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Expression of seasonality in Merinos d'Arles ewes of different genotypes at the *MT1* melatonin receptor gene

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Spontaneous ovulatory activity (SOA) in spring has been used to study the out-of-season breeding ability of Merinos d'Arles (MA) ewes. Within this breed, an association was found between more intense seasonality and genotype –/– at a MnlI restriction site (allele – for its absence v. + for its presence) in Exon II of the MT1 receptor gene. This study was designed to ascertain whether this association results in a direct effect of the MT1 genotype on the expression of seasonality in MA ewes. In the first year of the study, genotyping of 314 MA ewes at locus MnlI was carried out and resulted in frequencies of 43.0%, 44.9% and 12.1% for genotypes +/+, +/- and -/-, respectively. The SOA of these ewes was determined in early April of two consecutive years by assaying plasma progesterone concentrations in two blood samples taken 9 days apart. Groups of 30 ewes of each homozygous genotype (+/+ and -/-) were identified from this population and their SOA was followed by taking blood samples at regular intervals between January and mid-April of the second and third year of the study. In the second year, groups of ewes were managed together on rangelands, whereas in the third year each group was split into two subgroups given differential feed levels. The results clearly showed that genotype had no significant effect on SOA during the 2- to 3-month period preceding the introduction of rams for spring mating. In the second year of the study, in which the experimental procedure allowed a fair comparison of the fertility of ewes in spring mating, fertility was similar for both genotypes. The reciprocity of the association was not demonstrated and the MnlI polymorphic site could not be used as a genetic marker of selection for out-of-season breeding ability, at least not in the MA breed. The percentage of cycling ewes significantly decreased between January and April, and older ewes (5 or 6 years old depending on the year of the study) were more cyclic than younger ones (2 and 3 years old, respectively). The differential feeding level of ewes from early February did not significantly affect their SOA during the time period studied in the third year of the study.

Keywords: sheep, seasonality, ovulatory activity, melatonin receptor gene, feeding level

Implications

Seasonality of reproduction in sheep has a genetic component; however, the results of classical polygenic selection for the traits associated with spring breeding are limited. The use of polymorphic quantitative trait loci or associated genetic markers could improve the efficiency of selection. Various studies on different sheep breeds suggest that polymorphism in the *MT1* receptor gene may be a potential marker of selection. This study of Merinos ewes, tests the linkage between this polymorphism and the expression of seasonality. The results do not support the use of genotyping for *MT1* as a means of selection for aseasonality.

Introduction

Seasonality of reproduction remains an important constraint in temperate sheep breeds in which ovulatory activity generally is inhibited in spring. Measurements of spontaneous ovulatory activity (SOA) in early spring, before the introduction of males into the flock, has been used to study out-of-season breeding ability in several low seasonal sheep breeds such as Merinos d'Arles (MA; Hanocq *et al.*, 1999), Latxa (Beltran de Heredia *et al.*, 2002) and Chios (Avdi *et al.*, 2003). Very similar results with regard to SOA have been found in the above studies; this trait is repeatable and its heritability ranges from 0.12 to 0.22. In the MA breed, a strong association has been observed between breeding value for SOA in April and restriction polymorphism at position 605 in Exon II of the *MT1* melatonin

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receptor gene, corresponding to the presence (allele +) or absence (allele -) of a *MnlI* restriction site (Pelletier *et al.*, 2000). More intense seasonality was associated with genotype -/- . The discovery of this association extended previous results of Messer *et al.* (1997) and could point to a specific role of the *MT1* genotype in seasonal anoestrus; this study was designed to test this hypothesis. After genotyping MA ewes in a single flock in the first year of the study, groups of ewes from each homozygous genotype (+/+ and -/-) were identified and their SOA was assessed between the end of drying-off (January) and the beginning of spring mating (April) over two consecutive years. In the first year of this observation period (year two of the study), groups of ewes were managed together on rangelands (experiment 1). In the following year, they were kept in a sheepfold where half of the ewes of each genotype were fed at a higher level than the other half to also test the effect of feeding levels on SOA (experiment 2). This study provides results on genotype frequencies in MA, on SOA in early April for the genotyped ewes, and on the follow-up of SOA of +/+ ewes and -/- ewes between January and mid-April (before spring mating) under different experimental conditions.

Material and methods

Association study between genotype at the MnlI locus and SOA

Following genotyping of 314 MA ewes for the *MnlI* site early in 2003, their SOA was assessed in April of two consecutive years (2003 and 2004) by measuring peripheral progesterone levels twice in a 9-day period. These ewes from one flock belonging to an experimental farm, Le Merle (south-eastern France, latitude 43.5°N), were born in autumn lambing from 1998 to 2001, that is, aged 1 to 4 years at the beginning of 2003. The flock was established in the 1920s and at the time of the study included approximately 1100 pure MA ewes and 30 rams. Renewal was about 20% per year and the foreign breeding material introduced, consisting only of rams or semen, came from a regional breeding centre where male lambs from different sheep flocks were raised to provide breeding rams. These rams were the offspring of ewes qualified as 'ram dams' whose selection criteria were unrelated to seasonality with the exception that the male lambs had to be born in autumn to enter the breeding centre. Over the last 14 years, the percentage of ewes from identified sires has remained above 50%. Genotyping was performed on 314 DNA samples prepared from ewes using the procedure described in detail by Pelletier *et al.* (2000). The resulting genotypes were represented as +/+, +/- and -/- according to the presence (allele +) or absence (allele -) of the *MnlI* restriction site. In addition, the physiological status for SOA of the genotyped ewes (anovulatory or ovulatory) was determined in early April for 2 years (2003 and 2004). For this, we used the criteria established by Thimonier (2000), which hold that plasma progesterone concentrations in two blood samples taken 7 to 11 days apart are adequate for the detection of ovulatory activity at a given period in ewes with regular

ovarian cycles of 17 days on average and to discriminate the two physiological statuses. The most accurate results are obtained at an interval of 9 days between blood samples. Out of the 314 genotyped ewes, 312 were sampled twice in April 2003; however, only 289 were sampled in April 2004 because some had died or had been culled over the intervening year. Ewes with a progesterone level above the threshold of 1 ng/ml in at least one of the two consecutive blood samples were considered to be ovulatory or cycling. Progesterone concentrations were measured by radioimmunoassay using the technique described by Terqui and Thimonier (1974).

As a rule, all of the MA ewes involved in the study were mated for the first time at 18 months of age and were joined with rams between April and June in subsequent years. They were kept completely separate from males during the months between the annual spring mating.

Assessment of SOA over a long period in +/+ and -/- ewes

Experiment 1. In early 2004, two groups of 30 +/+ and 30 -/- ewes of similar age distribution (2, 3 and 5 years old) were identified from the population of genotyped ewes. The other sampling criteria for adults (3 and 5 years old) were fertility and the number of lambs suckled during the previous autumn lambing. The paternity of all of the ewes was not known; however, when possible, we tried to include ewes from different sires. From 15 January to 14 April, the SOA of ewes was assessed by measuring progesterone concentrations in blood samples taken 7 days apart. As the period of observation was long, the 7-day interval was chosen for logistical reasons, that is, to have a fixed day each week to take blood samples in the ewe groups. With this collection interval, the detection of ovulation is similar to that obtained with an interval of 9 days, especially when one can examine the progesterone levels in more than two consecutive blood samples to determine the status of SOA at a given period (Thimonier, 2000). The ewes were kept on separate rangelands to avoid repeated sorting of animals during the experiment, and their shearing was carried out over a single day at the end of March. At the end of blood sampling, the two groups of ewes were reunited on 15 April and three rams were introduced into this combined group for 1 month. They then joined the rest of the spring mating MA flock from mid-May to mid-June.

Experiment 2. In January of the following year (2005), 60 ewes from the population of genotyped ewes were divided into two groups of 30 ewes each; in each group, 15 ewes were +/+ and 15 ewes were -/-. These groups of ewes of similar age distribution (3, 4 and 6 years old) were balanced in terms of live weight (LW), body condition score (BCS), fertility and the number of lambs suckled during the previous autumn lambing. Several ewes involved in this experiment (15 +/+ and 7 -/-) were not the same as in the previous year due to the sampling procedure and the replacement of dead or culled animals. The two groups of ewes were

transferred to a sheepfold with natural light and, after a 15-day period of acclimatization together, placed in two adjacent pens with different feeding levels from 3 February to 14 April. One group was fed at a low level (L): 70% of maintenance energy requirements, with a diet composed of straw and pelleted alfalfa. The other one was fed at a high level (H): about 170% of maintenance energy through the addition of pelleted alfalfa and pelleted beet pulp. Daily rations for the H and L diets were formulated to increase the BCS by about 1 point in 60 days (H diet) or to maintain the LW and BCS at the level at which they were at the end of drying-off (L diet). The H diet was reduced to maintenance requirements beginning in late March to prevent excessive fattening at mating. Daily rations were established on the basis of standard feeding values for the ingredients (Jarrige, 1988). They were distributed, between midday and 1400 h, in racks allowing simultaneous access to all ewes. In each pen, the ewes had free access to water, salt blocks and a mineral–vitamin pre-mix.

Immediately before the different diets were introduced, SOA was determined by the progesterone levels in the two blood samples taken 9 days apart, using the rules of interpretation already defined for the two samples. SOA was then assessed through blood samples collected at 9- to 10-day intervals from 25 February to 14 April. Ewes were weighed at this interval and their condition scored at a double interval on a 5-point graded scale (Russel *et al.*, 1969) with 0.25 subdivisions. After an additional 12-day period of re-feeding (or feeding) at the maintenance requirement level in the sheepfold, the ewes were exposed to rams on pastures from 27 April to mid-June. For this experiment, shearing was delayed until the beginning of May, that is, during the mating period.

Periods of SOA

For the long-term experiments, determination of status (cycled or non-cycled) of a ewe at each blood sampling point of time was based on progesterone level at the time of sampling and additional information obtained from other progesterone measurements on the same ewe during the sampling period. Unlike the analysis of two consecutive blood samplings, interpretation of progesterone levels in sequential blood samples required taking into account the timing of observations. A measurement below the threshold of 1 ng/ml of progesterone (coded 0, non-cyclic or follicular phase) that followed a measurement above the threshold (coded 1, luteal phase) was therefore included systematically in the definition of periods of SOA. With the 7-day interval retained in experiment 1, the infrequent occurrence of two consecutive measurements below the threshold that both followed and preceded observations above the threshold also were included in periods of continuous activity. For example, in experiment 1, the vector of n observations for a ewe [1 0 1 1 0 0 1 0 0 0 1 1 0] was interpreted as [1 1 1 1 1 1 1 0 0 1 1 1]. In experiment 2, in which blood samples were taken 9 or 10 days apart, the vector [0 1 0 1 0 0] was interpreted as [1 1 1 1 0]. The first observation was used

to determine the SOA of ewes at the following date and analysis of modified data was carried out on $n-1$ variables.

Statistical procedures

The repeatability of SOA for the 2 years of measurements in April on all genotyped ewes was estimated by the coefficient ϕ , which is a measure of the degree of association between all-or-nothing traits as two binary variables (Yule, 1912).

The effects of genotype and feeding level on SOA variables (0/1 modified data) were investigated by a logistic regression of longitudinal data sets from experiments 1 and 2 using a marginal model, generalized estimating equations (GEE; Diggle *et al.*, 2002). GEE can be regarded as an extension of GLM for the analysis of correlated data. In the general framework of longitudinal analysis, GEE takes into account the dependency of repeated observations within subjects by specifying a 'working correlation structure'. In this case, the correction for within-ewe correlation was carried out by assuming an auto-regressive correlation structure for the repeated-in-time measurements. This option was retained in the model to obtain efficient estimates of the standard error (robust s.e.) of coefficients and hence efficient inference tests (Wald test), the Wald statistic being used for assessing significance of coefficients (Hosmer and Lemeshow, 2000). Under the null hypothesis, this statistic, also called z , will follow a standard normal distribution.

In these analyses, the date of observation was introduced as a linear continuous variable (coded from 0 for the first date to n for the last date) and genotype, age and diet as categorical variables. Interactions that are important for interpreting the effect of genotype, for example, genotype by date and genotype by diet, were introduced in a first step and were excluded from the final model if they had no significant effect (Wald test with a P -value >0.05). The genotype $-/-$, the first class of age (2 or 3 years old according to the studied data set) and the L diet were used as reference categories. Analyses were performed using R software: GEE function, binomial family, logit link, AR-M ($M=1$) correlation structure (R Development Core Team, 2007).

Other comparisons between genotypes (and feeding levels) on SOA percentage were carried out using χ^2 test and odds ratio (OR, and CI for its confidence interval). One-tailed t -test was used for the comparisons between feeding levels on ewe LW and ewe BCS.

Results

Genotypic frequencies and SOA in April

Frequencies of genotypes $+/+$, $+/-$ and $-/-$ in the population of 314 genotyped ewes were 43.0% ($n=135$), 44.9% ($n=141$) and 12.1% ($n=38$), respectively; this agrees with the Hardy–Weinberg equilibrium (p^2 , $2pq$, q^2 with $p=0.65$ and $q=1-p$) and suggests that the *MnlI* locus is not under selection in the MA population under investigation. With regard to this large sample of MA ewes (Table 1), there was no significant difference between genotypes in the percentage of ovulatory ewes in early April in

Table 1 Percentage of ovulatory ewes in early April in the MA genotyped for the MnlI site, according to genotype and year of measurement

Genotype	2003		2004	
	Number of ewes ¹	Ovulatory ewes (%)	Number of ewes ¹	Ovulatory ewes (%)
+/+	133	30.8	129	20.2
+/-	141	28.4	129	15.5
-/-	38	26.3	31	25.8

MA = Merinos d'Arles.

¹Number of ewes sampled on 2 and 11 April 2003 (n = 312); 5 and 14 April 2004 (n = 289).

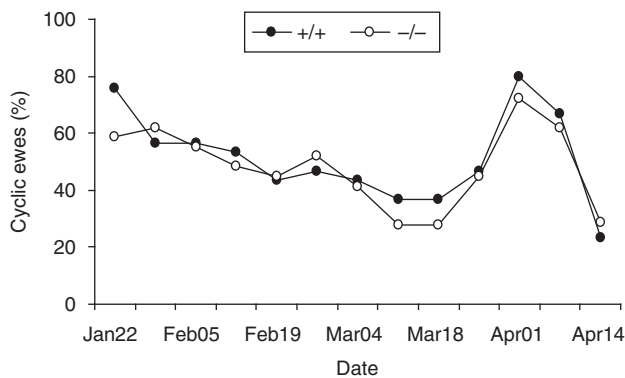


Figure 1 Changes in the percentage of cyclic ewes between January and mid-April for +/+ ewes (n = 30) and -/- ewes (n = 29 to 28) in experiment 1.

either year of measurement (χ^2 test: $P = 0.83$ and $P = 0.35$ in 2003 and 2004, respectively). Only 30 ewes were ovulating in early April in both of the 2 years, and none was of genotype -/- (17 +/+, 13 +/-). The repeatability of SOA for the two consecutive years was 0.28.

Time changes in SOA of homozygous ewes

Experiment 1. Two ewes of genotype -/- were eliminated during the experiment: one at the beginning, the other died before the last blood sampling. The representation of changes in the percentage of cyclic ewes from January to mid-April (Figure 1) displays a similar change in SOA over time in both groups of ewes (30 +/+ and 29 to 28 -/-). This change was marked by the occurrence of a temporary rise in ovulatory activity triggered prior to shearing on 29 March. In early April, which is the usual period of SOA measurement before rams are introduced, no difference was observed between genotypes. The overall percentage of cyclic ewes on 14 April was 25.9%.

Patterns of SOA for individual ewes (Figure 2) show that many ewes of both genotypes exhibited discontinuities in their ovulatory activity. In several of these ewes, no ovulatory activity was detected for periods of 2 to 3 weeks, suggesting the failure of a single cycle. The proportion of ewes continuously cycling at least until the apparent end of their SOA (11/30 and 6/29 for +/+ and -/- ewes, respectively) did not differ significantly between genotypes (OR = 2.22 with a 95% CI of 0.69, 7.12).

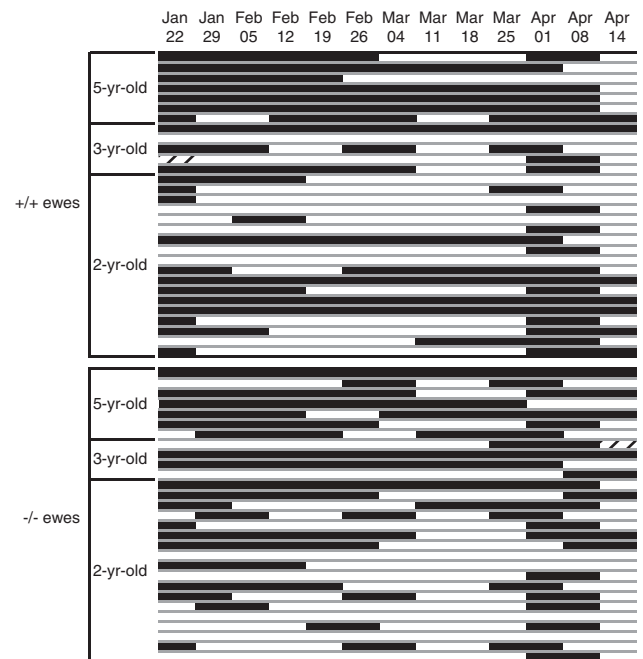


Figure 2 Periods of spontaneous ovulatory activity for individual ewes in experiment 1 sorted by age within genotype. Hatched areas indicate the absence of observation, open areas indicate periods of anovulatory activity.

The results of the GEE analysis of this data set showed in the first place that the interaction between genotype and date was not significant. Therefore, the final fitted model was reduced to the main effects of genotype, age and date variables (Table 2). The effect of genotype on SOA was not significant ($P = 0.68$). For the age effect, the 5-year-old ewes were much more likely to be cyclic than the 2-year-olds ($P < 0.001$), while no significant difference was observed between 3- and 2-year-old ewes ($P = 0.38$). The effect of date was highly significant ($P < 0.001$) with a negative estimate of the coefficient; this linear variable indicates an overall decrease of SOA among the ewes from January to mid-April.

Experiment 2. Changes in ewe LW and ewe BCS during this experiment are shown in Table 3 for the groups of ewes fed at high and low levels (15 or 14 +/+ and 15 -/- ewes per group; one +/+ ewe was eliminated early after the

constitution of the ewe groups). Average values for LW and BCS, which were equal between groups when they were constituted, differed significantly after 1 month of differential diets. From early February until the end of the experiment, the H diet induced an increase of 8.2 kg for LW and 0.8 point for BCS, while these traits remained approximately constant until the end of experiment for the ewes on the L diet.

For this controlled group feeding experiment, results of the GEE analysis of the SOA data set from 7 March to mid-April, when the ewes had been fed at different levels since early February, indicated similar trends as in experiment 1. The interaction between genotype and date as well as between genotype and diet were not significant; they consequently were excluded from the final model whose results are given in Table 4. These results confirmed the absence of effect of genotype on SOA ($P = 0.35$); the presence of effect of age, with the 6-year-old ewes significantly more cyclic than the 3-year-olds ($P < 0.01$); and the significant decrease in SOA among ewes during the period of study ($P < 0.001$). In addition, the result for the diet variable ($P = 0.32$) showed that the feeding level had no significant effect on the cyclicity of ewes within this period. The corresponding changes in SOA according to genotype (irrespective of diet) or to diet

(irrespective of genotype) are presented in Figures 3 and 4, respectively. At the end of this experiment on 14 April, the average percentage of cyclic ewes was 16.9%.

Fertility in spring mating

In experiment 1, in which ewes were joined with rams the day after the last blood sampling, there was no significant difference between genotypes in fertility in spring mating: 90% of $+/+$ ewes and 89.3% of $-/-$ ewes later lambed the following autumn. There also was no difference in prolificacy (1.32 and 1.28, respectively). During the first 15 days after joining, on the basis of autumn lambing, there were a few more $+/+$ ewes fertilized or conceiving than $-/-$ ewes (6 and 3 ewes, respectively), but this difference was not significant. Furthermore, the cumulative distribution of lambing dates did not differ significantly between genotypes ($P > 0.05$, non-parametric Kolmogorov–Smirnov two-sample test). In experiment 2, the comparison between genotypes of the fertility of ewes after the spring mating was not an objective because of the 12-day period of re-feeding of underfed ewes

Table 2 Estimated coefficients, s.e., Wald statistics and P-values for the GEE model fitted to SOA data set from experiment 1, including genotype and age categorical variables and date continuous variable

Variable	Category ¹	Coefficient	s.e. ²	Wald statistic (z)	P > z
Genotype	$+/+$	0.133	0.327	0.41	0.684
Age	3 years old	0.472	0.537	0.88	0.380
	5 years old	1.264	0.335	3.77	<0.001
Date		-0.096	0.024	-3.92	<0.001

GEE = generalized estimating equations; SOA = spontaneous ovulatory activity.
¹Reference categories for categorical variables are not included.
²Robust s.e. of the coefficients provided by the GEE method with the option of an auto-regressive structure of correlation within each ewe.
 z = coefficient/s.e., with P-value calculated from a normal table.

Table 4 Estimated coefficients, s.e., Wald statistics and P-values for the GEE model fitted to SOA data set from experiment 2 (7 March to 14 April), including genotype, age and diet categorical variables and date continuous variable

Variable	Category ¹	Coefficient	s.e. ²	Wald statistic (z)	P > z
Genotype	$+/+$	-0.478	0.506	-0.94	0.345
Age	4 years old	0.977	0.686	1.43	0.154
	6 years old	1.711	0.635	2.70	0.007
Diet	H level	0.522	0.522	1.00	0.318
Date		-0.368	0.091	-4.04	<0.001

GEE = generalized estimating equations; SOA = spontaneous ovulatory activity; H = high level.
¹Reference categories for categorical variables are not included.
²Robust s.e. of the coefficients provided by the GEE method with the option of an auto-regressive structure of correlation within each ewe.
 z = Coefficient/s.e. with P-value calculated from a normal table.

Table 3 Evolution of LW and BCS in the groups of ewes fed at H and L levels (170% and 70% of maintenance energy requirements, respectively) from early February: mean value (s.d.)

Feeding level	Variable	Date							
		19 January ¹	03 February ²	25 February	07 March	16 March	25 March	04 April	14 April
H	Number of ewes	30	30	30	30	30	30	30	30
	LW (kg)	46.5 (4.4)	45.5 (4.9)	50.3 (5.0)	51.6 (5.2)	53.1 (5.0)	53.7 (5.0)	52.7 (5.0)	53.7 (5.4)
	BCS	1.84 (0.19)	1.93 (0.23)		2.34 (0.37)		2.63 (0.38)		2.73 (0.40)
L	Number of ewes	30	29	29	29	29	29	29	29
	LW (kg)	46.5 (4.2)	44.7 (3.9)	43.7 (4.0)	43.5 (4.1)	43.6 (4.1)	42.8 (4.1)	42.5 (4.4)	42.9 (4.4)
	BCS	1.84 (0.14)	1.80 (0.17)		1.78 (0.19)		1.80 (0.17)		1.82 (0.19)
<i>t</i> -test ³	For LW	ns	ns	***	***	***	***	***	***
	For BCS	ns	ns		***		***		***

LW = live weight; BCS = body condition score; H = high level; L = low level.
¹Constitution of the groups of ewes.
²Start of differential feeding levels.
³Unilateral *t*-test by the hypothesis that the mean value for H diet was greater than the mean value for L diet.

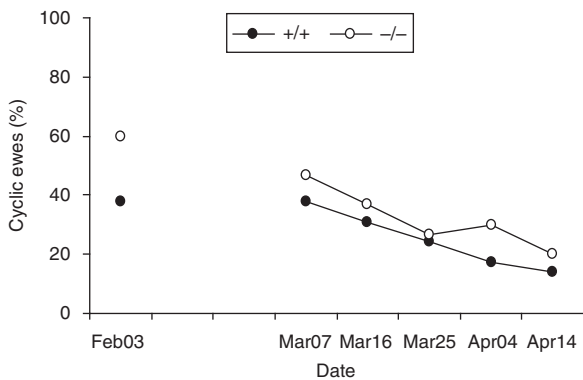


Figure 3 Changes in the percentage of cyclic ewes between February and mid-April for +/+ ewes ($n = 29$) and -/- ewes ($n = 30$) in experiment 2 (irrespective of diet).

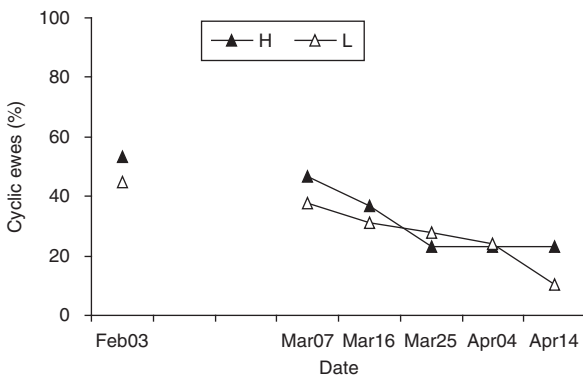


Figure 4 Changes in the percentage of cyclic ewes between February and mid-April for H level ($n = 30$) and L level ($n = 29$) ewes in experiment 2 (irrespective of genotype). Ewes were fed at different levels from 3 February.

before mating and the delayed shearing time, which had been postponed until after mating began.

Discussion

Pelletier *et al.* (2000) have shown that a mutation at position 612 in Exon II of the ovine *MT1* gene leads to the absence of a cleavage site for the *MnlI* enzyme at position 605 (allele -). They showed that this mutation was always found to be associated with a single allelic isoform among the different isoforms described for this exon. This haplotype is characterised by the specific and constant presence of four mutations, including the one (612) responsible for *MnlI* polymorphism, two other silent mutations at positions 453 and 891, and a mutation at position 706 that results in one base change in the amino acid sequence of the predicted *MT1* receptor protein (substitution of a valine by an isoleucine). Several independent results point out a potential functional role of this locus on the control of seasonality:

1. Within breeds, Pelletier *et al.* (2000) found a highly significant difference in the frequency of homozygotes -/- between two groups of MA ewes screened for their SOA in

spring. Ewes homozygous for the absence of the *MnlI* site were not observed in the group of ewes cycling in April over three consecutive years (1995 to 1997). This study conducted on the same flock showed that -/- ewes also were not found in the group of 30 ewes that cycled in April over two consecutive years (2003 and 2004). Furthermore, a significant effect of *MnlI* polymorphism on fertility in spring mating has been reported (Notter *et al.*, 2003) for adult ewes from a composite line (50% Dorset, 25% Rambouillet, 25% Finnsheep). Adult ewes with the genotype -/- had lower spring fertility. Significant differences between +/+ or +/-, and -/- genotypes in lambing frequency also were reported among Dorset ewes managed by an accelerated lambing system (Mateescu *et al.*, 2009).

2. Comparisons between breeds also support the functional role of the *MT1* genotype. Substantial changes in allelic frequencies at the *MnlI* site with the degree of seasonality of breeds have been described by several different authors (Messer *et al.* 1997; Pelletier *et al.* 2000; Notter and Cockett, 2005). For example, ewes of seasonal breeds had relatively higher frequencies of genotype -/- than ewes of low seasonal breeds. Thus, genotype -/- was not found in a large sample of ewes from the non-seasonal small-tailed Han breed in China (Chu *et al.*, 2003) and the following gradient of the percentage of -/- ewes was encountered in samples of several breeds: 9% in Blackbelly; 7% in Barbarine; 10% in MA; 31% in Ile-de-France; 35% in Suffolk; 100% in wild Mouflon (Trecherel, 2007).
3. Various physiological studies reinforce the assumption of a close relationship between the expression of seasonality and polymorphism of the *MT1* receptor gene. In MA -/- ewes, a higher density of melatonin receptors has been found in the pars tuberalis (Pelletier *et al.*, 2000) as well as in four other brain areas (Trecherel, 2007). One of these areas belongs to the pre-mammillary hypothalamus, which is an important site of melatonin action regulating the reproductive function in sheep (Malpaux *et al.*, 2001).
4. Moreover, the contrast between the unique allelic isoform of Exon II associated with higher seasonality in several breeds and large variability of other haplotypes suggests a common origin of this particular haplotype, which had not been lost through selection and dissemination due to the selective advantage it confers.

Despite these findings, the relationship between the studied part of Exon II of the *MT1* gene and the phenotype of seasonality remains unclear. First, this study indicates that in MA, no difference was observed between ewes of genotype +/+ and -/- in the rate of SOA in early spring or on fertility in spring mating. Second, various attempts failed to find a difference by various criteria (beginning, end or length of the breeding season) within the genotyped progeny (+/+ and -/-) of heterozygous sires in Ile-de-France (Hernandez *et al.*, 2005) and Latxa breeds (Ugarte *et al.*, 2007).

How may these two contradictory sets of findings be reconciled? It is unlikely that the relationship between the

expression of seasonality and polymorphism of the *MT1* gene found in MA (Pelletier *et al.*, 2000), OOS (Notter *et al.*, 2003) and Dorset (Mateescu *et al.*, 2009) ewes is casual. On the other hand, the mutated haplotype has been observed in enough animals of different breeds to exclude a sampling artefact. One hypothesis explaining this discrepancy would be the existence of another non-conservative mutation on an independent gene. Selection for spring ovulation would increase the frequency of favourable alleles at both sites, including the *Mnl* site. This would explain the results observed by Pelletier *et al.* (2000). In contrast, the screening of animals only for the presence or absence of the *Mnl* site would not have any influence on the proportions of alleles at the other site, and this therefore would be insufficient to induce a change in the expression of seasonality. Support for this hypothesis would come when such a non-conservative mutation has been identified; however, it has not yet been found.

With regard to environmental effects, it is important to note that the beginning of the unexpected rise of SOA in the first experiment (experiment 1) was observed on 25 March, that is, 4 days before shearing. No other change in management, handling or treatment may be associated with the increase of SOA on this date. The amplitude of the peak of SOA was probably related to shearing; however, its start before shearing might reflect spontaneous changes in the Mediterranean environment of grazing ewes with an increase in pasture availability and forage intake on rangelands after the colder months (January and February). Such events (shearing, re-growth on rangelands) could partially explain the inter-annual variations of the cyclicity rate observed in early April for MA ewes from first generations of selection lines for SOA at this time (Teyssier *et al.*, 2002). The introduction of controlled feeding levels in experiment 2 was also justified by different studies that have shown relationships between ovulatory activity and LW and BCS of ewes from Merino flocks (Hanocq *et al.*, 1999; Kleemann and Walker, 2005). In this study, the occurrence of a small divergence between diets at the end of the study period might suggest a delayed effect of feeding level on SOA (Teyssier *et al.*, 2006), but such a hypothesis was clearly challenged by the final analysis of results using a model suited to this type of data. The substantial differences in LW and BCS induced by differential feeding levels from February thus had no significant effect on SOA at the beginning of April. This agrees with results on Barbarine ewes (Khaldi, 1984), which have shown that significant variations of ewe LW between drying-off and the spring introduction of rams had no significant effect on the percentage of cyclic ewes before the beginning of mating, whereas initial differences in LW established during a pre-experimental period before drying-off significantly affected this percentage.

In conclusion, results of this study show that genotype at locus *Mnl* of the *MT1* receptor gene cannot be used alone as a genetic selection marker for spring (out-of-season) breeding in sheep, at least not in the MA breed. Further studies are required to define the role of this genomic region in the expression of seasonality. In addition, while the results

illustrate the responsiveness of SOA to events such as shearing and spontaneous variations in the Mediterranean environment of grazing ewes, they do not show a significant effect of differential feeding of sheepfold ewes after drying-off on their SOA before spring mating.

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