonesa population  
606 using Haploview. *MTNR1A* is in a reverse orientation on the genome, and the  
607 SNPs are ordered according to their position in the latest genome version  
608 (Oar4.0: GenBank acc. number NW\_014639035.1). The linkage disequilibrium  
609 colour scheme corresponds with the  $D'$  parameter, while the linkage  
610 disequilibrium values correspond to the  $r^2$  parameter. Haplotype blocks are  
611 indicated by dark lines. A strong LD ( $D' = 1$ ,  $\text{LOD} \geq 2$ ) is indicated by red, and  
612 lighter shades of pink indicate varying degrees of LD, with lighter shades  
613 displaying less than darker shades ( $D' < 1$ ,  $\text{LOD} \geq 2$ ). White indicates low LD ( $D'$   
614  $< 1$ ,  $\text{LOD} < 2$ ).

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**Table 1.** Information about the location and amino acid substitution effect of the identified SNPs in the *MTNR1A* gene and their genotypic and allelic frequencies. *MTNR1A* is in a reverse orientation on the genome, and the SNPs are ordered according to their positions in the latest genome version (Oar4.0: GenBank acc. number NW\_014639035.1).

| Location | Alias <sup>1</sup> | dbSNPs        | Oar 4.0<br>(NW_014639035.1) | Amino acid<br>substitution | Genotype | Genotype<br>frequencies | Allele | Allele<br>frequencies |
|----------|--------------------|---------------|-----------------------------|----------------------------|----------|-------------------------|--------|-----------------------|
| exon 2   | snp_1              | rs420016236   | g.15098857C>T               | 3' UTR<br>variant          | CC       | 0.597                   | C      | 0.78                  |
|          |                    |               |                             |                            | CT       | 0.366                   | T      | 0.22                  |
|          |                    |               |                             |                            | TT       | 0.037                   |        |                       |
|          | snp_2              | rs400830807   | g.15098860G>A               | 3' UTR<br>variant          | GG       | 0.544                   | G      | 0.742                 |
|          |                    |               |                             |                            | GA       | 0.392                   | A      | 0.258                 |
|          |                    |               |                             |                            | AA       | 0.064                   |        |                       |
|          | snp_3              | rs414185743   | g.15098868T>C               | 3' UTR<br>variant          | TT       | 0.322                   | T      | 0.568                 |
|          |                    |               |                             |                            | TC       | 0.493                   | C      | 0.432                 |
|          |                    |               |                             |                            | CC       | 0.185                   |        |                       |
| snp_4    | rs423194759        | g.15098916T>C | 3' UTR<br>variant           | TT                         | 0.322    | T                       | 0.568  |                       |
|          |                    |               |                             | TC                         | 0.493    | C                       | 0.432  |                       |
|          |                    |               |                             | CC                         | 0.185    |                         |        |                       |
| snp_5    | rs403826495        | g.15098968T>C | I348V<br>(ATA/GTA)          | TT                         | 0.322    | T                       | 0.568  |                       |
|          |                    |               |                             | TC                         | 0.493    | C                       | 0.432  |                       |
|          |                    |               |                             | CC                         | 0.185    |                         |        |                       |
| snp_6    | rs413084140        | g.15098976T>C | H345R<br>(CAT/CGT)          | TT                         | 0.322    | T                       | 0.568  |                       |
|          |                    |               |                             | TC                         | 0.493    | C                       | 0.432  |                       |
|          |                    |               |                             | CC                         | 0.185    |                         |        |                       |
| snp_7    | rs426523476        | g.15098996A>G | P338P<br>(CCC/CCT)          | AA                         | 0.182    | G                       | 0.57   |                       |
|          |                    |               |                             | AG                         | 0.496    | A                       | 0.43   |                       |
|          |                    |               |                             | GG                         | 0.322    |                         |        |                       |
| snp_8    | rs403212791        | g.15099004G>A | R336C<br>(CGC/TGC)          | GG                         | 0.511    | G                       | 0.724  |                       |
|          |                    |               |                             | GA                         | 0.425    | A                       | 0.276  |                       |
|          |                    |               |                             | AA                         | 0.064    |                         |        |                       |
| snp_9    | ss2137144054       | g.15099006T>G | K335T<br>(AAA/ACA)          | TT                         | 0.996    | T                       | 0.998  |                       |
|          |                    |               |                             | GT                         | 0.004    | G                       | 0.002  |                       |
|          |                    |               |                             | GG                         | 0        |                         |        |                       |

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|        |              |               |           |    |       |   |       |
|--------|--------------|---------------|-----------|----|-------|---|-------|
| snp_10 | rs416266900  | g.15099204G>T | A269D     | GG | 0.613 | G | 0.786 |
|        |              |               | (GCC/GAC) | GT | 0.346 | T | 0.214 |
|        |              |               |           | TT | 0.041 |   |       |
| snp_11 | rs429718221  | g.15099206G>A | P268P     | GG | 0.333 | G | 0.576 |
|        |              |               | (CCC/CCT) | GA | 0.486 | A | 0.424 |
|        |              |               |           | AA | 0.181 |   |       |
| snp_12 | rs404378206  | g.15099223C>T | V263I     | CC | 0.909 | C | 0.955 |
|        |              |               | (GTT/ATT) | CT | 0.091 | T | 0.045 |
|        |              |               |           | TT | 0     |   |       |
| snp_13 | rs417800445  | g.15099296C>T | R238R     | CC | 0.624 | C | 0.796 |
|        |              |               | (AGG/AGA) | CT | 0.342 | T | 0.204 |
|        |              |               |           | TT | 0.034 |   |       |
| snp_14 | rs427019119  | g.15099314C>T | L232L     | CC | 0.617 | C | 0.792 |
|        |              |               | (CTG/CTA) | CT | 0.35  | T | 0.208 |
|        |              |               |           | TT | 0.033 |   |       |
| snp_15 | rs407388227  | g.15099391C>T | V207I     | CC | 0.634 | C | 0.794 |
|        |              |               | (GTC/ATC) | CT | 0.321 | T | 0.206 |
|        |              |               |           | TT | 0.045 |   |       |
| snp_16 | rs420819884  | g.15099422C>T | V196V     | CC | 0.918 | C | 0.955 |
|        |              |               | (GTG/GTA) | CT | 0.074 | T | 0.045 |
|        |              |               |           | TT | 0.008 |   |       |
| snp_17 | rs430181568  | g.15099485C>T | P175P     | CC | 0.634 | C | 0.792 |
|        |              |               | (CCG/CCA) | CT | 0.317 | T | 0.208 |
|        |              |               |           | TT | 0.049 |   |       |
| snp_18 | rs406779174  | g.15099491G>A | Y173Y     | GG | 0.28  | G | 0.525 |
|        |              |               | (TAC/TAT) | GA | 0.49  | A | 0.475 |
|        |              |               |           | AA | 0.23  |   |       |
| snp_19 | ss2137144055 | g.15099575C>T | T145T     | CC | 0.922 | C | 0.959 |
|        |              |               | (ACG/ACA) | CT | 0.074 | T | 0.041 |
|        |              |               |           | TT | 0.004 |   |       |
| snp_20 | ss2137144056 | g.15099642C>A | G123V     | CC | 0.996 | C | 0.998 |
|        |              |               | (GGA/GTA) | CA | 0.004 | A | 0.002 |
|        |              |               |           | AA | 0     |   |       |
| snp_21 | rs419680097  | g.15099644C>A | T122T     | CC | 0.638 | C | 0.79  |
|        |              |               | (ACG/ACT) | CA | 0.305 | A | 0.21  |

|          |        |              |               |                   |       |       |       |       |
|----------|--------|--------------|---------------|-------------------|-------|-------|-------|-------|
|          |        |              |               |                   | AA    | 0.057 |       |       |
| exon 1   | snp_22 | ss2137144057 | g.15121956G>A | N16N<br>(AAT/AAC) | GG    | 0.696 | G     | 0.761 |
|          |        |              |               |                   | GA    | 0.13  | A     | 0.239 |
|          |        |              |               |                   | AA    | 0.174 |       |       |
| Promoter | snp_23 | rs406334919  | g.15122684T>C |                   | TT    | 0.699 | T     | 0.831 |
|          |        |              |               |                   | TC    | 0.265 | C     | 0.169 |
|          |        |              |               |                   | CC    | 0.036 |       |       |
|          | snp_24 | rs419743392  | g.15122766G>A |                   | GG    | 0.379 | G     | 0.601 |
|          |        |              |               | GA                | 0.445 | A     | 0.399 |       |
|          |        |              |               | AA                | 0.176 |       |       |       |
|          | snp_25 | rs400561563  | g.15122788C>T |                   | CC    | 0.544 | C     | 0.735 |
|          |        |              |               | CT                | 0.382 | T     | 0.265 |       |
|          |        |              |               | TT                | 0.074 |       |       |       |
|          | snp_26 | rs399461430  | g.15122829T>C |                   | TT    | 0.379 | T     | 0.601 |
|          |        |              |               | TC                | 0.445 | C     | 0.399 |       |
|          |        |              |               | CC                | 0.176 |       |       |       |
|          | snp_27 | rs412826644  | g.15122893G>A |                   | GG    | 0.915 | G     | 0.956 |
|          |        |              |               | GA                | 0.081 | A     | 0.044 |       |
|          |        |              |               | AA                | 0.004 |       |       |       |
|          | snp_28 | rs426266687  | g.15122902C>T |                   | CC    | 0.382 | C     | 0.605 |
|          |        |              |               | CT                | 0.445 | T     | 0.395 |       |
|          |        |              |               | TT                | 0.173 |       |       |       |
|          | snp_29 | rs402949406  | g.15122931C>A |                   | CC    | 0.386 | C     | 0.607 |
|          |        |              |               | CA                | 0.441 | A     | 0.393 |       |
|          |        |              |               | AA                | 0.173 |       |       |       |
|          | snp_30 | rs411931887  | g.15122934C>T |                   | CC    | 0.387 | C     | 0.607 |
|          |        |              |               | CT                | 0.441 | T     | 0.393 |       |
|          |        |              |               | TT                | 0.173 |       |       |       |
|          | snp_31 | rs406184829  | g.15123052T>C |                   | TT    | 0.695 | T     | 0.829 |
|          |        |              |               | TC                | 0.268 | C     | 0.171 |       |
|          |        |              |               | CC                | 0.037 |       |       |       |
|          | snp_32 | rs415456480  | g.15123097G>A |                   | GG    | 0.379 | G     | 0.601 |
|          |        |              |               | GA                | 0.445 | A     | 0.399 |       |
|          |        |              |               | AA                | 0.176 |       |       |       |
|          | snp_33 | rs428880789  | g.15123143G>A |                   | GG    | 0.379 | G     | 0.601 |

|        |             |               |    |       |   |       |
|--------|-------------|---------------|----|-------|---|-------|
|        |             |               | GA | 0.445 | A | 0.399 |
|        |             |               | AA | 0.176 |   |       |
| snp_34 | rs405080439 | g.15123157G>A | GG | 0.386 | G | 0.607 |
|        |             |               | GA | 0.441 | A | 0.393 |
|        |             |               | AA | 0.173 |   |       |
| snp_35 | rs429917252 | g.15123238C>T | CC | 0.699 | C | 0.831 |
|        |             |               | CT | 0.265 | T | 0.169 |
|        |             |               | TT | 0.036 |   |       |

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<sup>1</sup>Nomenclature used for each SNP in this work.



**Table 2.** Type III test for the SNP and SNP x age effects for the *MTNR1A* polymorphisms using the seasonality phenotype data from Rasa aragonesa ewes. The least square means and standard errors of the *MTNR1A* polymorphisms in the seasonality phenotype data in Rasa aragonesa ewes are also shown. Only significant SNPs after Bonferroni correction are shown. M=mature; Y=young; L=ewe lambs. snp\_29-snp\_30-snp\_34 and snp\_24-snp\_26-snp\_32-snp\_33 were linked ( $r^2=1$ ). Different letters indicate significant differences: a, b:  $P<0.05$ ; c, d:  $P<0.01$ ; and e, f:  $P<0.001$ .

| SNP                                  | Trait | P-value<br>SNP | LSmeans SNP        |                    |                     | P-value<br>SNPxAge | Age | LSmeans SNPxAge |               |              |
|--------------------------------------|-------|----------------|--------------------|--------------------|---------------------|--------------------|-----|-----------------|---------------|--------------|
|                                      |       |                | AA/CC/GG           | AC/CT/AG           | CC/TT/AA            |                    |     | AA/CC/GG        | AC/CT/AG      | CC/TT/AA     |
| snp_29<br>snp_30<br>snp_34           | TDA   | 0.01           | 68.72±7.33c        | 58.84±7.13cd       | 37.05±11.29d        | 0.012              | M   | 54.74±9.96      | 46.61±9.05    | 61.56±11.44  |
|                                      |       |                |                    |                    |                     |                    | Y   | 63.45±10.55     | 63.67±10.13   | 49.51±14.24  |
|                                      |       |                |                    |                    |                     |                    | L   | 87.96±10.96a    | 66.23±10.94ab | 0.09±25.91b  |
|                                      | P4CM  | 0.015          | 0.81±0.03a         | 0.85±0.03ab        | 0.93±0.04b          | 0.03               | M   | 0.85±0.03       | 0.88±0.03     | 0.83±0.04    |
|                                      |       |                |                    |                    |                     |                    | Y   | 0.82±0.04       | 0.82±0.04     | 0.88±0.05    |
|                                      |       |                |                    |                    |                     |                    | L   | 0.76±0.04       | 0.83±0.04     | 1.08±0.1     |
| snp_24<br>snp_26<br>snp_32<br>snp_33 | TDA   | 0.008          | 68.86±7.32c        | 58.48±7.15cd       | 36.58±11.26d        | 0.013              | M   | 55.83±10.04     | 46.21±9.03    | 90.81±11.37  |
|                                      |       |                |                    |                    |                     |                    | Y   | 63.16±10.55     | 63.37±10.13   | 49.19±14.23  |
|                                      |       |                |                    |                    |                     |                    | L   | 87.58±10.78a    | 65.84±10.76ab | -0.25±25.11b |
|                                      | P4CM  | 0.013          | 0.81±0.03a         | 0.85±0.03ab        | 0.93±0.04b          | 0.033              | M   | 0.85±0.04       | 0.89±0.03     | 0.84±0.04    |
|                                      |       |                |                    |                    |                     |                    | Y   | 0.82±0.04       | 0.83±0.04     | 0.88±0.04    |
|                                      |       |                |                    |                    |                     |                    | L   | 0.73±0.04       | 0.86±0.04     | 1.08±0.1     |
| snp_28                               | TDA   | 0.008          | TT<br>37.02±11.27c | CT<br>58.50±7.13cd | CC<br>69.10±7.31c   | 0.012              | M   | 61.60±11.42     | 45.77±9.06    | 56.00±9.93   |
|                                      |       |                |                    |                    |                     |                    | Y   | 49.44±14.22     | 63.61±10.12   | 63.41±10.54  |
|                                      |       |                |                    |                    |                     |                    | L   | 0.00±25.59a     | 66.13±10.74ab | 87.87±10.77b |
|                                      | P4CM  | 0.013          | 0.93±0.04a         | 0.85±0.03ab        | 0.81±0.03b          | 0.03               | M   | 0.83±0.04       | 0.89±0.03     | 0.85±0.04    |
|                                      |       |                |                    |                    |                     |                    | Y   | 0.88±0.05       | 0.83±0.04     | 0.82±0.04    |
|                                      |       |                |                    |                    |                     |                    | L   | 1.08±0.04       | 0.82±0.04     | 0.76±0.1     |
| snp_25                               | TDA   | 0.025          | CC<br>52.40±7.15b  | CT<br>65.52±7.54a  | TT<br>68.99±11.93ab | 0.104              | M   | 47.50±9.49      | 52.83±9.35    | 46.69±18.14  |
|                                      |       |                |                    |                    |                     |                    | Y   | 56.84±9.58      | 66.05±10.86   | 54.57±22.70  |
|                                      |       |                |                    |                    |                     |                    | L   | 52.85±10.60     | 86.66±12.09   | 105.7±16.09  |
|                                      | P4CM  | 0.005          | 0.88±0.03d         | 0.80±0.03c         | 0.80±0.04cd         | 0.089              | M   | 0.88±0.04       | 0.86±0.04     | 0.88±0.07    |
|                                      |       |                |                    |                    |                     |                    | Y   | 0.85±0.04       | 0.82±0.04     | 0.83±0.09    |
|                                      |       |                |                    |                    |                     |                    | L   | 0.90±0.04       | 0.73±0.05     | 0.71±0.6     |

**Table 3.** Type III test for the SNP effects on the *MTNR1A* gene using the seasonality phenotype data from Rasa aragonesa ewes. The least square means and standard errors for the SNP effects on the *MTNR1A* polymorphisms are also shown. Only significant SNPs after Bonferroni correction are shown. Different letters indicate significant differences: a, b:  $P < 0.05$ ; c, d:  $P < 0.01$ ; and e, f:  $P < 0.001$ .

| SNP    | Trait | P-value<br>SNP | LSmeans SNP   |               |                |
|--------|-------|----------------|---------------|---------------|----------------|
|        |       |                | GG            | GA            | AA             |
| snp_2  | TDA   | 0.0001         | 66.62±7.91a,c | 48.79±7.88b   | 16.25±15.15d   |
|        | P4CM  | 0.003          | 0.81±0.03a    | 0.87±0.03b    | 0.96±0.06b     |
|        | OCM   | 0.015          | 0.49±0.03a    | 0.55±0.03ab   | 0.64±0.07b     |
| snp_8  | TDA   | <0.0001        | 73.08±7.25c,e | 53.57±7.36a,d | 20.85±13.13b,f |
|        | P4CM  | 0.001          | 0.80±0.03a,c  | 0.86±0.03b    | 0.94±0.05b,d   |
|        | OCM   | 0.022          | 0.49±0.03a    | 0.54±0.03ab   | 0.64±0.06b     |
| snp_17 | TDA   | 0.037          | 78.41±14.37   | 59.38±8.40    | 50.71±7.94     |
|        | P4CM  | 0.073          | 0.76±0.06     | 0.83±0.03     | 0.86±0.03      |
|        | OCM   | 0.018          | 0.39±0.06a    | 0.50±0.04ab   | 0.55±0.03b     |
| snp_18 | TDA   | 0.0002         | 72.25±9.01e   | 58.05±7.78c   | 33.28±9.69f,d  |
|        | P4CM  | 0.02           | 0.46±0.04a    | 0.50±0.03ab   | 0.57±0.04b     |
|        | OCM   | 0.0002         | 72.25±9.01e   | 58.05±7.78c   | 33.28±9.69f,d  |

**Table 4.** Type III test for the haplotype and haplotype x age effects for blocks 3 and 4 on the *MTNR1A* gene promoter region using the seasonality phenotype data from Rasa aragonesa ewes. The least square means and standard errors for the haplotype and haplotype x age effects for the *MTNR1A* haplotypes are also shown. Only significant SNPs after Bonferroni correction are shown. Different letters indicate significant differences: a, b:  $P < 0.05$ ; c, d:  $P < 0.01$ ; and e, f:  $P < 0.001$ .

| Trait | Block <sup>1</sup> | Haplotype      | Freq. | SNP effect <sup>2</sup> |              |              | SNP x age effect <sup>2</sup> |         |     |             |               |              |
|-------|--------------------|----------------|-------|-------------------------|--------------|--------------|-------------------------------|---------|-----|-------------|---------------|--------------|
|       |                    |                |       | P-value                 | 0 copy       | 1 copy       | 2 copies                      | P-value | Age | 0 copy      | 1 copy        | 2 copies     |
| TDA   | 3                  | h3 (TG)        | 0.61  | 0.010                   | 35.52±11.35c | 57.61±7.29cd | 67.32±7.43d                   | 0.013   | M   | 58.98±11.51 | 44.94±9.23    | 52.56±10.22  |
|       |                    |                |       |                         |              |              |                               |         | Y   | 48.2±14.35  | 62.36±10.26   | 62.15±10.67  |
|       |                    |                |       |                         |              |              |                               |         | L   | -0.61±25.7a | 65.53±10.87ab | 87.27±10.89b |
|       | 4                  | h1 (TCACTGGGC) | 0.61  | 0.011                   | 35.1±11.32c  | 57.64±7.29cd | 66.94±7.41d                   | 0.016   | M   | 58.10±11.34 | 44.38±9.35    | 52.86±10.23  |
|       |                    |                |       |                         |              |              |                               |         | Y   | 47.96±14.32 | 62.13±10.23   | 61.91±10.64  |
|       |                    |                |       |                         |              |              |                               |         | L   | -0.76±25.7a | 66.40±10.78ab | 86.94±10.89b |
| P4CM  | 3                  | h3 (TG)        | 0.61  | 0.016                   | 0.93±0.04a   | 0.85±0.03ab  | 0.082±0.03b                   | 0.036   | M   | 0.84±0.04   | 0.89±0.03     | 0.86±0.04    |
|       |                    |                |       |                         |              |              |                               |         | Y   | 0.88±0.05   | 0.83±0.04     | 0.83±0.04    |
|       |                    |                |       |                         |              |              |                               |         | L   | 1.08±0.10   | 0.83±0.04     | 0.76±0.04    |
|       | 4                  | h1 (TCACTGGGC) | 0.61  | 0.015                   | 0.93±0.04a   | 0.85±0.03ab  | 0.82±0.03b                    | 0.031   | M   | 0.84±0.04   | 0.89±0.04     | 0.86±0.04    |
|       |                    |                |       |                         |              |              |                               |         | Y   | 0.88±0.05   | 0.83±0.04     | 0.83±0.05    |
|       |                    |                |       |                         |              |              |                               |         | L   | 1.08±0.10   | 0.83±0.04     | 0.69±0.07    |

<sup>1</sup> Block 3: snp\_23 - snp\_24 and Block4: snp\_26 - snp\_28 - snp\_29 - snp\_30 - snp\_31 - snp\_32 - snp\_33 - snp\_34 - snp\_35.

<sup>2</sup> M=mature; Y=young; L=ewe lambs. 0 copy: LSmeans and SE for 0 copy of the haplotype; 1 copy: LSmeans and SE for 1 copy of the haplotype; and 2 copies: LSmeans and SE for 2 copies of the haplotype.

**Table 5.** Type III test for the haplotype effects of blocks 1 and 2 in exon 2 on the *MTNR1A* gene using the seasonality phenotype data from Rasa aragonesa ewes. The least square means and standard errors for the haplotype effect for the *MTNR1A* gene are also shown. Only significant SNPs after Bonferroni correction are shown. Different letters indicate significant differences: a, b:  $P < 0.05$ ; c, d:  $P < 0.01$ ; and e, f:  $P < 0.001$

| Trait | Block <sup>1</sup> | Haplotype      | Freq. | SNP effect <sup>2</sup> |               |             |                |
|-------|--------------------|----------------|-------|-------------------------|---------------|-------------|----------------|
|       |                    |                |       | P-value                 | 0 copy        | 1 copy      | 2 copies       |
| TDA   | 1                  | h3 (CATCTTG)   | 0.26  | 0.0001                  | 66.62±7.91a,e | 48.79±7.88b | 16.25±15.15b,f |
|       | 2                  | h2 (AGGCCCCAC) | 0.26  | 0.0004                  | 65.13±7.85a,e | 50.33±7.96b | 13.78±15.42a,f |
| P4CM  | 1                  | h3 (CATCTTG)   | 0.26  | 0.0029                  | 0.81±0.03a    | 0.87±0.03b  | 0.96±0.06b     |
|       | 2                  | h2 (AGGCCCCAC) | 0.26  | 0.014                   | 0.82±0.03a    | 0.86±0.03ab | 0.97±0.06b     |
| OMC   | 1                  | h3 (CATCTTG)   | 0.26  | 0.015                   | 0.49±0.03a    | 0.55±0.03b  | 0.64±0.06b     |
|       | 2                  | h2 (AGGCCCCAC) | 0.26  | 0.023                   | 0.49±0.03a    | 0.54±0.03ab | 0.64±0.07b     |

<sup>1</sup> Block1: snp\_1 - snp\_2 - snp\_3 - snp\_4 - snp\_5 - snp\_6 - snp\_7; Block2: snp\_8 - snp\_10 - snp\_11 - snp\_13 - snp\_14 - snp\_15 - snp\_17 - snp\_18 - snp\_21

<sup>2</sup> 0 copy: LSmeans and SE for 0 copy of the haplotype; 1 copy: LSmeans and SE for 1 copy of the haplotype; and 2 copies: LSmeans and SE for 2 copies of the haplotype.

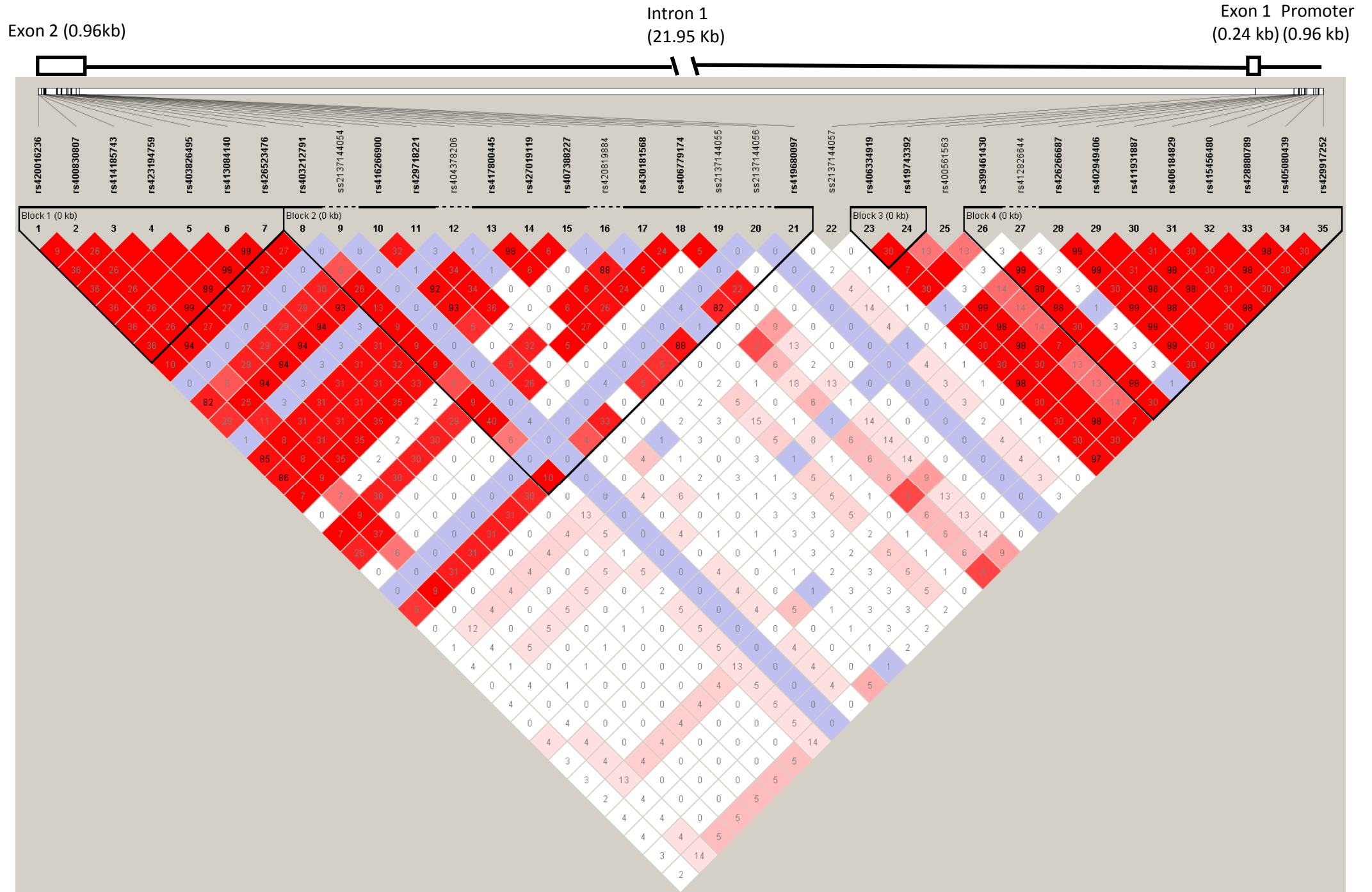
**Table 6.** Type III test for the haplotype effects of block 5 on the *MTNR1A* gene using the seasonality phenotype data from Rasa aragonesa ewes. The least square means and standard errors for the haplotype effect on the *MTNR1A* gene are also shown. Only significant haplotypes after Bonferroni correction are shown. Different letters indicate significant differences: a, b:  $P < 0.05$ ; c, d:  $P < 0.01$ ; and e, f:  $P < 0.001$ . Alleles associated with lower TDA and higher P4CM and OMC values for each SNP according to SNP association results are shown in bold.

| Trait | Haplotype                    | Freq. | SNP effect <sup>2</sup> |                   |                    |                     |
|-------|------------------------------|-------|-------------------------|-------------------|--------------------|---------------------|
|       |                              |       | P-value                 | 0 copy            | 1 copy             | 2 copies            |
| TDA   | h4( <b>AACAACCTCTAAA</b> )   | 0.17  | 0.0387                  | 63.17 $\pm$ 7.88a | 48.77 $\pm$ 8.44b  | 37.25 $\pm$ 21.15ab |
|       | h12 (GGCGATCTCTAAA)          | 0.02  | 0.029                   | 56.83 $\pm$ 7.42a | 98.48 $\pm$ 17.77b | --                  |
|       | h13 ( <b>AACAGTTCACGGG</b> ) | 0.05  | 0.0014                  | 59.20 $\pm$ 7.45c | 30.74 $\pm$ 11.94d | --                  |
| P4CM  | h4( <b>AACAACCTCTAAA</b> )   | 0.17  | 0.014                   | 0.81 $\pm$ 0.03a  | 0.87 $\pm$ 0.03b   | 0.97 $\pm$ 0.08b    |
|       | h12 ( GGCGATCTCTAAA)         | 0.02  | 0.040                   | 0.83 $\pm$ 0.03a  | 0.71 $\pm$ 0.07b   | --                  |
|       | h13 ( <b>AACAGTTCACGGG</b> ) | 0.05  | 0.026                   | 0.83 $\pm$ 0.03a  | 0.92 $\pm$ 0.04b   | --                  |
| OMC   | h10 ( <b>AACAGCTCACGGG</b> ) | 0.003 | 0.048                   | 0.50 $\pm$ 0.03a  | 0.61 $\pm$ 0.03b   | --                  |
|       | h13 ( <b>AACAGTTCACGGG</b> ) | 0.05  | 0.0014                  | 0.50 $\pm$ 0.03c  | 0.65 $\pm$ 0.05ad  | --                  |

<sup>1</sup> Block 5: snp\_2 - snp\_8 - snp\_17 - snp\_18 - snp\_24 - snp\_25 - snp\_26 - snp\_28 - snp\_29 - snp\_30 - snp\_32 - snp\_33 - snp\_34

<sup>2</sup> 0 copy: LSmeans and SE for 0 copy of the haplotype; 1 copy: LSmeans and SE for 1 copy of the haplotype; and 2 copies: LSmeans and SE for 2 copies of the haplotype.

Fig.1.



**Highlights**

- Significant SNPs in promoter and exon 2 of the MTNR1A were found.
- Haplotype analyses showed that the effects found were caused by the SNPs in exon 2.
- SNP rs403212791 in exon 2 gene is associated to reproductive seasonality
- The SNP rs403212791 leads a change of an Arginine to Cysteine (R336C).
- The SNP rs403212791 was not previously reported associated for reproductive seasonality.