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Original article

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Abstract – The gene encoding the MT1 melatonin receptor in sheep has a restriction fragment length polymorphism (RFLP) site to the MnlI enzyme whose incidence is associated to the expression of seasonality in several breeds. The aim of this study was to examine the relationship between this genetic marker and the physiological effects of MT1 receptor gene polymorphism on several seasonal functions in Île-de-France ewes. The study was performed using 12 pairs of half-sib adult Île-de-France ewes. Within each pair, ewes were selected on the basis of their genotype at the MnlI RFLP site: group +/+ and –/– (presence and absence of MnlI restriction site, respectively). No difference in the dates of the beginning, the end or the length of the breeding season was observed between groups during the two-year study. The seasonal changes in prolactin secretion were not different between groups. Similarly, wool growth rate and primary follicle activity, measured for one year, varied with the time of the year in the same way in the two groups. Our study therefore failed to show any relationship between MT1 polymorphism and reproductive seasonality in Île-de-France ewes. This suggests that the influence of this polymorphism on the regulation of seasonal function is dependent upon the breed and/or environmental conditions. The MT1 polymorphism can explain only a small part of the genetic variability of seasonal functions and the implication of other genes must be investigated.

polymorphism / MT1 receptor gene / reproduction / seasonal functions / genetic control / Île-de-France ewes

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1. INTRODUCTION

Many mammals display profound physiological and behavioural adaptations to cope with seasonal changes in the environment [1]. Photoperiod plays a key role in regulating these annual physiological functions such as reproduction [2], wool growth [3], hair growth cycles [4] and appetite and voluntary food intake [5]. The annual cycle in reproduction results from changes in gonadotropin secretion while that of wool growth appears to depend upon prolactin secretion [6, 7]. In mammals, day length is transduced by the pineal gland by means of a 24-h melatonin secretory rhythm. The concentrations of this hormone are low in the blood and the cerebro-spinal fluid (CSF) during the day and conversely elevated at night. The duration of elevated melatonin level is directly related to the length of the night and this feature of the rhythm mediates the effect of photoperiod on seasonal functions [8].

Two high affinity melatonin receptor subtypes have been cloned and characterised in mammals: MT1 and MT2 (previously known as Mel 1a and Mel 1b, respectively) [9]. The MT1 subtype is thought to mediate the seasonal responses to melatonin [9, 10].

Exon II of the gene encoding the MT1 receptor in sheep has two restriction fragment length polymorphism (RFLP) sites, one for MnlI and the second for RsaI enzymes [11]. The MnlI RFLP site is characterised by a mutation leading to the absence (–) of the specific MnlI cleavage site at position 605 of the coding sequence, which leads to a characteristic pattern of digestion by this enzyme. Interestingly, the incidence of the MnlI RFLP was found to be higher in the Suffolk, a seasonal breed, than in the less seasonal white-faced crossbred ewes [11]. In addition, an association between genotype –/– (i.e. is animals carrying the mutation on both chromosomes) and seasonal anovulatory activity has been described [12]. Also, fertility for autumn lambing, that is fertilisation in the spring, is reduced in animals genotyped –/– in Virgine Tech out-of season mating (OOS) line (a crossbreed line of 50% Dorset, 25% Rambouillet and 25% Finnsheep) [13]. Altogether these data collected from isolated physiological events (spring spontaneous ovulatory activity and fertility after mating in the spring) suggest that a genotypic effect associated with the MnlI RFLP site could account for the variability observed in the expression of seasonality both between and within breeds. The mutation leading to the suppression of the MnlI site is silent and cannot explain the associated phenotype differences; consequently, it has to be viewed as a genetic marker for a quantitative trait locus (QTL) responsible for seasonal variability. The results cited above are therefore the consequence of another polymorphism in the same gene or at a close locus with strong linkage disequilibrium between the two loci. Interestingly, the silent mutation linked to the RFPL site is always found associated with a non conservative mutation at position 706 which could be a causal mutation responsible for variability in seasonal reproduction [12].

The polymorphism of the MT1 receptor gene could also be relevant to the genetic control of other seasonal functions, particularly those controlled by seasonal changes in prolactin secretion. Indeed, the physiological role of the MT1 receptor in mediating effects of melatonin on reproduction has not been clearly established. Binding sites and transcripts for MT1 receptors are detected in the pre-mammillary hypothalamus, the site of melatonin action to control reproduction in sheep [14], but whether MT1 mediates the effects of melatonin on reproductive activity has yet to be determined. In contrast, the MT1 receptor expressed in the pars tuberalis (PT) of the pituitary, has been shown to mediate the effect of melatonin on seasonal changes in prolactin secretion [15–17]. In addition, in the PT, the number of binding sites has been shown to be significantly higher in –/– ewes when compared to +/+ ewes [12] using the radioligand 2-[^{125}I]-iodomelatonin. These data

reinforce the hypothesis that MT1 polymorphism could be linked to seasonal regulation of prolactin secretion.

The present study was therefore designed to examine the association between MT1 receptor gene polymorphism and seasonal functions as well as annual hormone secretion for two years. Specifically, our aim was to compare reproductive activity, wool growth and wool follicle activity, melatonin and prolactin secretions in two groups of Île-de-France (IF) ewes selected on the basis of their genotype at the MnlI RFLP site. The choice of the IF breed was motivated by two decisive factors, firstly, seasonal reproductive activity has been well characterised in this breed [18] and secondly, the management of the internal flock allowed access to genealogical data for these ewes [19].

2. MATERIAL AND METHODS

The experimental procedure reported in this study was carried out in accordance with Authorisation 37801 for Animal Experimentation and Surgery from the French Ministry of Agriculture, according to the European Community Council Directive 86/609/EEC.

2.1. Animals and management

The study was performed on twenty-four sexually mature Île-de-France ewes (about 5 years old at the start of this study). Ewes were daughters of 12 heterozygous sires for the MT1 receptor gene (+/–); for each sire, one +/+ and one –/– daughter were selected. Genotypes were determined according to the MnlI enzyme restriction pattern [12]. The ewes were therefore divided into two groups of half-sibs according to their genotype: 12 ewes genotyped +/+ and 12 ewes genotyped –/–. However, one ewe of the –/– group that was misgenotyped at the first instance, was in fact heterozygous +/– and was consequently discarded from the study. In addition, two ewes, one in each group, died during the experiment and were excluded from the analysis.

The experiment was carried out during two consecutive years (from 20 July 2000 to 2 August 2002). The two groups of ewes (+/+ and –/–) were placed in two light-controlled rooms in INRA of Nouzilly, France (47º N). Lighting was given by fluorescent bulbs providing approximately 300 lux at the level of the animal's eyes during the day, and total darkness at night. Both rooms were placed on the same photoperiodic schedule mimicking the natural changes at this latitude (from 8.0 to 16.0 h light per 24 h at the winter and summer solstice, respectively). The temperature was not controlled.

During the first year (from June 2000 to May 2001) ewes were fed daily with hay, barley and wheat; during the second year (from June 2001 to July 2002), pellets were used to facilitate the management. Both diets provided the same energy and protein amount. Ewes had free access to water and mineral licks. Every four weeks (wk), the ewes were weighed. The height at withers was also measured in order to calculate a body mass index (BMI) [20].

2.2. Blood sampling

In order to analyse the seasonal changes both in ovarian activity (by measuring progesterone in plasma) and in prolactin secretion, blood samples were collected, throughout the experiment, twice weekly from the jugular vein and always at the same time of day (9:00 h). The nycthemeral profiles of melatonin and prolactin secretions were assessed in January, March, July and October 2001. Blood samples were collected every hour for 25 h by venepuncture of the left jugular vein. At night, blood samples were collected under a dim-red light (< 1 lux at 20 cm) with care taken to avoid any direct illumination of the animal's eyes. Plasma was obtained and stored at –20 °C.

2.3. Wool follicle activity and wool growth sampling

Biopsies of the skin were carried out monthly (from April 2001 to April 2002).

Biopsies were taken from the right mid-side region, following subcutaneous injection of 0.5 mL of local anesthesia (Lidocaine 1.0%). Skin samples were treated according to the classical histological methods and embedded in paraffin wax. Serial transverse sections of 8 µm were mounted on microscope slides and stained using the ROAN trichome method [21]. The biopsies obtained were only analysed at two month intervals from 7 ewes of each group, in order to calculate the percentage of primary wool follicles in the growing stage (anagen) [4]. On the opposite mid-side to the skin snip biopsy, a wool patch (approximately 10×10 cm) was established in order to assess fleece growth rate. Wool was clipped from the patch with Oster clippers every month from July 2001 to July 2002 and was weighed.

2.4. Hormone assay

Plasma progesterone was assayed by the radioimmunoassay method described by Terqui and Thimonier [22]. When progesterone concentration was lower than 0.75 ng·mL^{-1} of plasma, the female was considered to be in the follicular phase of the cycle or in anovulation.

Plasma prolactin concentration was determined in duplicate using the radioimmunoassay method of Kann (1971) [23]. The sensitivity of the assay was 2.5 pg·mL^{-1} and the intra assay coefficient of variation was 9.2%. Melatonin concentrations were determined as previously described [24] using an antibody first raised by Tillet and colleagues [25]. The sensitivity of the assay was 4.0 pg·mL^{-1}. The mean intra- assay coefficient of variation was 11.3%.

2.5. Data analysis

The onset of the breeding season corresponded to the first ovulation of the season determined by the date of the first occurrence of a positive progesterone sample. The end of the breeding season corresponded to the last ovulation of the season determined by the date of the last occurrence of a pos-

itive progesterone sample. Mean duration of ovulatory activity is the number of days between first and last ovulation in the same breeding season and corresponded to the length of the breeding season (for the 2 years and the 2 groups). All variables were analysed by analysis of variance (ANOVA) using the GLM procedure of SAS (Inst. Inc. Cary, NC) and adapted from Littell et al. [26]. The considered factors were: genotype, ewes nested under genotype, season (8 seasons in the study), interaction between genotype and season and interaction between season and ewes nested under genotype. These factors were taken into consideration according to their significance for the studied variable. When the main factor was significant (Fisher test $P < 0.05$) the comparison between the difference of least squares (LS) means was done using the same error term as in the estimate of the global effect in the variance analysis.

The number of days from the summer solstice to the date of the onset of ovarian activity and the number of days from the winter solstice to the date of the offset of ovarian activity were used (after log transformation) to analyse the beginning and the end of ovarian activity. The number of days between the onset and offset of ovarian activity and number of ovarian cycles during the year were used to analyse the length of the breeding season. In order to take into account the two years of experimentation, these data were analysed with a repeated measure model in which the interaction between ewe and group was used as the error term to test the effect of genotype.

BW and BW/h^2 (where h = height at withers in cm) were analysed. It was previously verified that h was not different between the 2 genotypes, 79 ± 3 and 76 ± 5 cm for genotype +/+ and –/– respectively, $P = 0.12$). The data collected in the same season (winter, spring, summer and fall) were considered as a repetition of the same measure and were analysed with an ANOVA model taking into account the two levels of
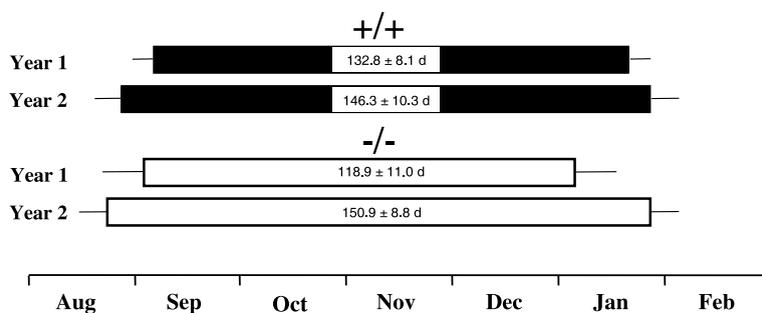
Figure 1. Mean (± SEM) dates of onset and offset of the breeding season during a two-year study in Île-de-France ewes characterised by the absence (–/–; open bars) or the presence (+/+; black bars) of an MnlI cleavage site at position 605 of the gene encoding the MT1 receptor on both chromosomes. Occurrence of ovulation was assessed from measurement of progesterone in blood samples obtained twice weekly. Figures within each bar indicate the mean number of days (± SEM) corresponding to the length of the breeding season.

repetition: year and season. The error term was chosen according to Danielie [27].

To analyse the changes in prolactin secretion measured in two weekly samples, the data collected in the same season (winter, spring, summer and fall) were considered as a repetition of the same measure and were analysed (after log transformation) as annual BW variation.

For the percentages of anagen, the classical arcsin transformation was used to normalise the percentage and the same ANOVA model was used as for annual prolactin variations.

For melatonin and prolactin 24-h profiles, log-transformed concentrations were analysed and those of the blood samples collected during the light or the dark phase were considered as the measure of the same phenomenon so the main factors of the variance analysis model were the light or dark phase, seasons of the blood sampling, genotypes and ewes nested under genotypes. The appropriate interactions were chosen as the error term. The duration of melatonin nocturnal secretion was calculated using the criteria previously described [28]. Data are presented as mean ± SEM. Differences were considered to be significant at $P < 0.05$.

3. RESULTS

3.1. Ovarian activity

Changes in ovarian activity throughout the study are summarised in Figure 1. The mean date of the onset of the breeding season (first elevated progesterone concentration) was not different between genotypes ($P = 0.52$), or between year ($P = 0.26$).

The date of offset of the breeding season (last elevated progesterone concentration) was not different between genotypes ($P = 0.37$) nor was the interaction between genotype and year ($P = 0.46$). The length of breeding season (Fig. 1) was not different between genotypes ($P = 0.88$), nor between years ($P = 0.12$). The interaction between genotype and year was not significant ($P = 0.60$).

The number of ovarian cycles did not vary between genotypes ($P = 0.48$), nor between years ($P = 0.21$). No interaction was detected between genotype and year. In group –/–, 7.3 ± 0.6 and 9.0 ± 0.5 cycles were observed in year 1 and 2, respectively while, in group +/+, 8.5 ± 0.5 and 9.0 ± 0.6 were detected at the same times (interaction group × year, $P = 0.48$).
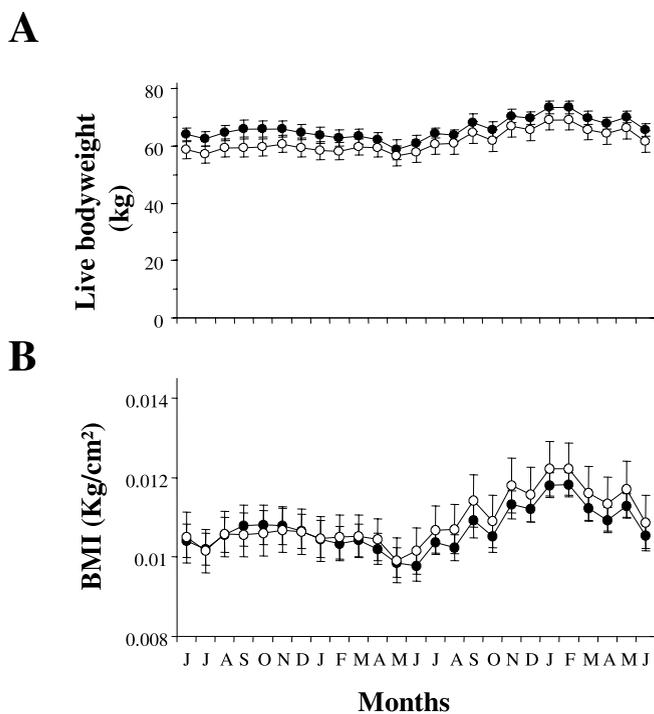
A

B

Months

Figure 2. Mean (± SEM) body weight (BW, **A**) and body mass index (BMI, **B**) during a two-year study (July 2000 to June 2002) in Île-de-France ewes characterised by the absence (–/–, open circles) or the presence (+/+, closed circles) of the MnlI cleavage site at position 605 of the gene encoding the MT1 receptor on both chromosomes. Body weight was measured once monthly and body mass index was the ratio between body weight and height at wither.

3.2. BW and BMI

BW and BMI were not different between groups (Fig. 2; BW: 65.3 ± 9.1 and 61.5 ± 11.6 kg for groups +/+ and –/–, respectively; BMI: 10.7 ± 1 $g \cdot cm^{-2}$ and 10.9 ± 2 $g \cdot cm^{-2}$ for groups +/+ and –/–, respectively). Both these parameters varied over time ($P < 0.001$) with an increase during year 2 but these variations did not differ between groups (Fig. 2).

3.3. Prolactin secretion

The seasonal changes in prolactin concentration were assessed in two ways: twice weekly samples throughout the experiment (Fig. 3) and hourly samples for 24 h at 4 times of year 1 (Fig. 4, right panels). As

expected, prolactin showed dramatic seasonal changes with the highest levels observed around the summer solstice and the lowest ones around the winter solstice (Fig. 3). Regardless of the way the samples were obtained, no evidence for an effect of the genotype on these seasonal variations was found since the ANOVA did not reveal any effect of group, nor an interaction between group and time.

3.4. Wool growth and wool follicle activity

Wool growth variations were observed for one year during the study (April 2001– April 2002) (Fig. 5) ($P < 0.001$). Wool production was maximal in March (3.27 ± 0.07 and 3.27 ± 0.19 $g \cdot month^{-1}$ for genotype +/+
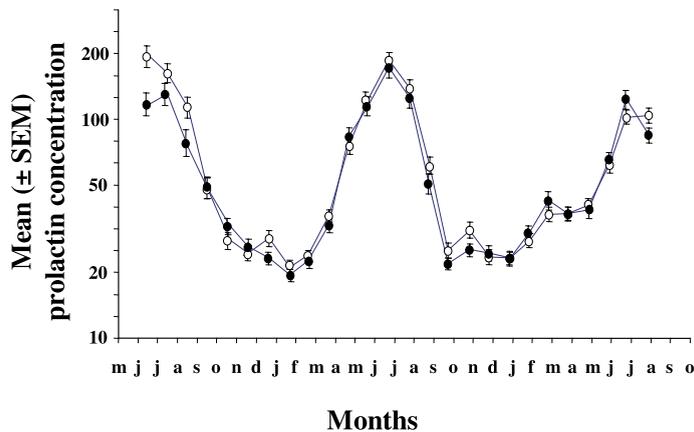
Figure 3. Mean (± SEM) prolactin concentration during a two-year study (July 2000 to June 2002) in Île-de-France ewes characterised by the absence (–/–, open circles) or the presence (+/+, closed circles) of the MnlI cleavage site at position 605 of the gene encoding the MT1 receptor on both chromosomes. Blood samples were collected twice weekly and all values obtained during a given month were averaged for clarity of presentation.

and –/–, respectively) and minimal in August $(1.36 \pm 0.07$ and 1.59 ± 0.09 g) $(P = 0.01)$. These time-related changes were not different between genotype +/+ and –/– $(P = 0.83)$.

Similarly, the percentage of primary wool follicles in the growing stage (anagen) displayed large changes throughout the year $(P < 0.01)$. In April, almost all of the primary follicles were found to be in the anagen stage (growth phase). Conversely, the greatest percentage of inactive primary follicle (telogen) was found in October (94 and 90% for genotype +/+ and genotype –/– respectively) $(P = 0.01)$. These time-related variations in primary follicle activity did not differ between genotypes (effect of genotype: $P = 0.52$; interaction genotype × time: $P = 0.10$).

3.5. Melatonin 24-hour profile

At the four times of assessment, plasma melatonin concentration (Fig. 4, left panels) was low during the light phase, increased during the dark phase, remained elevated until the end of this one, and plummeted after lights-on in both groups. The duration of the nocturnal melatonin secretion differed between months, reflecting in all occasions the duration of darkness $(P = 0.001)$. The amplitude of the melatonin peak did not differ between months $(P = 0.23)$. No effect of genotype on any parameter of melatonin secretion was detected.

4. DISCUSSION

Identification of major genes or QTL affecting the control of seasonal reproduction has not been carried out and the first approach to characterise individual loci that could be implicated in the control of this important physiological function has been the RFLP of the melatonin receptor [11, 12], the hormone that plays an important role in reproductive processes [14]. Two groups of a seasonal breed, Île-de-France ewes (half sib) were used in the present study and the unique genetic difference between these two groups came from the segregation of the two allelic forms of the
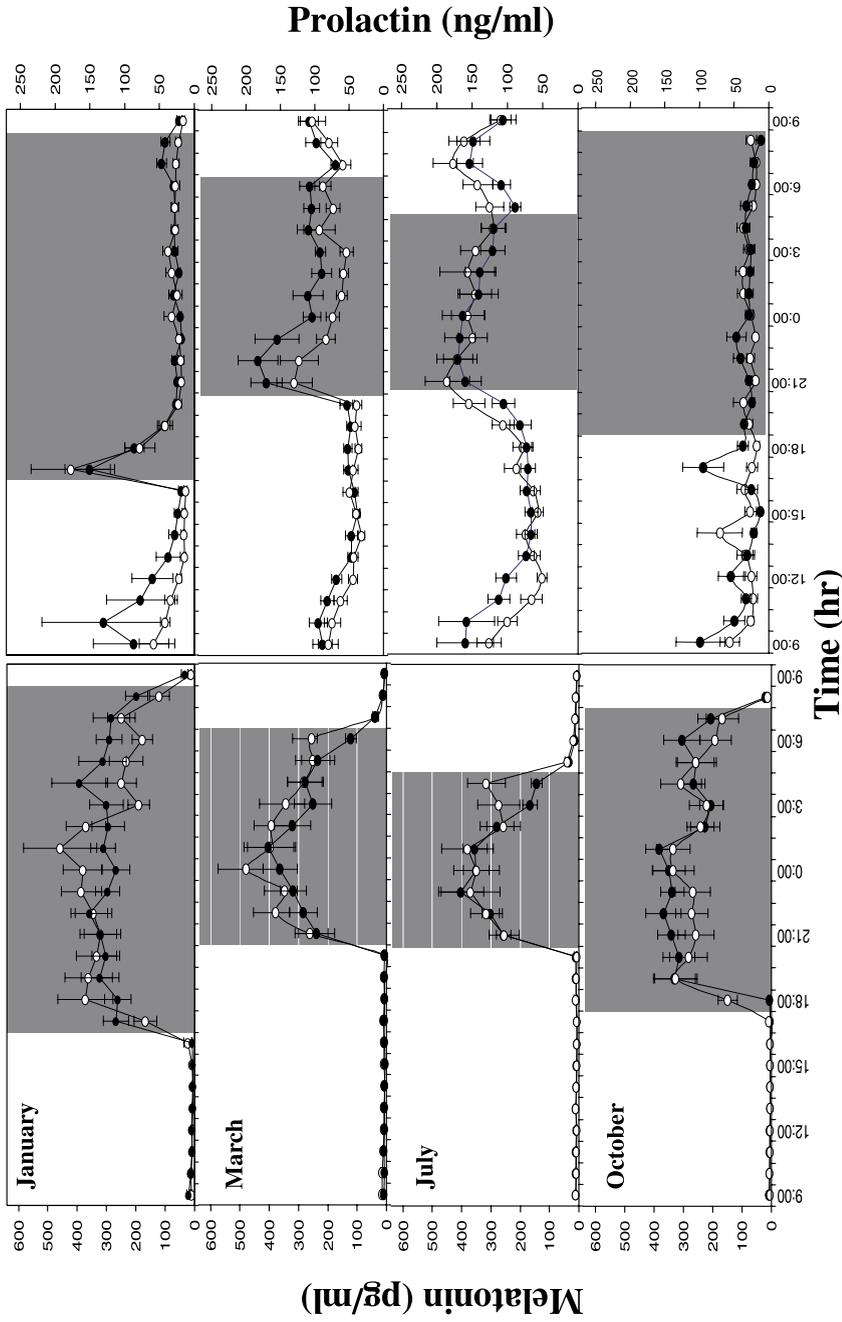
Figure 4. Mean (± SEM) melatonin (left panels) and prolactin (right panels) concentration at four times of the year in Île-de-France ewes characterised by the absence (−/−, open circles) or the presence (+/+, closed circles) of the MnlI cleavage site at position 605 of the gene encoding the MT1 receptor on both chromosomes. Blood samples were collected hourly for 24 h. The night is represented by shaded areas.
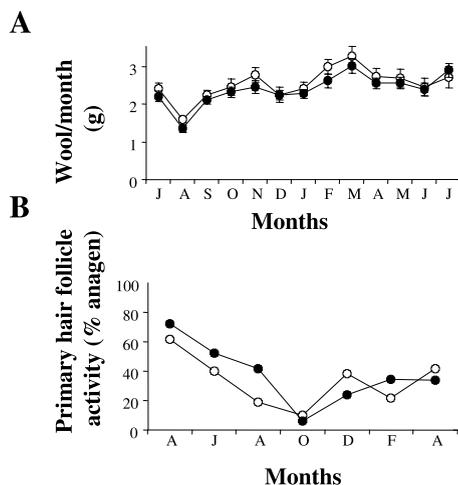
A

B

Figure 5. Mean (± SEM) wool growth rate (panel **A**) and mean percentage of active primary hair follicles (panel **B**) during a 1-year period (July 2001 to July 2002) in Île-de-France ewes characterised by the absence (–/–, open circles) or the presence (+/+, closed circles) of the MnlI cleavage site at position 605 of the gene encoding the MT1 receptor on both chromosomes. Wool growth was estimated from a 10 × 10 cm patch in which wool clipped once monthly was weighed. The percentage of the primary wool follicle in the growing stage (anagen) was determined from a skin biopsy obtained monthly and analysed twice monthly on 7 ewes of each group.

RFLP at position 605 of the coding sequence of the MT1 receptor gene. This site is characterised by the absence of an Mnl1 enzyme restriction site, leading to –/– homozygous genotypes when this silent mutation occurs on both chromosomes. Animals differing at this locus may also differ at closely linked loci i.e. the associated non-conservative mutation at position 706. In this study, we tested the hypothesis that this MT1 RFLP is linked to the genetic control of seasonal physiological functions. Overall, our results do not support this hypothesis by failing to show any direct effects of the two allelic forms of the MT1 receptor gene (genotype) on the seasonal pattern of ovulatory activity, prolactin secretion, wool growth, activity of wool follicle and melatonin secretion in Île-de-France ewes.

The aim of our study was to assess precisely the seasonal variations in reproduction in two groups of half-sib ewes differing by their genotype at the MnlI RFLP site by a thorough and continuous study that lasted two years. This study was performed in Île-de-France ewes because of the previous detailed description of seasonality in this breed and the possibility to measure reliably in controlled conditions the reproductive parameters. Surprisingly, no difference in the dates of onset or offset and in the length of the breeding season was detected between the animals of the two genotypes. Our study therefore contrasts with previous results [11–13] and shows that the relationship between MT1 polymorphism and reproductive seasonality may change with the breed of animals and/or the environmental conditions.

The variable effect of MT1 gene polymorphism according to the breed can be explained by several reasons. First, the mutation at the MnlI cleavage site can be viewed as a marker for a QTL, polymorphic only in some breeds, for instance Merinos d'Arles, while homozygous in others, for instance, Île-de-France. Secondly, even for a polymorphic QTL in the Île-de-France breed, the family sires involved in the experiment could be homozygous at this QTL. Thirdly, it is possible that, more seasonal breeds such as Île-de-France, express genes that exert a strong inhibition of reproduction and therefore obliterate the expression of the polymorphism at the MnlI site. In contrast, in less seasonal breeds such as Merinos d'Arles or OOS lines, such inhibitory genes may not be expressed letting MT1 polymorphism express its influence. Alternatively to genetic background, the variable influence may be explained by an interaction with environmental factors. An interaction between genetics and the environment in order to define the characteristics of seasonality has been demonstrated in various instances [1, 12, 29]. For instance, in

rams, nutrition has a strong influence on the expression of seasonality in the Merino breed while its influence is limited in a more seasonal breed such as the Suffolk breed [30, 31]. Genetic effects, i.e. differences in seasonality between breeds, are therefore larger when nutritional levels and body weight are higher; alternatively, it is reduced when nutrition is limiting [32]. It is therefore possible that the effect of MT1 polymorphism is more or less expressed according to the condition of the animals, particularly body weight. Such an influence is suggested in our study by the fact that the end of the breeding season tended to be different between groups during the first year of our study and not during the second one when animals had gained weight. A better understanding of the demonstrated influence of MT1 polymorphism on seasonality will therefore require the precise analysis of the relationship between genotype for this gene, and the influence of environmental factors, such as nutrition.

An important point of our study is the analysis of the relationship between MT1 polymorphism and non reproductive physiological parameters, prolactin secretion and wool growth features. However, no influence of the two allelic forms of the MT1 receptor gene on the seasonal fluctuation of prolactin secretion was observed. This absence of difference was obtained despite the fact that prolactin secretion was measured in two complimentary ways: two weekly samples to obtain a continuous measurement and 24-h samples to obtain a precise estimate at several time-points. The absence of difference in prolactin secretion is further substantiated by the lack of effects of the genotype on the seasonal rhythms of wool growth and wool follicle activity, physiological features that are controlled by the seasonal secretion of this hormone. Nevertheless, our data showed the existence of annual variations in the amplitude of wool growth in Île-de-France ewes. This breed was developed from a cross of Leincester and Merino sheep [18], the latter exhibiting a less pronounced seasonal wool growth [33, 34]. It will be interesting to study whether MT1 receptor polymorphism is involved in wool production in breeds of wool sheep, such as the Merino breed showing low seasonality in the wool growth that could be altered by nutrition level [35, 36].

In conclusion, our study demonstrated that, in the Île-de-France sheep breed, the two allelic forms of the MT1 receptor gene (genotype) have no direct effect on the seasonal pattern of various seasonal functions: ovulatory activity, prolactin secretion, wool growth, wool follicle activity and melatonin secretion. The influence of this polymorphism on the regulation of seasonal function could therefore be dependent upon the breed and/or environmental conditions.

ACKNOWLEDGEMENTS

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