

MTNR1A gene polymorphisms and reproductive recovery after seasonal anoestrus in different Mediterranean sheep breeds

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2 Mediterranean sheep breeds

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14 **ABSTRACT**

15 The aims of this study were 1) to investigate the effect of *MTNR1A* gene polymorphisms on
16 reproductive performance in ewes of one Italian and two Slovenian dairy sheep breeds (Sarda,
17 Istrian Premenka and Boska, respectively) which were located at different latitudes, and 2) to
18 highlight if the different season of the male placement with females that was utilized in the different
19 breeding systems in Sardinia (Italy) and Slovenia resulted in different effects of these
20 polymorphisms on reproductive functions. Reproductively mature ewes ($n = 100$) from each breed
21 were utilized to conduct the study. To evaluate the reproductive efficiency, lambing dates and
22 number of lambs born were recorded per ewe; additionally, the duration in days from ram
23 placement with ewes to lambing (DRPEL), litter size and the fertility rate were determined based on
24 lambing dates. In each breed, there were eight nucleotide variations within the *MTNR1A* gene exon
25 II, two of which (g.17355358 and g.17355171), respectively, resulted in a valine to isoleucine, and
26 alanine to aspartic acid substitution, in amino acid sequence. The SNPs at position g.17355452 and
27 g.17355458 were determined to have effects on reproductive performance. Genotypes C/C and C/T
28 at g.17355452 in Bovska and Sarda and genotype A/A at g.17355458 in Istrian Pramenka were
29 associated with a greater fertility and a lesser duration in days from ram placement with ewes to
30 lambing. These findings confirmed that the nucleotide sequences of the *MTNR1A* gene could affect
31 reproductive functions of Mediterranean sheep.

32
33 **Keywords:** *MTNR1A*; Polymorphism; Sheep; Reproductive Performances

34

35 **1. Introduction**

36 Melatonin is an indoleamine produced in a variety of tissues including the pineal gland
37 (Venegas et al., 2012). Synthesis of pineal melatonin is tightly controlled by the circadian light/dark
38 cycle, with there being inhibition of melatonin synthesis and release during the light period. The
39 day/night oscillation of melatonin in blood represents a peripheral clock involved in the regulation

40 of various circadian and circannual rhythms including seasonal reproduction (Reiter, 1991).
41 Melatonin has functions through binding and activation of specific receptors in the hypothalamus,
42 both at the suprachiasmatic nucleus - which is the site of the circadian clock - for circadian effects,
43 (Weaver et al., 1996), and in preammillary hypothalamus (PMH) for reproductive effects (Migaud
44 et al., 2005).

45 Melatonin has effects by binding to high-affinity, G-protein-coupled receptors (Dubocovich
46 and Takahashi, 1987; Reppert et al., 1994). In mammals, two high-affinity melatonin receptors have
47 been identified, termed MT1 and MT2, but only MT1 is associated with modulation of reproductive
48 functions (Dubocovich et al., 2003; Weaver et al., 1996). The largest concentration of melatonin
49 MT1 receptors has been detected in the Pars tuberalis (PT) of the hypothalamus, while there is a
50 lesser concentration of MT1 receptors in the PMH (Chabot et al., 1998; Malpoux et al., 1998).

51 Melatonin actions in the PT are associated both with the control of prolactin secretion and in
52 sensitivity to photoperiodic changes (Dardente, 2007). It, however, remains to be precisely
53 established as to how the photoperiodic signal perceived in the PT is transmitted to the medial basal
54 hypothalamus (MBH), where the nuclei regulating GnRH secretion are located, however, this signal
55 is likely modulated by thyroid hormones and Kisspeptin (Dardente, 2007). The MT1 receptor is
56 encoded by the *MTNR1A* gene, in which several polymorphic sites have been recognized in
57 different mammalian species, including sheep, goats and buffalo (Luridiana et al., 2012; Messer et
58 al., 1997). In sheep, two single nucleotide polymorphisms (SNPs) at position g.17355458G>A
59 (rs406779174, ex 606/RsaI) and g.17355452C>T (rs430181568, ex 612/MnII) have been associated
60 with different seasonal reproductive traits (Carcangiu et al., 2009; Chu et al., 2006; Mateescu et al.,
61 2009; Notter and Cockett, 2005). In the Ile de France sheep breed, however, there is no association
62 between these SNPs and reproductive functions. There was an association of those SNPs with
63 reproductive seasonality that might be breed-specific or might be affected by environmental
64 conditions (Hernandez et al., 2005). Different aspects of reproductive seasonality have been
65 examined in several studies where there was a focus on the effects of *MTNR1A* gene

66 polymorphisms. Results in many of the studies were inconsistent due to the different methodologies
67 utilized or the different periods of the year when the studies were conducted. Pelletier et al. (2000),
68 reported that there were reproductive functions as indicated by fluctuating progesterone
69 concentrations only in April, in Merino d'Arles, and reported that the rs430181568 SNP (previously
70 612/MnII) is associated with seasonal patterns of reproduction. By evaluating lambing dates,
71 Carcangiu et al., 2009; and Chu et al., 2006, reported that ewes with the C/C genotype at
72 rs430181568 and the A/A at rs406779174 loci had reproductive functions during periods of the year
73 when ewes of the breed evaluated were anoestrus. In Rasa Aragonesa breed, when there was
74 evaluation of ram-ewe mating patterns, Martínez-Royo et al. (2012) determined that ewes with the
75 A/A genotype at the rs406779174 locus had greater fertility between January and August compared
76 to those with the G/A or G/G genotypes. In Indian breeds, Saxena et al. (2014) reported that both of
77 these loci were associated with ewes breeding during periods of the year when ewes of these breeds
78 were typically anoestrus. In Slovenian dairy and meat sheep breeds, Starič et al. (2020) reported that
79 there was an association of these two SNPs with the re-initiation of reproductive functions in the
80 autumn, after seasonal anoestrus (reproductive recovery). The results for all these studies indicated
81 there was an association of *MTNR1A* gene in modulating seasonal effects on reproduction of ewes
82 in the breeds evaluated, but the determination of the effects in the various studies on reproductive
83 variables were evaluated using different methods. To obtain comparable data, therefore, it is
84 necessary to conduct studies using the same methods for determination of effects on reproductive
85 functions.

86 Based on the findings of previous studies, the hypothesis was tested that the *MTNR1A* gene
87 polymorphism affects reproductive functions in sheep. Consequently, the aims of this study were 1)
88 to investigate the effect of *MTNR1A* gene polymorphisms on reproductive performance, in the same
89 year, after placement of rams with ewes, of two Slovenian and one Italian dairy sheep breed (Istrian
90 Premenka and Boska, and Sarda, respectively) that were located at different latitudes, and 2) to
91 determine if the season of male placement with ewes was associated in the different breeding

92 systems utilized in Sardinia (Italy) and Slovenia had effects on how these polymorphisms affected
93 reproductive functions.

94

95 **2. Material and methods**

96 Ewes ($n = 300$) of dairy-type breeding were utilized in the present study. The ewes were of
97 three different breeds, Bovska and Istrian Pramenka – two Slovenian autochthonous dairy breeds -
98 and Sarda, the main Italian autochthonous dairy breed. The Sarda ewes were produced and located
99 in North West of Sardinia - Italy - while the Bovska and Istrian Pramenka ewes were produced and
100 located in the Soča valley and in the Vremščica mountain area, respectively, which are Slovenian
101 regions. For the characteristics of these breeds, see the report of Starič et al. (2020). For each breed,
102 100 reproductively mature ewes were used, with an average age of 4.3 ± 0.8 years for the Bovska
103 breed, 4.5 ± 0.7 years for the Istrian Pramenka and 4.1 ± 0.6 years for the Sarda breed. Bovska and
104 Istrian Pramenka ewes grazed during the day in a paddock where there were gramineous and
105 leguminous grasses, and were provided a supplement of 200 g of corn grain during the periods
106 when the ewes were milked twice daily. The animals were penned in a barn during the night where
107 there were water and hay provided *ad libitum*. The Sarda ewes, grazed leguminous and gramineous
108 grasses during the daylight hours and were provided 300 g per ewe daily concentrated commercial
109 food (crude protein 20.4% and 12.5 MJ ME/kg DM) during the period of the afternoon milking. The
110 ewes were penned during the night hours, and had free access to hay and water during this period.
111 Lambing generally occurs in March/April for the Bovška ewes, in February/March in the Istrian
112 Pramenka, and from September to January in the Sarda breed. Rams of the three breeds were placed
113 with ewes of the flocks at different times of the year, depending on the different farming systems.
114 For the two Slovenian breeds, rams were placed with ewes in the autumn so that in May the ewes
115 could be moved to the mountain area to graze, when lambs had developed to the extent they could
116 easily stay with their mothers during the transit to the mountains in the region used for summer
117 grazing. In Sardinia, however, the rams are commonly placed with ewes in May so that lambing can

118 occur mainly in October, when there is natural lush grass which ensures adequate milk production
119 for optimal lamb development. Furthermore, autumn lambing ensures that there can be a lactation
120 period from October to July because in Sardinia winters are generally mild from a climatic
121 perspective, thus favouring a long lactation period in a semi-extensive management system. For the
122 present study, rams were placed with ewes on 1 October, 25 September and 15 May in the Bovska,
123 Istrian Pramenka, and Sarda flocks, respectively, with a male/female ratio of one ram to 25 ewes.
124 Rams were removed from the ewe flocks after 60 days from the time of placement with ewes. The
125 lambing dates and the number of lambs born were recorded for each ewe. From the recorded data,
126 the data for the following reproductive variables were collected: fertility (number of ewes that
127 lambed per number of ewes with which there was ram placement), litter size (number of lambs born
128 per ewe that lambed) and the duration in days from ram placement with ewes to lambing (DRPEL).

129

130 *2.1. Sampling and genotyping*

131 An individual 10 ml blood sample was collected using jugular venepuncture procedures,
132 utilising a vacuum tube with EDTA (ethylenediaminetetraacetic acid) as anticoagulant (BD
133 Vacutainer Systems, Plymouth, UK). A total of 300 blood samples were collected, 100 from each of
134 the three breeds.

135 The nucleic acid extraction was performed using a commercial genomic DNA extraction kit
136 (NucleoSpin® Blood, Catalogue No., Macherey-Nagel, Germany). The concentration and purity
137 values of the extracted DNA were evaluated using spectrophotometric procedures. The average
138 concentration was 132 ng/μl, and a 260/280 ratio of 1,80 was considered suitable for the analysis.

139 Polymerase chain reaction (PCR) was performed utilising 150 ng of genomic DNA using
140 MAXYGENE II Thermal Cycler (Axygen® Tewksbury, MA, USA). The primers used were the
141 following: Forward: 5'-TGTGTTTGTGGTGAGCCTGG-3'; Reverse: 5'-
142 ATGGAGAGGGTTTGC GTTTA-3', as reported by Reppert et al. (1994). The PCR reaction was
143 performed as reported by Luridiana et al. (2015). The PCR products were separated using

144 electrophoresis procedures on a 1.5% agarose gel added with 9 µl of RedSafe Dna Stain 20.000X
145 (iNtROn Biotechnology, Inc., Sangdaewon-dong, Seongnam-si, Gyeonggi-do, Korea), processed in
146 parallel with 100 bp DNA marker (Invitrogen, Carlsbad, USA) in TAE 1% Buffer, at steady voltage
147 of 110 V for 30 minutes. The gel was then visualized using a UV light transilluminator (UVItec,
148 Cambridge, UK).

149 The PCR products were purified (multiscreen filter plates - Millipore) and then sequenced in
150 forward and reverse direction, by a commercial service. Before the Sanger sequencing (ABI PRISM
151 3730 DNA Analyzer, Applied Biosystems), the samples were prepared using the Big Dye
152 Terminator sequencing kit v3.1 (Applied Biosystems). The alignment of the resulting sequences and
153 the comparison with the latest version of the sheep genome - Oar_rambouillet_v1.0 - GenBank
154 Assembly Accession Number: GCF_002742125.1 - was performed using the BLAST program
155 (www.ncbi.nlm.nih.gov/blast/). To perform nucleotide sequence alignments, the ClustalW, within
156 “Accessory Application” of BioEdit Sequence Alignment Editor software
157 (<http://www.mbio.ncsu.edu/BioEdit/BioEdit.html>) was used, selecting the IUB weight matrix (for
158 DNA) scoring matrix.

159

160 *2.2. Statistical analysis*

161 All the statistical analyses were conducted using R statistical software (Version 4.0.4 R Core
162 Team 2021 R: A language and environment for statistical computing. R Foundation for Statistical
163 Computing, Vienna, Austria. URL <https://www.R-project.org/>). To analyse the association between
164 each genotype and fertility rate or number of ewes lambled in 10-day periods (from 150 to 200 days
165 after ram placement with ewes) a Chi-square test was used. The differences among breeds in
166 fertility rate were analysed using a Chi-square test. To assess for normal distribution and
167 homogeneity of variances of the duration in days from ram placement with ewes to lambing
168 (DRPEL) and the litter size, the Shapiro and Lavene tests were used, respectively. The ranges in

169 DRPEL and in litter size were 60 and 1, respectively. The duration in days from ram placement
170 with ewes to lambing (DRPEL) and litter size were analysed using the following linear model:

$$171 \quad Y_{jkm} = \mu + G_j + R_{kj} + e_{jkm}$$

172 where Y_{jkm} is the trait measured for each animal (DRPEL or litter size), μ is the overall
173 mean of each trait in considered ewes, G_j is the fixed effect of each genotype (three levels), R_{kj} is
174 the random effect of the ram (four levels within genotype), and e_{jkm} is the random residual effect of
175 each observation. Data of DRPEL and litter size are expressed as least-square mean \pm SEM. There
176 were considered to be mean differences when there was a $P < 0.05$.

177

178 **3. Results**

179 The PCR product resulted in a single fragment of 824 bp in length, corresponding to the
180 major part of the *MTNR1A* gene exon II. From the sequence analysis of the amplified fragment in
181 the Bovska, Istrian Pramenka and Sarda breeds, there were eight nucleotide variations detected
182 (Table 1), additionally, in two Bovska ewes a single nucleotide variant (SNV), at position
183 g.17355517G>A, was identified. All the nucleotide variations were reported according to the latest
184 sheep genome version (Oar_rambouillet_v1.0 - GenBank assembly accession number:
185 GCA_002742125.1). Three of the SNPs (g.17355458G>A, g.17355452C>T and g.17355358C>T),
186 which are the most frequently evaluated in many breeds for the association with seasonal
187 reproduction, did not result in a Hardy-Weinberg equilibrium in all the three breeds ($P < 0.05$). In
188 addition, two of these SNPs (g.17355452C>T and g.17355358C>T) were always associated so that
189 these were considered to be unique SNP. The SNP at the position g.17355358C>T (rs407388227)
190 resulted in an amino acid change (Ile/Val). The SNPs g.17355452C>T and g.17355458G>A were
191 of different allelic and genotype frequencies in the three breeds evaluated, as reported in Table 2.

192 In the three breeds, the C allele was the most frequent both in the g.17355452 and
193 g.17355358 loci, and, consequently C/C resulted the most frequent genotype. Instead, for the locus

194 at position g.17355458, in the three breeds evaluated, G was the most frequent allele and, therefore,
195 was also the genotype (Table 2).

196 In Bovška and Sarda ewes, the results of the statistical analysis indicated there were C/C and
197 C/T genotypes, at the positions g.17355452 and g.17355358 that were associated with greater
198 fertility ($P<0.05$), compared with the T/T genotype. Furthermore, the Bovška and Sarda ewes with
199 the C/C genotype at the previously described positions, had a shorter DRPEL than ewes with the
200 T/T genotype ($P<0.05$). Comparison of results for fertility rate between these two breeds as related
201 to the C/C and C/T genotypes indicated the Bovska ewes were more fertile than Sarda ewes (P
202 <0.05). There was no association between genotypes and litter size neither in ewes of the Bovška
203 nor Sarda breed (Tables 3 and 4). There was no association between the SNP at position
204 g.17355458 and the values for reproductive variables both in ewes of the Bovska and Sarda breed
205 (Table 3 and 4). Otherwise, in the Istrian Pramenka ewes the A/A genotype, at g.17355458 position,
206 was positively associated with a greater fertility ($P<0.05$) and a shorter DRPEL ($P<0.05$) compared
207 to the ewes with the other two genotypes (Table 5).

208 In the Istrian Pramenka ewes, litter size was also not affected by the different
209 polymorphisms (Table 5). The distribution in percentage of lambing, every 10 days, in the Bovska
210 and Sarda breed indicated the ewes with the C/C or C/T genotype at g.17355452 and g.17355358
211 positions, initiated reproductive functions earlier after a period of anoestrus, and consequently
212 lambed earlier in the lambing season, compared to the ewes with the T/T genotype (Table 6 and 7).
213 Furthermore, the Bovska ewes with the T/T genotype had the greatest occurrence of lambing
214 approximately 10 days earlier than ewes of the Sarda breed. In the Istrian Pramenka breed, the
215 percentage distribution of lambing every 10 days indicated ewes with the A/A genotype at position
216 g.17355458 initiated lambing earlier in the lambing season compared to the ewes of the other two
217 genotypes (Table 8).

218

219 **4. Discussion**

220 The three breeds of dairy sheep evaluated had the same eight SNPs within the exon II of the
221 *MTNR1A* gene; additionally, only in two Bovska ewes there was an additional nucleotide variation
222 detected, but due to its extremely small frequency, it was considered a SNV rather than a
223 polymorphism. These results are consistent with those reported in previous studies by Starič et al.
224 (2020) and Luridiana et al. (2020). Furthermore, these SNPs were also reported to be present in
225 other sheep breeds, with some differences among the breeds which may be related to the analysed
226 sequence length of the *MTNR1A* gene (Cosso et al., 2021; Pelletier et al., 2000; Saxena et al., 2014).
227 The results of the present study, also confirmed that the SNPs at positions g.17355452 and
228 g.1755358 are fully linked and these data are consistent with the findings of Starič et al. (2020) and
229 Mura et al. (2019). In the present study, the reproductive functions in the Istrian Pramenka breed
230 were affected by the SNP at position g.17355458 of the *MTNR1A* gene, while in the Bovska and
231 Sarda breeds there were no effects on reproductive functions of the polymorphisms at positions
232 g.1755452 and g.1755358. In the Bovska and Sarda breed, ewes with C/C or C/T genotype at locus
233 g.17355452 and g.1755358 were more fertile and had a shorter DRPEL than ewes with T/T
234 genotype. These findings are consistent with those in previous studies with Slovenian and other
235 European sheep breeds (Luridiana et al., 2016; Starič et al., 2020). Considering the different dates
236 of ram placement with ewes in the present study, however, it was expected that there would be
237 considerable differences between the two sheep breeds. In the Bovska breed, the rams were placed
238 with the ewes on 1 October (complete breeding season), while in the Sarda breed this placement of
239 rams with ewes was on 15 May (breeding during the season when breeding does not typically
240 occur). Surprisingly, the differences in reproductive response to ram placement with ewes in the
241 two breeds were very few, therefore, effects of ram placement with ewes was similar at both
242 seasons of the year. In the Bovska breed there was a greater fertility of ewes with the C/C and C/T
243 genotypes, than in Sarda ewes (for C/C genotype 92% compared with 80%, and for C/T 88%
244 compared with 82%, in Bovska and Sarda ewes, respectively), however, it is important to consider
245 that the rams were placed with the Bovska ewes during their natural breeding season, while rams

246 were placed with Sarda ewes during their seasonal anoestrous period. It, therefore, is expected that
247 the Bovska ewes were already having oestrous cyclic functions at the initiation of the breeding
248 season, leading to the earlier response to the rams compared with the Sarda ewes. Likely, this could
249 be the reason for the difference in fertility in the ewes of the two breeds. It was expected that Sarda
250 ewes would be in the transitional phase from being anoestrus to initiation of oestrous cyclic patterns
251 during which the reproductive axis gradually regains full functionality (Fabre-Nys et al., 2015;
252 Mura et al., 2019). The lesser fertility detected in Sarda compared to Bovska ewes, therefore,
253 probably depended on the lesser capacity of these ewes to promptly respond to the ram stimuli
254 during the initial period when rams were placed with ewes, maybe because the “male effect” alone
255 was not adequate to induce onset of oestrous cyclic patterns. When all of the findings in the present
256 study are considered, the Sarda and Bovska ewes with the C/C and C/T genotype had a greater
257 reproductive response when placed with rams even when the evaluations occurred during different
258 periods of the year when ewe reproductive functions were markedly different. Wang et al. (2017)
259 reported that the *MTNR1A* gene is involved in the development and maturation of the ovarian
260 follicle. It could be hypothesized that the two previously described genotypes are involved in
261 regulating ovarian functions, explaining why ewes with these two genotypes are more fertile. The
262 ovary, however, produces or has an uptake in the follicle of melatonin which is thought to sustain
263 ovarian follicle functions and inhibit apoptosis (Tamura et al., 2008). Furthermore, there are
264 melatonin receptors in different portions of the ovarian follicle, as reported by Wang et al. (2014),
265 and it is well known that the MT1 receptor is involved in modulation of physiological processes in
266 the ovarian follicle (Wang et al., 2017). The “silencing” of this receptor results in a lesser
267 expression of anti-apoptotic genes and a decrease in the antioxidant effect of melatonin. Melatonin
268 also modulates the expression of genes involved in steroidogenesis, in the conversion of
269 progesterone to androgens, and in luteinisation of granulosa cells (He et al., 2016; Lima et al.,
270 2015). Hence, it is reasonable to hypothesize that the different genotypes at the *MTNR1A* gene can
271 influence the transmission of the melatonin signal, as reported by Trecherel et al. (2010), and thus

272 can affect the growth and sustenance of the follicle functions. The greater fertility of Bovska and
273 Sarda ewes with the C/C and C/T genotype is certainly due to an enhanced ovarian sensitivity to the
274 melatonin signal.

275 Ewes of the two breeds with the T/T genotype at loci g.1755452 and g.1755358 have some
276 differences in fertility and peak time of lambing during the lambing season. The Bovska ewes with
277 the T/T genotype were more fertile than Sarda T/T ewes (73% compared with 66%, respectively;
278 Table 3 and 4). In addition, for Bovska T/T ewes there was the peak occurrence of lambing peak at
279 day 181 to 190, while for Sarda T/T ewes that was at day 191 to 200 after ram placement with the
280 ewes (Table 6 and 7). This genotype is most prevalent in non-domesticated sheep (*Ovis gmelini*
281 *musimon*) which are very sensitive to the photoperiod in regulation of reproductive functions
282 (Carcangiu et al., 2010). In these non-domesticated sheep, the spring lambing period ensures the
283 survival of the species, enhancing the probability for optimal conditions for offspring development.
284 It, therefore, can be hypothesized that in both the Sarda and Bovska breeds, the ewes with the T/T
285 genotype are less sensitive to the stimuli resulting when there is placement with rams, compared to
286 the ewes with the other two genotypes. This was more evident in the Sarda breed because the period
287 when rams were placed with ewes was a period when ewes were expected to be anoestrus. In the
288 present study, the greater fertility rate in the ewes with the C/C or C/T genotype confirmed the
289 hypothesis about these ewes being lesser sensitive to photoperiod than ewes with the other
290 genotype. Presumably, there was a shorter period of anoestrus which led to a greater response when
291 rams were placed with the ewes as compared with ewes having the T/T genotype, which may have
292 longer periods of seasonal anoestrus. The different hypothalamic sensitivity to photoperiodic signals
293 could be the reason for the different reproductive responses among the ewes with the three
294 genotypes.

295 In the Istrian Pramenka breed, however, only the polymorphism at position g.17355458 had
296 an effect on reproductive functions, which is consistent with findings in the same breed by Starič et
297 al. (2020) and Martínez-Royo et al. (2012) for the Aragonesa breed. In particular, ewes with the

298 A/A genotype were more fertile and had a shorter DRPEL than ewes with the A/G and G/G
299 genotypes (Table 5). This association is difficult to explain because this polymorphism does not
300 result in an amino acid change and, therefore, there is not a change in the conformation of the
301 protein which could affect signal transmission as occurs when there is the polymorphism at position
302 g.17355358 (Trecherel et al., 2010). It, however, could also be that this polymorphism is linked to
303 another polymorphism not yet discovered within the same gene, or within other genes involved in
304 the regulation of reproductive activity. It is reasonable to consider that in a system as complex as
305 that modulating reproductive functions, several genes are involved such as the *Fec*, or *Kiss-1* genes
306 that could have combined functions with the protein encoded by the *MTNR1A* gene in regulating
307 reproductive efficiency. In future research, therefore, it would be interesting to investigate the
308 associations among the proteins encoded for by the previously described genes and how these
309 proteins may interact to modulate reproductive functions in ewes of different sheep breeds.

310

311 **5. Conclusion**

312 In conclusion, for the *MTNR1A* gene polymorphisms there is an association with initiation
313 of reproductive functions following seasonally induced anoestrus in ewes of all the three breeds
314 evaluated. In addition, although the period of ram placement with ewes of the Bovska breed was
315 different compared with the ewes of the Sarda breed, the effect of the polymorphism on initiation of
316 reproductive cyclic functions after a period of seasonal anoestrus was evident in ewes of both of
317 these breeds. Bovska ewes were more fertile than Sarda ewes, but the effect of the polymorphism
318 occurred in both of breeds. In both breeds, the ewes with the C/C or C/T genotype at position
319 g.17355452 and g.17355358 of the *MTNR1A* gene were more fertile and had a shorter DRPEL than
320 the ewes with the T/T genotype. Instead, the SNP that was associated with having effects on
321 initiation of oestrous cycles after a period of seasonal anoestrus in the Istrian Pramenka breed was
322 that at position g.17355458. In this breed ewes with the A/A genotype were more fertile and had a
323 shorter DRPEL than ewes with the A/G and G/G genotypes. The findings of the present study could

324 be used in animal breeding programs for reproductive genetic improvement in dairy sheep. To more
325 precisely elucidate the mechanism through which there is the effect of *MTNR1A* gene
326 polymorphisms on reproductive functions, other studies need to be conducted. In particular, there
327 should be studies focused on ovarian functions ascertaining whether the polymorphisms at this gene
328 can have effects on the maturation of the ovarian follicle. These types of studies would be useful for
329 clarification of the effect of these polymorphisms on initiation of reproductive functions in ewes
330 during the transition period of ewes from being seasonally anoestrus to the time when ewes are
331 oestrous cyclic.

332

333 **Author contribution**

334 All co-authors have contributed equally to the research (Conceptualization, Data curation;
335 Formal analysis; Funding acquisition; Investigation; Methodology; Project administration;
336 Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing
337 - review & editing) as well as to article preparation. All co-authors have approved the final draft of
338 this article.

339

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348 **References**

349 Carcangiu, V., Mura, M.C., Vacca, G.M., Dettori, M.L., Pazzola, M., Daga, C., Luridiana, S., 2010.

350 Characterization of the melatonin receptor gene MT1 in mouflon (*Ovis Gmelini Musimon*) and
351 its relationship with reproductive activity. *Mol. Reprod. Dev.* 77, 196.
352 <https://doi.org/10.1002/mrd.21125>

353 Carcangiu, V., Mura, M.C., Vacca, G.M., Pazzola, M., Dettori, M.L., Luridiana, S., Bini, P.P.,
354 2009. Polymorphism of the melatonin receptor MT1 gene and its relationship with seasonal
355 reproductive activity in the Sarda sheep breed. *Anim. Reprod. Sci.* 116, 65–72.
356 <https://doi.org/10.1016/j.anireprosci.2009.01.005>

357 Chabot, V., Caldani, M., De Reviere, M.M., Pelletier, J., 1998. Localization and quantification of
358 melatonin receptors in the diencephalon and posterior telencephalon of the sheep brain. *J.*
359 *Pineal Res.* 24, 50–57. <https://doi.org/10.1111/j.1600-079X.1998.tb00365.x>

360 Chu, M.X., Cheng, D.X., Liu, W.Z., Fang, L., Ye, S.C., 2006. Association between melatonin
361 receptor 1A gene and expression of reproductive seasonality in sheep. *Asian-Australasian J.*
362 *Anim. Sci.* 19, 1079–1084. <https://doi.org/10.5713/ajas.2006.1079>

363 Cosso, G., Luridiana, S., Pulinas, L., Curone, G., Pich, G., Carcangiu, V., Mura, M.C., 2021.
364 Melatonin treatment in rams and their replacement with novel treated rams advance first
365 lambing and increase fertility in sarda ewe lambs. *Animals* 11.
366 <https://doi.org/10.3390/ani11051227>

367 Dardente, H., 2007. Does a melatonin-dependent circadian oscillator in the pars tuberalis drive
368 prolactin seasonal rhythmicity? *J. Neuroendocrinol.* 19, 657–666.
369 <https://doi.org/10.1111/j.1365-2826.2007.01564.x>

370 Dubocovich, M.L., Rivera-Bermudez, M.A., Gerdin, M.J., Masana, M.I., 2003. Molecular
371 pharmacology, regulation and function of mammalian melatonin receptors. *Front. Biosci.*
372 <https://doi.org/10.2741/1089>

373 Dubocovich, M.L., Takahashi, J.S., 1987. Use of 2-[125I]iodomelatonin to characterize melatonin
374 binding sites in chicken retina. *Proc. Natl. Acad. Sci. U. S. A.* 84, 3916–3920.
375 <https://doi.org/10.1073/pnas.84.11.3916>

376 Fabre-Nys, C., Kendrick, K.M., Scaramuzzi, R.J., 2015. The “ram effect”: New insights into neural
377 modulation of the gonadotropic axis by male odors and socio-sexual interactions. *Front.*
378 *Neurosci.* <https://doi.org/10.3389/fnins.2015.00111>

379 He, C., Ma, T., Shi, J., Zhang, Z., Wang, J., Zhu, K., Li, Y., Yang, M., Song, Y., Liu, G., 2016.
380 Melatonin and its receptor MT1 are involved in the downstream reaction to luteinizing
381 hormone and participate in the regulation of luteinization in different species. *J. Pineal Res.*
382 279–290. <https://doi.org/10.1111/jpi.12345>

383 Hernandez, X., Bodin, L., Chesneau, D., Guillaume, D., Chemineau, P., Malpaux, B., Migaud, M.,
384 2005. Relationship between MT1 melatonin receptor gene polymorphism and seasonal
385 physiological responses in Île-de-France ewes. *Reprod. Nutr. Dev.* 45, 151–162.
386 <https://doi.org/10.1051/rnd:2005012>

387 Lima, G.N., Maganhin, C.C., Simões, R.S., Pinheiro Baracat, M.C.N., Da Sasso, G.R.S., Portugal
388 Fuchs, L.F., De Simões, M.J., Baracat, E.C., Soares Jú Nior, J.M., 2015. Steroidogenesis-
389 related gene expression in the rat ovary exposed to melatonin supplementation. *Clinics* 70,
390 144–151. [https://doi.org/10.6061/clinics/2015\(02\)12](https://doi.org/10.6061/clinics/2015(02)12)

391 Luridiana, S., Cosso, G., Pulinas, L., Di Stefano, M.V., Curone, G., Carcangiu, V., Mura, M.C.,
392 2020. New polymorphisms at MTNR1A gene and their association with reproductive
393 resumption in sarda breed sheep. *Theriogenology* 158, 438–444.
394 <https://doi.org/10.1016/j.theriogenology.2020.10.006>

395 Luridiana, S., Mura, M.C., Daga, C., Cosso, G., Bodano, S., Farci, F., Zidda, F., Carcangiu, V.,
396 2016. Influences of melatonin treatment, melatonin receptor 1A (MTNR1A) and kisspeptin
397 (KiSS-1) gene polymorphisms on first conception in Sarda ewe lambs. *Reprod. Fertil. Dev.* 28,
398 750–756. <https://doi.org/10.1071/RD14120>

399 Luridiana, S., Mura, M.C., Daga, C., Farci, F., Di Stefano, M. V., Zidda, F., Carcangiu, V., 2015.
400 Melatonin treatment in spring and reproductive recovery in sheep with different body
401 condition score and age. *Anim. Reprod. Sci.* 160, 68–73.

402 <https://doi.org/10.1016/j.anireprosci.2015.07.004>

403 Luridiana, S., Mura, M.C., Pazzola, M., Paludo, M., Cosso, G., Dettori, M.L., Bua, S., Vacca, G.M.,
404 Carcangiu, V., 2012. Association between melatonin receptor 1A (MTNR1A) gene
405 polymorphism and the reproductive performance of Mediterranean Italian buffaloes. *Reprod.*
406 *Fertil. Dev.* 24, 983–987. <https://doi.org/10.1071/RD11297>

407 Malpoux, B., Daveau, A., Maurice-Mandon, F., Duarte, G., Chemineau, P., 1998. Evidence that
408 melatonin acts in the premammillary hypothalamic area to control reproduction in the ewe:
409 Presence of binding sites and stimulation of luteinizing hormone secretion by in situ
410 microimplant delivery. *Endocrinology* 139, 1508–1516.
411 <https://doi.org/10.1210/endo.139.4.5879>

412 Martínez-Royo, A., Lahoz, B., Alabart, J.L., Folch, J., Calvo, J.H., 2012. Characterisation of the
413 Melatonin Receptor 1A (MTNR1A) gene in the Rasa Aragonesa sheep breed: Association with
414 reproductive seasonality. *Anim. Reprod. Sci.* 133, 169–175.
415 <https://doi.org/10.1016/j.anireprosci.2012.06.018>

416 Mateescu, R.G., Lunsford, A.K., Thonney, M.L., 2009. Association between melatonin receptor 1A
417 gene polymorphism and reproductive performance in Dorset ewes. *J. Anim. Sci.* 87, 2485–
418 2488. <https://doi.org/10.2527/jas.2008-1688>

419 Messer, L.A., Wang, L., Tuggle, C.K., Yerle, M., Chardon, P., Pomp, D., Womack, J.E., Barendse,
420 W., Crawford, A.M., Notter, D.R., Rothschild, M.F., 1997. Mapping of the melatonin receptor
421 1a (MTNR1A) gene in pigs, sheep, and cattle. *Mamm. Genome* 8, 368–370.
422 <https://doi.org/10.1007/s003359900444>

423 Migaud, M., Daveau, A., Malpoux, B., 2005. MTNR1A melatonin receptors in the ovine
424 premammillary hypothalamus: Day-night variation in the expression of the transcripts. *Biol.*
425 *Reprod.* 72, 393–398. <https://doi.org/10.1095/biolreprod.104.030064>

426 Mura, M.C., Luridiana, S., Pulinas, L., Di Stefano, M.V., Carcangiu, V., 2019. Reproductive
427 response to male joining with ewes with different allelic variants of the MTNR1A gene. *Anim.*

428 Reprod. Sci. 200, 67–74. <https://doi.org/10.1016/j.anireprosci.2018.11.012>

429 Notter, D.R., Cockett, N.E., 2005. Opportunities for detection and use of QTL influencing seasonal
430 reproduction in sheep: a review. *Genet. Sel. Evol.* 37, S39. [https://doi.org/10.1186/1297-9686-](https://doi.org/10.1186/1297-9686-37-s1-s39)
431 37-s1-s39

432 Pelletier, J., Bodin, L., Hanocq, E., Malpaux, B., Teyssier, J., Thimonier, J., Chemineau, P., 2000.
433 Association between expression of reproductive seasonality and alleles of the gene for Mel(1a)
434 receptor in the ewe. *Biol. Reprod.* 62, 1096–1101. <https://doi.org/10.1095/biolreprod62.4.1096>

435 Reiter, R.J., 1991. Pineal melatonin: Cell biology of its synthesis and of its physiological
436 interactions. *Endocr. Rev.* 12, 151–180. <https://doi.org/10.1210/edrv-12-2-151>

437 Reppert, S.M., Weaver, D.R., Ebisawa, T., 1994. Cloning and characterization of a mammalian
438 melatonin receptor that mediates reproductive and circadian responses. *Neuron* 13, 1177–1185.
439 [https://doi.org/10.1016/0896-6273\(94\)90055-8](https://doi.org/10.1016/0896-6273(94)90055-8)

440 Saxena, V.K., Jha, B.K., Meena, A.S., Naqvi, S.M.K., 2014. Sequence analysis and identification of
441 new variations in the coding sequence of melatonin receptor gene (MTNR1A) of Indian
442 Chokla sheep breed. *Meta Gene* 2, 450–458. <https://doi.org/10.1016/j.mgene.2014.05.005>

443 Starič, J., Farci, F., Luridiana, S., Mura, M.C., Pulinas, L., Cosso, G., Carcangiu, V., 2020.
444 Reproductive performance in three Slovenian sheep breeds with different alleles for the
445 MTNR1A gene. *Anim. Reprod. Sci.* 216. <https://doi.org/10.1016/j.anireprosci.2020.106352>

446 Tamura, H., Takasaki, A., Miwa, I., Taniguchi, K., Maekawa, R., Asada, H., Taketani, T.,
447 Matsuoka, A., Yamagata, Y., Shimamura, K., Morioka, H., Ishikawa, H., Reiter, R.J., Sugino,
448 N., 2008. Oxidative stress impairs oocyte quality and melatonin protects oocytes from free
449 radical damage and improves fertilization rate. *J. Pineal Res.* 44, 280–287.
450 <https://doi.org/10.1111/j.1600-079X.2007.00524.x>

451 Trecherel, E., Batailler, M., Chesneau, D., Delagrangé, P., Malpaux, B., Chemineau, P., Migaud,
452 M., 2010. Functional characterization of polymorphic variants for ovine MT1 melatonin
453 receptors: Possible implication for seasonal reproduction in sheep. *Anim. Reprod. Sci.* 122,

454 328–334. <https://doi.org/10.1016/j.anireprosci.2010.10.007>

455 Venegas, C., García, J.A., Escames, G., Ortiz, F., López, A., Doerrier, C., García-Corzo, L., López,
456 L.C., Reiter, R.J., Acuña-Castroviejo, D., 2012. Extrapineal melatonin: Analysis of its
457 subcellular distribution and daily fluctuations. *J. Pineal Res.* 52, 217–227.
458 <https://doi.org/10.1111/j.1600-079X.2011.00931.x>

459 Wang, F., Tian, X., Zhang, L., Gao, C., He, C., Fu, Y., Ji, P., Li, Y., Li, N., Liu, G., 2014.
460 Beneficial effects of melatonin on in vitro bovine embryonic development are mediated by
461 melatonin receptor 1. *J. Pineal Res.* 56, 333–342. <https://doi.org/10.1111/jpi.12126>

462 Wang, S.J., Liu, W.J., Wang, L.K., Pang, X.S., Yang, L.G., 2017. The role of Melatonin receptor
463 MTNR1A in the action of Melatonin on bovine granulosa cells. *Mol. Reprod. Dev.* 84, 1140–
464 1154. <https://doi.org/10.1002/mrd.22877>

465 Weaver, D.R., Liu, C., Reppert, S.M., 1996. Nature’s Knockout: The Mel1b Receptor Is Not
466 Necessary for Reproductive and Circadian Responses to Melatonin in Siberian Hamsters. *Mol.*
467 *Endocrinol.* 10, 1478–1487.

468

469

470 **Table 1**

471 SNPs in the three breeds evaluated (Bovska, Istrian Pramenka and Sarda) and resulting amino acid changes
 472 in the *MTNR1A* gene according to the latest genome version Oar_rambouillet_v1.0 (GenBank assembly
 473 accession number: GCA_002742125.1)

SNP position	Nucleotide change	Amino acid change
g.17355611	C->A	none
g.17355458	G->A	None
g.17355452	C->T	None
g.17355358	C->T	Val→Ile
g.17355281	C->T	None
g.17355263	C->T	None
g.17355173	G->A	None
g.17355171	G->T	Ala→Asp

474

475

476

477 **Table 2**

478 Allele and genotype frequency of the *MTNR1A* gene SNPs in the three sheep breeds evaluated in
 479 the present study, according to the latest genome version Oar_rambouillet_v1.0 (GenBank assembly
 480 accession number: GCA_002742125.1)

481

Position	Allele frequency				Genotype frequency					
	g.17355452		g.17355458		g.17355452			g.17355458		
Breed	C	T	G	A	C/C	C/T	T/T	G/G	G/A	A/A
Bovška	0.67	0.33	0.60	0.40	0.50	0.35	0.15	0.43	0.34	0.23
Istrian Pramenka	0.75	0.25	0.64	0.36	0.63	0.25	0.12	0.52	0.25	0.23
Sarda	0.78	0.22	0.66	0.34	0.68	0.20	0.12	0.53	0.26	0.21

482

483

484

485 **Table 3**

486 Fertility rate, number of days from date of ram placement with ewes to lambing (DRPEL) and litter size for
 487 ewes of the different genotypes of the Bovška breed ($n = 100$); ^{a, b} within the row, values without a common
 488 superscript differ ($P < 0.05$).

489

SNP	g.17355452C>T			g.17355458G>A		
	Genotype	C/C	C/T	T/T	G/G	G/A
Fertility rate	92.0 ^a	88.0 ^a	73.0 ^b	87.0	83.0	85.0
DRPEL	172.3±11.6 ^a	174.4±11.9 ^a	185.9±11.6 ^b	173.4±13.1	176.4±13.2	180.5±13.5
Litter size	1.07±0.03	1.08±0.03	1.09±0.01	1.09±0.02	1.08±0.03	1.07±0.01

490

491

492 **Table 4**

493 Fertility rate, number of days from date of ram placement with ewes to lambing (DRPEL) and litter size for
 494 ewes of the different genotypes of the Sarda breed ($n = 100$); ^{a, b} within the row, values without a common
 495 superscript differ ($P < 0.05$).

496

SNP	g.17355452C>T			<i>P</i>	g.17355458G>A			<i>P</i>
	Genotype	C/C	C/T		T/T	G/G	G/A	
Fertility rate	80 ^a	81.0 ^a	66.0 ^b	*	76.0	74.0	78.0	ns
DRPEL	170.7±11.6 ^a	172.1±12.6 ^a	190.7±12.8 ^b	*	177.5±12.7	176.1±12.3	179.8±12.3	ns
Litter size	1.08±0.03	1.09±0.03	1.07±0.01	ns	1.08±0.02	1.09±0.03	1.04±0.01	ns

497

498

499 **Table 5**

500 Fertility rate, number of days from date of ram placement with ewes to lambing (DRPEL) and litter size for
 501 the different genotypes in the ewes of the Istrian Pramenka breed ($n = 100$); ^{a, b} within the row, values
 502 without a common superscript differ ($P < 0.05$).

503

SNP	g.17355452C>T			<i>P</i>	g.17355458G>A			<i>P</i>
	Genotype	C/C	C/T		T/T	G/G	G/A	

Fertility rate	82.0	79.0	84.0	ns	77.0 ^a	76.0 ^a	91.0 ^b	*
DRPEL	178.5±12.6	180.3±13.0	181.4±11.1	ns	186.2 ± 12.7 ^a	180.5± 11.6 ^a	172.6±11.8 ^b	*
Litter size	1.11±0.03	1.09 ±0.01	1.10 ±0.02	ns	1.12±0.04	1.09±0.01	1.07± 0.01	ns

504

505

Table 6

506

Percentage of lambing distribution each 10 days during the observation period in the Boska breed (*n*

507

= 100) based on the ewe genotype at position g.17355452. ^{a, b} within the column, values without a

508

common superscript differ (*P*<0.05).

	150-160	161-170	171-180	181-190	191-200	201-210
C/C	10.9 ^a	45.7 ^a	21.7	13.0 ^a	6.5	2.2 ^a
C/T	6.5 ^a	48.4 ^a	25.8	9.7 ^a	3.2	6.5 ^a
T/T	0.0 ^b	9.1 ^b	18.2	45.5 ^b	9.1	18.2 ^b

509

510

511

Table 7

512

Percentage of lambing distribution each 10 days during the observation period in the Sarda breed (*n*

513

= 100) based on the ewe genotype at position g.17355452. ^{a, b} within the column, values without a

514

common superscript differ (*P*<0.05).

	150-160	161-170	171-180	181-190	191-200	201-210
C/C	11.1 ^a	50.0 ^a	22.2 ^a	7.4 ^a	7.4 ^a	1.9 ^a
C/T	6.3 ^a	56.3 ^a	25.0 ^a	6.3 ^a	6.3 ^a	0.0 ^a
T/T	0.0 ^b	0.0 ^b	12.5 ^b	25.0 ^b	50.0 ^b	12.5 ^b

515

516

517

Table 8

518

Percentage of lambing distribution each 10 days during the observation period in the Istrian

519

Pramenka breed (*n* = 100) based on the ewe genotype at position g.17355458. ^{a, b} within the column,

520

values without a common superscript differ (*P*<0.05).

	150-160	161-170	171-180	181-190	191-200	201-210
C/C	10.0 ^a	15.0 ^a	22.5	22.5 ^a	20.0 ^a	10.0
C/T	10.5 ^a	15.8 ^a	15.8	42.1 ^a	5.3 ^a	10.5
T/T	19.0 ^b	38.1 ^b	23.8	4.8 ^b	4.8 ^a	9.5

521