Seasonality of reproduction in sheep: a) Experimental project for QTL detection influencing seasonality; b) preliminary results of an experimental genetic selection for seasonality

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In several breeding systems involving sheep, seasonality of reproduction induces disequilibrium between the availability of animal products and consumer demand, and also disequilibrium between needs of animals and pastoral availability. The use of hormonal and/or photoperiodic treatments allows avoiding seasonality but only partly and for technical, economical and ethical reasons, producers are obliged to reduce the use of these treatments. An efficient alternative is the genetic improvement for out-of-season breeding ability.

In the low seasonal Merinos d’Arles (MA) breed, the measurement of spontaneous ovulatory activity (SOA) in April by progesterone measurement, before any reproduction event, has been used for studying the out-of-season breeding ability of ewes. A divergent selection for or against the SOA was undertaken in MA to evaluate the actual possibility of selection based on SOA. Very preliminary results showed the interest and the efficiency of this selection criterion, but also the difficulty to select for this trait and to maintain the divergence.

Development of QTL analyses for spontaneous out-of-season ovulatory activity will allow identifying genetic polymorphisms directly involved in the control of the out-of-season breeding ability. The backcross design will involve Texel (seasonal) and MA breeds and the 60k SNP chip available in sheep. Polymorphisms that will be identified could be used to develop future efficient selection for SOA and to reduce seasonality of several breeds of sheep.

Keywords: seasonality, sheep, ovulatory activity, QTL, genetic selection
SUMMARY 2

In several breeding systems, and particularly for sheep, seasonality of reproduction induces disequilibrium between the availability of animal products and consumer demand, and also disequilibrium between needs of animals and pastoral availability. Several alternatives have been developed to overcome seasonality. The use of hormonal and/or photoperiodic treatments allows avoiding seasonality but only partly and for technical, economical and ethical reasons, producers are obliged to reduce the use of these treatments. An efficient alternative is the genetic improvement for out-of-season breeding ability. Genetic improvement of seasonality is of great importance for sheep breeding sustainability in harsh environment in which animals have to assure economical viability by being well adapted and highly productive. Genetic selection for out-of-season breeding ability will contribute to improve synchronisation between needs of animals and pastoral availability and increase length of production period.

Literature shows that useful additive genetic variability in duration of the breeding season is present among and within breeds. In the low seasonal Merinos d’Arles (MA) breed, the measurement of spontaneous ovulatory activity (SOA) in April by progesterone measurement, before any reproduction event, has been used for studying the out-of-season breeding ability of ewes. Previous estimation of genetic parameters for SOA indicated this trait can be used in selection but showed low heritability value. In addition, SOA measurement is technically difficult and only feasible in experimental unit. A divergent selection was undertaken in MA to evaluate the actual possibility of selection based on SOA. Two lines of about 100 ewes are selected for or against the spontaneous ovulatory activity measured in early April without any male stimulation. Very preliminary results showed the interest and the efficiency of this selection criterion, but also the difficulty to select for this trait and to maintain the divergence.

Causal mutations for seasonality have not been identified. Several studies suggested that polymorphism in the melatonin receptor gene affects sheep seasonality, but one or several other genes might be involved in the large differences observed. Genomic approach appears thus necessary to develop future efficient selection for seasonality by using polymorphisms that will be identified through QTL detection for seasonality.

A backcross design will be used to perform QTL detection and will involve seven families of 80 backcross females produced by mating F1 crossed males Texel-Mérinos d’Arles with Texel (seasonal) and MA (low seasonal) females from pure breeds. Two aspects of seasonality of reproduction will be considered jointly in this program: - Spontaneous ovulatory activity will be assessed after puberty and during two adult sexual seasons through progesterone level in blood samples; - Response to ram effect will be evaluated at the end of each sexual season by joining sexually active males with females and measured by the ovarian response (progesterone level) at different time after joining. Genotyping will be performed using the 60k SNP chip available in sheep. The access to QTLs and functional mutations associated with seasonal breeding will improve the understanding of the genetic mechanisms underlying of reproduction rhythms and presents the opportunity to better manage the breeding period and reproduction in sheep production.
The present manuscript is divided into two distinct parts. The first part (A) deals with an experimental project planned to undertake QTL detection underlying seasonality of reproduction in sheep. This project has been submitted to the animal genomic call for proposals of the French National Research Agency (Agence Nationale pour la Recherche, ANR) (this year, 2010). Here, we present first the context, innovative aspect of this project and the state of art about seasonality. Then, we describe the experimental protocol planned for undertaken QTL mapping and the expected results. The second part of this report (B) deals with preliminary results of an experimental genetic selection for seasonality in sheep. We present the selection process that has been used and data set available. Statistical models that will be used for genetic analyses are described. This second part will constitute a draft for a future journal article.

A- Experimental project for QTL detection influencing seasonality of reproduction in sheep

I- Context

A very large majority of farm animals express seasonal variations in their production traits. This pattern is the consequence of constraints due to a more or less marked seasonal reproductive interval in farm animal species such as sheep, goats and horses. Like their wild counterparts, most seasoned farm animals normally give birth at the end of winter-early spring, the most favourable period for their progeny to survive. In a recent survey (“Commmission Scientifique Ovine et Caprine” 2006) among sheep and goat breeders, overcoming the constraint of seasonality was one of the three most important preoccupations that producers expressed to researchers.

1- Economical context

As a consequence of seasonality, sheep products are marketed seasonally and these seasonal variations result in disequilibrium between the availability of animal products and consumer demand. These changes in the availability of fresh derived animal products constitute an organisational challenge to all stakeholders of the production chain and induce
high variation in the price paid to producers and in the activity of the industry to process the products. The demand for constant supply of animal products needs to be balanced against the biological constraint of the animal. Moreover, in several sheep breeding systems, seasonality of reproduction limits efficiency of production. The French market is highly dependent on importations since France produces only 43% of the French consumption of meat mutton. Therefore, diversification and intensification of sheep production to meet consumer demands for meat mutton, both locally and in global markets, provides both motivation and opportunity to modify the traditional lambing schedules. This involves extending the breeding period.

A large number of sheep breeding systems in France already use the out-of-season breeding ability of ewes. Extensive breeding systems involving rustic breeds are all dependent on the pastoral resources often limited in time and/or in quantity. In such systems, seasonality induces disequilibrium between needs of animals and pastoral availability. One way to overcome the constraint of pastoral availability is to delay the lambing period to cover needs of lambs after suckling with pastoral resources. Other breeding systems performing three lambings throughout two years also use the out-of-season breeding ability. Such breeding systems performing out-of-season breeding have poor results concerning reproduction efficiency and particularly low fertility, thus limiting production. Improvement of the out-of-season breeding ability will contribute to improve reproduction efficiency during the out-of-season period and will allow improving production and economical viability of such breeding systems. This will also allow avoiding large variation in production level and price paid to producer throughout the year.

2- Environmental and societal issues

Several alternatives have been developed to overcome constraints induced by seasonality and to induce fertile matings outside the normal breeding season (Cognié, 1988). Hormonal treatments are widely used to short-cut the seasonal inhibition of reproduction but they may have negative consequences on the future efficiency of reproduction (Bodin et al., 1997), on its cost and on the image of the product. The market in Europe tends to reject animal products that have been exposed to hormones. This societal demand and actual requirements from the European sanitary authorities to revise the registration files of some old veterinary products may lead to reduce or forbid their use. Consequently, we must be prepared to provide alternative means. Photoperiodic treatments are also efficient and allow the timing of the period of reproductive activity to be modified in both males and females (Chemineau et al., 1996; Chemineau et al., 2007; Chemineau et al., 2008). However, these
treatments require a long term flock management (several months of photoperiodic treatments). In addition, they have strong limitation in that they time-shift the period of fertility but do not at all prevent animals to go through alternation of sexual activity and rest. Finally, even if this technique is non-invasive, one can ask if it fully respects animal welfare considerations. The use of the ram effect is another solution to induce ovulatory activity, but this method is labour, time consuming and difficult to carry out in farm while breeders strongly request to reduce the amount of time devoted to breeding. Genetic component of sexual activity has long been considered as a potential approach to control the onset of the breeding season. Some previous genetic studies suggest the genetic improvement for seasonality can be an efficient alternative and comfort the need to undertake further investigations on genetic determinism of sexual seasonality.

An interesting characteristic of seasonality that should be considered is that the out-of-season breeding ability is a natural trait spontaneously expressed in some sheep breeds. Thus, the genetic approach based on the use of this natural trait would probably be better accepted by consumer. The development of a genetic selection of animals showing long breeding period is a more sustainable process for livestock systems than the repeated use of hormonal treatments (i.e., reduction of inputs at farm level and reduction of spreading of synthetic steroids into the environment). Biodiversity is another aspect of sustainable development that would benefit from such selection. Uncover of QTL controlling seasonality and diffusion of corresponding favourable alleles in populations will help maintaining and development rustic breeds reared in harsh environment (mountains, low rainfall, and poor soil quality). Rustic breeds have to be well adapted and highly productive to assure economical viability of such breeding system, and this involves synchronization of the breeding period with the pastoral resources often limited in time and in quantity in such systems.

3- Innovative aspect

The first innovative aspect of the project relies on the trait that will be studied. The out-of-season breeding ability is a difficult trait to measure for practical and technical reasons (interaction with reproduction, cost and workload on a large number of ewes in private farms) and only feasible in experimental farm. Moreover, measuring the sexual seasonality may conceal different traits. For instance, studies based on fertility performance in spring mix, in an indistinguishable manner, ewes that are already cycling and ovulate spontaneously from those that were in anoestrous but induced by joining with rams (ram effect). Physiological
mechanisms involved in the fertility of these two groups widely differ. The originality of our project is not only to avoid this confusion but also to consider both traits in the same design. Enlarging the period of sexual activity is the main objective, but in some conditions it could be worthwhile to increase the response to a ram effect. Our challenge is to find QTLs for both traits.

The second innovative aspect of the project is the use of the most relevant genomic tools which will be available when analysis will start. Thus we will attempt to find directly those genes of which the polymorphism affects the variability of seasonality and which could be directly used for genetic improvement. At the present time, we plan in our project to use the 60K SNP chip available in sheep to locate precisely QTLs, as well as tools and statistical methods that are currently developed and tested in different projects in the context of the use of this chip. The field of genomics and genome technologies is currently in a period of very rapid development. During the project, technologies will have moved on and costs changed. If we are confident that we can fulfil the objectives we have set with the 60K SNP chip and the requested funds, it can be envisaged that likely developments will mean that the project can deliver significantly more than proposed. For example, if cost of sequencing fall in price sufficiently we may be able to use this alternative to fine mapping. For this reason, some tasks are deliberately not definitive about which methodology will be used; in fact it would be unwise and short-sighted to be definitive.

II- Seasonality in sheep

1- Photoperiodic control

The breeding season of most species is defined by seasonal variations in their ovulation frequency (presence or absence of ovulation), spermatogenic activity (from moderate decrease to complete absence of sperm production), gamete quality (variations in sperm quality, fertilization and embryo survival) and also sexual behaviour. The principal intimate mechanism that triggers the onset of the breeding season relates to a complex combination of endogenous circannual rhythm driven and synchronized by light and melatonin (Chemineau et al., 2008). In temperate latitudes, sheep is one of the best examples of seasonally breeding species. Thus naturally, the ovulatory activity is generally inhibited in spring and therefore sheep mainly bred in the autumn, when the duration of day light...
decreases. In extensive and semi-intensive production systems, this mechanism protects the lambs from the winter by giving birth in early spring and allows them to grow in more favourable conditions.

2- Genetic variability and criteria for measuring the out-of-season breeding ability

The understanding of the genetic control underlying of reproduction rhythms and the access to QTLs and functional mutations associated with seasonal breeding remains limited in all species and by the fact in sheep.

Literature shows that useful additive genetic variability in duration of the breeding season are present among and within sheep breeds (Avdi et al., 1993; Hanocq et al., 1999). Several researchers have shown differences in the duration of the breeding season of sheep raised in the same region (Avdi et al., 1993). For example, Texel ewes are highly seasonal while Merinos d’Arles ewes present more discrete expression of seasonality (Hafez, 1952; Santiago-Moreno et al., 2000). Breeds raised in the subtropics and in the tropics generally present a low seasonality or cycle all the year round with no anovulatory period (for review, see (Chemineau et al., 2008)). Unfortunately, a marked seasonality is expressed in these breeds when subjected to the large photoperiodic variations and temperate climates of northern countries, preventing the possible use of these breeds in flocks under temperate latitude (Chemineau et al., 2004). In sheep, Texel and Merinos d’Arles breeds are known to be the most divergent breeds for seasonality trait. A preliminary quantitative evaluation of sexual seasonality has been recently done in order to measure the difference between Merinos d’Arles and Texel breeds (Teyssier et al., 2009). Preliminary results showed the difference was huge since pure Texel started their sexual season between late September and late October while Merinos ewes were already in oestrus in July.

Genetic component of sexual activity has long been considered as a potential approach to control the onset of the breeding season. Some previous genetic studies suggest the genetic improvement for seasonality can be an efficient alternative to increase length of the breeding period and selection of sheep with a reduced sexual seasonality is feasible (for review, see (Notter and Cockett, 2005)).

The Virginia Tech out-of-season mating line was developed using classical selection procedure for fertility in May and June matings (AlShorepy and Notter, 1997). Single trait selection was based on the ability of the ewes to mate and conceive in natural, single-sire
matings over an 8-week period beginning May 1. The selected ewes have a dramatic reduction in duration of seasonal anoestrus and many ewes continue to cycle during spring and summer (AlShorepy and Notter, 1997; Notter and Cockett, 2005). However, significant ewe age effects on fertility and response to selection were observed and highlight the difficulty in evaluating seasonality in young ewes (Notter and Cockett, 2005). Finally this experiment showed difficulties and limits linked to use fertility in spring as a selection criterion.

Fertile ewes in spring gather those which are already cycling and present spontaneous ovulatory activity (SOA) with those which are on their seasonal anoestrous but respond to a male effect. Both events imply different physiological and genetic mechanism. In France (INRA) and other European countries which collaborate with, genetic studies (i.e., genetic parameters estimation, experimental selection…) about reproductive issues focussed on SOA. In the low seasonal Merinos d’Arles breed, the measurement of the spontaneous ovulatory activity (SOA) in April, before any reproduction event, has been used for studying the out of season breeding ability of ewes. This measurement was based on the blood progesterone levels in two blood samples taken 10 days apart in early spring, before any contact with rams (Terqui and Thimonier, 1974). Hanocq et al (Hanocq et al., 1999) reported high heritability and repeatability estimates (0.20 and 0.30, respectively) for spontaneous out-of-season ovulatory activity in the Merinos d’Arles breed. Similarly, a high proportion of ewes ovulating outside the normal breeding season has been reported in the Chios breed in Greece (Avdi et al., 2003) and in Latxa breed in Spain (Beltran de Heredia et al., 2002). For these 3 breeds, heritability of SOA was similarly found to be relatively high (0.20 to 0.37).

Estimation of genetic parameters for SOA thus indicated this trait can be used in selection.

A divergent selection was undertaken in Merinos d'Arles to evaluate the actual possibility of selection based on SOA. Two lines of about 100 ewes are selected for or against the spontaneous ovary activity measured in early April without any male stimulation. Results showed the interest and the efficiency of this selection criterion, but the difficulty to select for this trait (Teyssier et al., 2002; Bodin et al., 2005; Teyssier et al., 2010).

3- Candidate gene approach

Candidate gene approach can be developed to research locus or genes involved in a function. This approach assumes that a gene known to be involved in the physiology of the trait of interest could present in the nucleotide sequence polymorphism causing variation in the trait. Sequencing of this gene in number of different animals allows searching for
polymorphisms and variations in the nucleotides sequences found are tested for association with variation in the quantitative trait.

Causal mutations responsible for variability of sexual seasonality have not yet been identified. Nevertheless, an interesting result found in Merinos d’Arles ewes lines described above was a strong association between SOA in April and the presence (+) or absence (−) of an MnlI restriction site in the exon II of the gene that encodes melatonin receptor (MT1) (Pelletier et al., 2000). Similarly, this polymorphism was significantly associated with fertility in spring mating in the Virginia Tech out-of-season-breeding line (genotype “-/-” showing lower spring fertility) (Notter et al., 2003). Furthermore, substantial changes in allelic and genomic frequencies at the MnlI site with the degree of seasonality of breeds have been described by different authors (Messer et al., 1997; Pelletier et al., 2000; Notter et al., 2003; Trécherel, 2007). Various physiological studies reinforce the assumption of a close relationship between the expression of seasonality and the polymorphism of the MT1 receptor gene. A higher density of melatonin receptors have been found in the pars tuberalis of –/– ewes than in +/+ ewes (Pelletier et al., 2000). A higher receptor density was also observed in four brain areas of –/– ewes (Trécherel, 2007). One of these areas belongs to premammillary hypothalamus which is the site of the melatonin action to regulate reproductive function in sheep (Malpaux et al., 1998; Migaud et al., 2005).

In spite of these arguments, the relationship between the studied part of Exon II of the MT1 gene and the phenotype of seasonality is still not clear. First of all, in Merinos d’Arles, no difference was observed between ewes of genotype +/+ and –/– on the rate of SOA in early spring and on fertility in spring mating. Secondly, various attempts failed to find a difference on 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). These results suggest that the polymorphism in the MT1 gene affects sheep seasonality but is not sufficient and one or several other genes which remain to be found might explain the large differences encountered. This is consistent with inconvenient in the candidate gene approach that comes from the fact that there are usually a large number of candidate genes involved in the regulation of a trait, multiplying the number of sequencings needed and the number of associations studies that must be carried out. Moreover, list of candidate genes involved in a function is usually non exhaustive and causal mutation can occur in a gene that would not have been regarded a priori as an interesting
Figure 1: Experimental design used to produce the backcross population
gene for the trait considered. This comforts the need to undertake QTL detection for seasonality using large numbers of ewes in adapted experimental designs.

4- QTL mapping approach

An alternative to the candidate gene approach to research locus or genes involved in a function is the QTL mapping approach. QTL mapping consists to identify chromosome regions associated with variation in phenotypic traits assuming that genes which affect a quantitative trait are unknown. A recent study was undertaken to find QTL for seasonality in sheep. It was done on 159 backcross Dorset-East Friesen ewes with 120 microsatellites markers. However, only very large chromosomal regions were reported associated with variation in aseasonal reproduction (Mateescu and Thonney, 2010).

III- Experimental protocol for QTL mapping

The project is made up of 5 scientific tasks defined by the discipline and/or specific analyses undertaken in each task: breeding, phenotyping, genotyping, quantitative genetics, bioinformatics.

1- Generation of backcross ewes and animal breeding

The first task is aimed to create and rear the experimental animals (i.e., backcross females) which will be phenotyped and genotyped to perform QTL detection.

One way to perform QTL detection in outbred populations is to cross between lines or breeds with extreme phenotypes in order to produce F1 heterozygous at QTL(s). Due to the variability of the association between markers and QTLs in outbred populations, LD can be then found only within families. The experimental designs (i.e., backcross or F2) employed to produce families from the F1 animals are determined by the species characteristics. In sheep, due to physiological constraints of the species (fertility, prolificity, viability, sex ratio), production of large number of F1 and F2 ewes would have taken a long time (several years) and increased consistently the costs. For scientific and practical reasons, a design based on female families issued of F1 sires back-crossed with both parental breeds has been chosen (Figure 1). Choice of the Texel and Merinos as parental breeds has been done according to
**Figure 2a:** Power of QTL detection in a sire design for various sizes of QTL effect (columns: 0.25, 0.5 and 1.0 phenotypic standard deviation) and various values of heterozygosity of QTL (first raw, 1; second raw, 0.5) (h2, heritability; hetero, heterozygosity of QTL). For each combination, several number of sires and daughters per sire were tested.
results of the literature and preliminary estimations of sexual seasonality specifically undertaken for this project on ewes of these breeds in the experimental farm Le Merle (Teyssier et al., 2009). Since the two types of backcrosses will be produced between Texel and Merinos breeds, genes could be identified whatever their type of inheritance (dominant, codominant or recessive).

Power of QTL detection:

In order to estimate the total sample size, power of QTL detection has been estimated in a sire design by taking into account the heritability of the trait (0.2), the heterozygosity of the QTL (i.e. the probability that the sire is heterozygous, 0.5 or 1.0), the type I error accepted (0.05), the magnitude of the QTL effect (0.25, 0.5, or 1.0 \sigma_p), the recombination fraction between marker and QTL (0.02) and the structure of the family (i.e., number of sires equal to 5, 8, 11, 14 and number of daughters per sire ranging from 10 to 110) (Figure 2a). Consistently with literature, for a given number of sires and daughters, power of QTL detection increase with the increase of the size of the QTL effect (i.e. 0.25 to 1 phenotypic standard deviation tested) and with the increase in the heterozygosity of QTL. High power of detection (around 100%) was reached with small sample size when heterozygosity of the QTL was equal to 1.0 and the size of QTL was equal to 1 \sigma_p. Conversely, low power of detection (lower than 50%) was reached with large sample size when heterozygosity of the QTL was equal to 0.5 and the size of QTL was equal to 0.25 \sigma_p. An heterozygosity of QTL equal to 0.5 (50% of heterozygous sires) can be considered to mimic a recessive or dominant case in a backcross design, because in such situation of gene inheritance only half of the backcross design will be informative. A more detailed estimation of power was done in the intermediary situation considering a size of QTL equal to 0.5 \sigma_p and an heterozygosity of QTL equal to 0.5. Power of QTL detection was estimated for a number of sires ranging between 5 and 14 and a number of daughters ranging between 10 and 110 per sire (Figure 2b). The power of QTL detection increased with the family size and the number of families. Power reached a plateau with large family size (around 100 daughters) when number of sires was high (for instance 14 sires). We also showed that for a fixed total sample size, power decreased with an increase in the number of sires and conversely power increased with the number of daughters per sire. Power was greater for a few large families than for many small ones. These results were consistent with those found by Soller and Genezi (1978). These results showed there was an optimum between the number of families and number of progenies per family. The use of 7 sire families constituted of 80 progenies will allow reaching a power of detection of 80%.
Figure 2b: Power of QTL detection in a sire design for a size of QTL effect equal to 0.5 $\sigma_P$ and an heterozygosity of QTL equal to 0.5. (h2, heritability; hetero, heterozygosity of QTL). Power was estimated for a number of sires ranging between 5 and 14 and a number of daughters per sire ranging between 10 and 110.

Total size $\approx$ 600

80 %
This value would be probably higher by taking into account the linkage disequilibrium in this estimation.

**Animal management:**

Merinos d’Arles females have already been artificially inseminated with Texel males at the experimental farm “Le Merle” to produce F1 crossed males (Figure 1). Seventeen F1 crossed males are available at "Le Merle" and will be trained for semen production. Seven F1 crossed males will be used to inseminate purebred Texel ewes on private farms (Figure 1). One thousand artificial inseminations will be realized in autumn 2010 to produce approximately 300 backcross females ¾ Texel and ¼ Merinos. The same 7 F1 crossed males will be used in the backcrossing to the purebred Merinos by artificial insemination (Figure 1). One thousand artificial inseminations will be realized in October 2010, half at the experimental farm Le Merle and half in private farms, to produce approximately 300 backcross females ¾ Merinos and ¼ Texel. Backcross females of both backcross types will be bought at weaning and then brought to the experimental farm “Le Merle” (Salon de Provence, France) where they will be reared in shed and phenotyped during all the duration of the project (3 years). They will be isolated from sires to avoid reproductive disturbances which could affect sexual seasonality. However for testing their response to a male effect they will be joined each year at the end of the control period with sexually active harnessed males. Ewes will have access to pasture and will be managed in several sub-mobs for which the sire family and the backcross type will be taken into account.

2- **Phenotypic measurements**

The second task will consist in performing the phenotypic measurements in the experimental farm for spontaneous ovulatory activity and ovulatory response to ram effect.

The backcross females will be reared without reproductive events and over four consecutive years in order to record reproductive traits linked to seasonality without disturbances due to reproduction and during puberty and two adult sexual seasons (constituting thus two repetitions). Their maternal ability will be assessed on one lambing obtained at the end of the program. Firstly, spontaneous ovulatory activity (SOA) will be assessed from the onset of puberty the first year and then from mid-April to February for sexual adult seasons (Figure 3) through progesterone measurement in blood samples collected at 7-day intervals. A concentration of
Figure 3: Measurements of spontaneous ovulatory activity (SOA) and ovulatory response to the ram effect.
progesterone higher than 1ng/ml at least in one of three consecutive blood samples indicates ovulatory activity (Thimonier, 2000). Secondly, ewes will be joined to sexually active harnessed rams at the onset of April for 10 days to measure ovulatory response to ram effect (Figure 3). Introduction of rams in flocks of ewes that are in anovulatory anoestrus induces a silent ovulation (without oestrus) in 2 to 4 days followed either by a ovulatory cycle of normal duration (i.e., 17 days) or by a ovulatory cycle of short duration (i.e., 6 days) (Thimonier et al., 2000). To control this response, blood samples will be collected every day for 10 days to characterize the first ovulatory response and to search for short ovulatory cycle.

Progesterone concentration will be measured by enzyme immunoassay (EIA) using the technique described by Canépa et al. (2008). Although blood samples will be collected over the full experiment, only samples of the beginning and the end of the individual breeding season and samples for characterising the response to the male effect will be processed. For that, progesterone analysis will start in august 2011, then analysis will be stopped progressively when females attain puberty (females exhibiting at least two consecutive ovulatory cycles). To characterise the end of the first breeding season, progesterone analysis will start again in December 2011, then analysis will be stopped progressively when females attain the anovulatory status (progesterone levels under 1 ng/ml in at least 6 consecutive blood samples). To characterise the beginning of the second and third breeding season, progesterone analysis will be start immediately after the end of the male effect (mid-April), then analysis will be stopped progressively when females exhibit at least two consecutive spontaneous ovulatory cycles. To characterise the end of the second and third breeding season we will proceed as explained for the first breeding season.

Complementary measurements for growth traits will be also performed. Live weight of backcross females will be recorded at weaning and regularly until 120 days to detect QTL for post-weaning growth. After the third sexual season (end of the protocol), backcross females will be submitted to a ram effect and joined with fertile rams to produce lambs. The maternal ability will be recorded by measuring growth of lambs before weaning by weighting lambs at birth, 30, 60 and 90 days. QTL detection for early growth traits will be carrying out using these recorded data. Live weight and body condition score of backcross females will be also recorded regularly over the four years to evaluate the relation with the reproduction efficiency.
3- Genotyping and verification of Mendelian transmission

Blood samples will be collected from backcross females and F1 males for DNA extraction and genotyping. Blood samples will be stored frozen until DNA extraction. DNA will be extracted and used to genotype the set of 60 000 SNPs markers using the 60K SNP chip developed in sheep by the International Sheep Genomics Consortium. Genotyping will be performed according Illumina instructions using a fully automated liquid handling genotyping protocol. Genotyping quality will be checked through quality control process of the Labogena platform. The Illumina “infinium” genotyping technology is available at Labogena platform which is in addition fully trained in high throughput samples manipulations.

Monomorphic, ungenotyped and unmapped SNPs will be removed. Quality control criteria, i.e. SNP call rate, Hardy-Weinberg equilibrium and minor allele frequency will be calculated. SNPs for which more than 5% of the genotyped individuals exhibited Mendelian inheritance discordance with their parents will be discarded. Leaving SNP with Mendelian inheritance discordances will be corrected considering missing genotype. When enough genotypes will be available within family, imputation will be performed to infer some major ungenotyped sires genotypes. Mendelian transmission of marker alleles will be verified using pedigree information (within each sire family) and corrected or missing genotype will be considered in case of error.

The genotypes and their related molecular information will be stored in a dedicated databank Genetic information related to this project will be added to genotypes data (molecular information of the chip, pedigree information, and information about the experimental protocol …). This will provide a user friendly access to the data produced in this project, available via a web browser and will also provide links to complementary molecular information for in silico investigations.

4- QTL detection

The objective of task 4 is to detect and obtain precise location of QTLs underlying reproductive traits (and growth traits).

To perform QTL detection analyses, several approaches will be considered. Firstly, linkage analyses will allow estimating QTL effect using marker by maker or multimarker approaches.
The evidence for linkage between a marker and a QTL can be based upon analysis of variance. Linear model include sire effects and the effect of the marker allele transmitted within sire (Soller and Genizi, 1978; Luo, 1993). Difference between mean performances in progeny groups receiving alternate marker alleles is an effect of a putative QTL genetically linked to the marker.

Linkage analysis (LA) by interval mapping is a powerful tool to find out regions of the genome associated with phenotypic variation hosting putative QTLs. LA can be performed with methods describing the effect of the QTL on the phenotype through differences of trait distribution conditionally to the QTL genotype: mixture of distributions (Elsen et al., 1999), regression analysis (Haley and Knott, 1992; Mott et al., 2000). This approach is efficient (high power, simple implementation) for designs based on a few number of large families (sire or grandsire designs) or crosses (F2, backcrosses) and thus well adapted to our QTL experimental design.

QTL detection will be performed using different softwares available such as QTL Express (Seaton et al., 2002) and QTLMAP (Elsen et al., 1999), which will be adapted to the high number of markers. QTLMAP software offers useful options for the type of analyses needed in this project (multiQTL and multi-trait approaches) (Gilbert and Le Roy, 2007).

A second method (joint analysis LDLA) based on the combination of LA and Linkage Disequilibrium will be used to map finer QTLs than using only linkage analysis. Because of crossbreeding experiments where within breed and between breeds LD coexists, the existed LDLA methods will be adapted to take into account linkage disequilibrium from the both maternal breeds. However, the only use of linkage disequilibrium will not be assumed because of these different linkage disequilibrium origins in the backcross populations.

Tests considering simultaneously linkage association and linkage disequilibrium (LD) are more robust and will be performed. Several proposals exist. The extensions of association tests (QTDT, FBAT, PBAT; (Abecasis et al., 2000; Hernandez-Sanchez et al., 2003; Lange et al., 2004)) combine evidence of the association analysis and the transmission of markers throughout a pedigree. These are single-marker test, easy to use, but not as general as a multimarker interval mapping approach. Extensions of linkage analysis to include linkage disequilibrium try either to model the distribution of linkage disequilibrium among markers and the QTL (Farnir et al., 2002; Perez-Enciso, 2003) or to model covariances among individuals assuming that similarity among markers implies similarity in QTL genotype (Almasy et al., 1999; Cardon and Abecasis, 2000; Meuwissen and Goddard, 2000). A recent
The proposal is to consider LDLA by linear regression (Legarra and Fernando, 2009). Software exists in INRA to fit these models. However, some additional developments have to be done to take into account the breed origin of the LD (Merinos or Texel) in the present project.

5- Meta-analyses/SNP validation and identification of candidate genes in silico

For each trait, polymorphisms found in the present study will be compared to results previously obtained for the same trait by functional or structural genomic analyses. The second objective of this task is to look for and propose a list of positional candidate genes for the most interesting QTLs fine mapped in the QTL mapping. The work will consist in choosing pertinent QTLs based on significance and precision of the related confidence interval, amplitude of the QTLs effects. For these candidate genomic regions, candidate genes will be proposed using comparative mapping.

The work will consist in choosing pertinent QTLs based on significance and precision of the related confidence interval, amplitude of the QTL effects. For these candidate genomic regions, candidate genes will be proposed using comparative mapping. We will benefit from the refinement of the existing bovine-ovine comparative map thanks to the announced ovine genome sequence together with a high density SNP map previously. Complementarily, functional annotations available for positional candidate genes will be used to check which positional candidate genes match functional candidate genes and conversely. Functional annotations include Pubmed literature, gene ontology data, species comparison, transcriptomic data, proteomic data, and knockout data in rodents…

A Gene Ontology (GO) annotation could provide a controlled vocabulary to describe gene and gene product attributes in any organism. AmiGO provides an interface to search and browse the ontology and annotation data provided by the GO, it would provide the biological process, cellular component and molecular function. The KEGG database (Kyoto Encyclopedia of Genes and Genomes) is a collection of manually drawn pathway maps representing our knowledge on the molecular interaction and reaction networks. More complete software will be also used such as Ingenuity Pathways Analysis (IPA). Ingenuity system is a web-based interface and provides computational algorithms to identify and dynamically generate significant biological networks and pathways that are particularly enriched with our genes of interest called “focus genes”. IPA also gives information on biological functions and canonical pathways. The main advantages of IPA are that the bibliographical links that are present in the database are manually curated by biologists and it
is used by many scientists. The main drawback is that the references that are curated are mostly on human, mouse and rat species. Cytoscape is an open source bioinformatics software platform for visualizing molecular interaction networks and biological pathways and integrating these networks with annotations, gene expression profiles and other state data.

IV- Expected results and deliveries

This project will lead to accumulate phenotypic data on seasonality and growth. This large phenotyping will increase our knowledge on non conventional traits in two pure breeds and their crosses. However, they are considered as breed models and the result will be useful for other breeds. This work will also help to understand genetic mechanisms underlying seasonality and to postulate candidates genes through in silico studies of the most significant QTL located in small genomic regions. A part of this project will allow validating polymorphisms through meta-analyses of seasonality and growth traits. Small intervals for positions of QTLs with very accurate locations, large effects and well known candidate genes that will be found in this project could be used in further investigations aiming to identify causal mutations.

1-2. Collection of blood samples over three years for each ewe will be constituted. Progesterone measurements during puberty and two adult seasons will be obtained for each ewe. Spontaneous ovulatory activity data will allow determining, for each group of backcross females, the following reproductive seasonal parameters: puberty age, date of the beginning and the end of the breeding season, breeding season length, percentage of sheep showing ovulatory cycles during the anoestrus period. Measurement of the ovulatory response to the male effect during anoestrus of backcross females will allow characterising the first ovulatory response, identifying short ovulatory cycles and determining if the ram effect induce or not cyclic ovulatory activity.

3. A collection of identified DNA samples will be available for genotyping. Data tables containing animal identifications and corrected SNP genotypes for the set of 60000 SNPs will be available for QTL detection. Data of this project and related information will be accessible (for partners) via a user friendly web site.
4. The deliveries will be likelihood ratio test profiles testing the presence of a QTL on the genome, rejection thresholds at genome-wise and chromosome-wise levels, confidence intervals. The joint analyses LDLA will provide a map with fine and robust QTL locations.

5. The search of positional candidate genes will result in detailed comparative maps including human, bovine, and ovine species for some genomic regions and a list of positional candidate genes related to seasonality.

Conclusion

Development of QTL analyses for spontaneous out-of-season ovulatory activity and ovulatory response to male effect will allow identifying genetic polymorphisms directly involved in the control of the out-of-season breeding ability. The experimental design involves seasonal and less seasonal sheep breeds and recent genomic tools that will allow finding very small genomic regions. Polymorphisms that will be identified could be used in selection scheme to reduce seasonality of several breeds of sheep and in a larger extent may serve other seasonal species. Genomic approach appears particularly interesting to develop future efficient selection for SOA and presents the opportunity to better manage the breeding period and reproduction in sheep production.

This project has been submitted to the animal genomic call for proposals of the French National Research Agency, a founding agency, (Agence Nationale pour la Recherche, ANR) (this year, 2010) and is currently under peer review selection process. Projects are selected based on their scientific quality as well as on their economic relevance for industries. This project has also been submitted to private sheep breeders. They have approved this project and accepted to support 44% (344 574€) of the cost of the project.
B- Preliminary results of an experimental genetic selection for seasonality in sheep

I- Introduction

The breeding season of most species is defined by seasonal variations in their ovulation frequency, spermatogenic activity, gamete quality and also sexual behaviour. The principal intimate mechanism that triggers the onset of the breeding season relates to a complex combination of endogenous circannual rhythm driven and synchronized by light and melatonin (Chemineau et al., 2008). In temperate latitudes, the breeding season classically corresponds to the period of decrease in day length. Sheep is one of the best examples of seasonally breeding species. Thus naturally, the ovulatory activity in sheep is generally inhibited in spring and therefore sheep mainly bred in the autumn, when the duration of day light decreases.

In several breeding systems involving sheep, seasonality of reproduction induces disequilibrium between the availability of animal products and consumer demand, and also disequilibrium between needs of animals and pastoral availability. Several alternatives have been developed to overcome seasonality. The use of hormonal and/or photoperiodic treatments allows avoiding seasonality (Cognié, 1988; Chemineau et al., 1996) but only partly and for technical, economical and ethical reasons, producers are obliged to reduce the use of these treatments. An efficient alternative is the genetic improvement for out-of-season breeding ability. Genetic improvement of seasonality concerns the whole sheep industry because it could be used in several breeding systems to increase length of production period and reduce seasonal market of sheep products. Genetic improvement of seasonality is also particularly of great importance for sheep breeding sustainability in harsh environment in which animals have to assure economical viability of the breeding system by being well adapted and highly productive. Thus genetic selection for out-of-season breeding ability will contribute to improve synchronisation between needs of animals and pastoral availability in such harsh environment.
Useful additive genetic variability in duration of the breeding season is present among and within breeds (Hafez, 1952) Some previous genetic studies suggest the genetic improvement for seasonality can be an efficient alternative to increase length of the breeding period and selection of sheep with a reduced sexual seasonality is feasible (for review, see (Notter and Cockett, 2005)). Expression of the out-of-season breeding ability differs according to the criteria used for its measurement. The Virginia Tech out-of-season mating line was developed using classical selection procedure for fertility in May and June matings (AlShorepy and Notter, 1997). Single trait selection was based on the ability of the ewes to mate and conceive in natural, single-sire matings over an 8-week period beginning May 1. The selected ewes have a dramatic reduction in duration of seasonal anoestrus and many ewes continue to cycle during spring and summer (AlShorepy and Notter, 1997; Notter and Cockett, 2005). However, significant ewe age effects on fertility and response to selection were observed and highlight the difficulty in evaluating seasonality in young ewes (Notter and Cockett, 2005). Finally this experiment has showed the difficulty linked to the use of fertility as a selection criterion.

Fertile ewes in spring gather those which are already cycling and present spontaneous ovulatory activity (SOA) with those which are on their seasonal anoestrus but respond to a male effect. Both events imply different physiological and genetic mechanisms. In the low seasonal Merinos d’Arles breed, the measurement of the spontaneous ovulatory activity (SOA) in April by progesterone measurement, before any reproduction event, has been used for studying the out of season breeding ability of ewes. Hanocq et al. (1999) reported heritability and repeatability estimates of 0.20 and 0.30, respectively, for spontaneous out-of-season ovulatory activity in the Merinos d’Arles breed. Similarly, a high proportion of ewes ovulating outside the normal breeding season has been reported in the Chios breed in Greece (Avdi et al., 2003) and in Latxa breed in Spain (Beltran de Heredia et al., 2002). For these 3 breeds, heritability of SOA was similarly found to be relatively high (0.20 to 0.37).

A divergent selection was undertaken in Merinos d’Arles to evaluate the actual possibility of selection based on SOA. Two lines of about 100 ewes are selected for or against the spontaneous ovulatory activity measured in early April without any male stimulation. We present here the selection process that has been used and data set available. Statistical models that will be used for genetic analyses are described. Very preliminary results of this selection are presented.
II- Materials and Methods

1- Animals and Management

The Merinos d’Arles ewes included in the experiment were animals of the experimental flock of the experimental farm “Domaine du Merle (southeastern France). Merinos d’Arles breed shows interesting aptitudes for adaptation to its environment and for aseasonality. These abilities are fully exploited in the breeding system in which the main mating period is spring. MA ewes are mated once a year from mid-April to early June, before transhumance to the Alpine mountains in summer. Replacement ewes are mated for the first time at the age of 18 months. A large number of ewes were hormonally synchronized by a progestagen treatment to ensure better fecundity and joined with vasectomized rams during 14 to 15 days and then with fertile rams during 16 to 18 days. After transhumance, the ewes lamb at the experimental farm in autumn. Weaning took place in January.

2- Cyclicity measurement

In order to specifically examine the spontaneous ovulatory activity, measurement of the ovulatory activity was done before any reproduction event (i.e., before hormonal synchronisation and/or ram introduction). Two jugular blood samples per ewe were collected at an interval of 8-10 days during the first two weeks of April. Following centrifugation at 2,000 g for 15 min at 4°C, plasma samples were separated and stored deep frozen at -20°C until measurement of progesterone concentration by RIA using a specific technique described by Terqui & Thimonier (1974). Ewes with at least one sample in which concentration of progesterone was higher than 1ng/ml were considered as in ovulatory activity. An ovulatory activity score of 1 was thus assigned for such ewes and a score of 0 otherwise.

Other traits:
Lambing date and prolificacy were recorded. Fertility was expressed as a percentage and was computed as the number of ewes which lambed in autumn compared to the number of ewes mated in spring. The live weight of all ewes was recorded just before the mating period in April.
Table 1: Repartition of ewes according to their year of birth for each year of cyclicity measurement. Generation number is indicated by G0, G1, G2, G3 and G4. Low number of animals are indicated between brackets.

<table>
<thead>
<tr>
<th>Year of cyclicity measurement</th>
<th>Year of birth</th>
<th>G0</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>End of generation structure</th>
<th>Estimation of genetic value</th>
</tr>
</thead>
</table>
3- Selection process and data set

First, in 1997 a total of 933 MA ewes, daughters of 176 rams were included in the experiment. Genetic values were estimated through a BLUP animal model from these ewes collected between 1995 and 1997 one, two or three times as the result of the replacement of culled or removed animals (Table 1). This BLUP animal model considered the physiological status (combining age and number of lambing) and live weight before mating as fixed effects and a random effect of animals. A random effect associated with the ewes was added in the model as permanent effect to take into account repeated records. Two groups of ewes (and rams) were chosen as the extremes of the overall genetic value distribution (Generation 0). This included 101 ewes frequently cycling in spring (group H) and 100 ewes that rarely cycled in spring (group L). These G0 ewes were mated with 5 G0 rams from the same selection group to produce females and males of the following generation (G1). The generations G1, G2 (G1 x G1), G3 and G4 were respectively born in 1998, 2000, 2002 and 2004 (Table 1). The G1, G2, G3 and G4 females were collected for the first time in April 2000, 2002, 2004 and 2006 respectively. During the selection process, genetic values have been estimated in 2005, 2006 and 2008.

The whole data set consisted of 4050 ovulatory activity records (0 or 1) measured in the first two weeks of April in the 15 consecutives years (i.e. from 1995 to 2009) of the experiment. All the adult ewes in the flock were blood sampled. Ewes could be studied usually over 1.5 to 8.5 years as the result of the replacement of culled or removed animal that generally occurred at 9 years-old.

4- Data analysis

4.1- Statistical model for descriptive statistics

The line effect and potential factors affecting the variation of the ovulatory activity, such as the year of test, the age of ewes and the live weight of ewes just before the mating period were studied. Live weight of ewes was analysed through five classes defined with thresholds of 41, 45, 49 and 55kg. A logistic model including fixed effects described above was fit with SAS software (v9) to test effects on spontaneous ovulatory activity.

4.2 Statistical models for genetic analyses
Ovulatory activity follows a discrete distribution (0 or 1). The most adapted statistical method of analysis would have been to analyse it at categorical data using a non-linear model. Two models: a linear and non-linear univariate mixed models will be used to analyse present data.

The linear model considers the trait as continuous and normally distributed. With this model it is possible to separately include the individual animal and permanent environmental effects, and also all available genetic relationships via an animal model. The linear model will be fit as:

$$Y_{ijklmn} = \mu + \text{line}_i + \text{year}_j + \text{age}_k + \text{weight}_l + \text{animal}_m + p_{mn} + e_{ijklmn}$$

(1)

where:

- $Y$ = spontaneous out-of-season ovulatory activity record (0, 1);
- $\mu$ = overall mean;
- line = fixed effect of the $i$th line of ewe;
- year = fixed effect of the $j$th year of measurement of SOA;
- age = fixed effect of the $k$th age of the ewe;
- weight = fixed effect of the $l$th weight of the ewe class;
- animal = random effect of the $m$th ewe (Variance = $\sigma_a^2$)
- $p$ = random effect of the $n$th permanent environment of the $m$th ewe (Variance= $\sigma_p^2$);

The permanent environment effect will be included to take into account repeated records.

- $e$ = random residual effect (Variance = $\sigma_e^2$)

Random factors are assumed normally distributed with the following expectation and variance-covariance structure:

$$\begin{pmatrix} a \\ p \\ e \end{pmatrix} \sim N \left( \begin{pmatrix} 0 \\ 0 \\ 0 \end{pmatrix}, \begin{pmatrix} A\sigma_a^2 & 0 & 0 \\ 0 & \sigma_p^2 & 0 \\ 0 & 0 & \sigma_e^2 \end{pmatrix} \right)$$

where $a$ is the random effect vector of animals, $p$ is the random effect vector of permanent environment associated with the ewe, $e$ is the random residual effect vector, $A$ is the numerator relationship matrix including animals; $\sigma_a^2$, $\sigma_p^2$ and $\sigma_e^2$ are the animal, permanent environment and residual variances, respectively.

The three variance components will be estimated with a single trait restricted maximum likelihood (REML) analysis fitted to the linear animal model described in equation 1. The
ASREML programme will be used. Genetic parameters, heritability ($h^2$) and repeatability ($r$), will be calculated from the estimated variance components as follows:

$$h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_p^2 + \sigma_e^2} \quad r = \frac{(\sigma_a^2 + \sigma_p^2)}{\sigma_a^2 + \sigma_p^2 + \sigma_e^2}$$

The best linear unbiased predictor (BLUP) for the fixed and the random effects of equation 1 will be computing using the variance components from the REML analysis.

The non-linear model provides a most accurate way to analyse discrete traits and uses the threshold model assuming that the observed binary (0, 1) variable is associated with an underlying normally distributed variable. Due to computational limitations, a sire effect is fitted in the model (Gianola and Foulley, 1983). All genetic relationships between sires are considered. The model used for the analysis will be:

$$Y_{ijklmn} = \mu + \text{line}_i + \text{year}_j + \text{age}_k + \text{weight}_l + \text{sire}_m + e_{ijklmn}$$

where:

- $Y$ = the underlying variable associated with the spontaneous out-of-season ovulatory activity record;
- $\mu$, line, year, age, weight = overall mean and fixed effects described in model 1;
- sire = random effect of the $m$th sire of the $n$th ewe (Variance = $\sigma_s^2$);
- $e$ = random residual effect (Variance = $\sigma_e^2$)

The expectation and variance-covariance structure will be as follows:

$$\begin{bmatrix} s \\ e \end{bmatrix} \sim \mathcal{N} \left( \begin{bmatrix} 0 \\ 0 \end{bmatrix}, \begin{bmatrix} \mathbb{A}_s \sigma_s^2 & 0 \\ 0 & 1 \end{bmatrix} \right)$$

where $s$ is the random effect vector of sires, $e$ is the random residual effect vector, $\mathbb{A}_s$ is the numerator relationship restricted to the sires and $\sigma_s^2$ is the sire variance. Estimates of the effects of the model and of variance components will be obtained as described by Gianola and Foulley (1983). Heritability for the underlying variable (liability) associated with SOA will be calculated as follows:

$$h_l^2 = \frac{4\sigma_s^2}{\sigma_s^2 + 1}$$

Heritability estimated on the underlying scale may be related to the heritability measured on the observed scale ($h^2$) using the expression proposed by Robertson and Lerner (1949):
Table 2: Number of MA ewes and spontaneous cyclicity (%) in early spring of the MA ewes in the selected lines L and H from 1995 to 2009.

<table>
<thead>
<tr>
<th>Year measurement</th>
<th>Ewes number</th>
<th>Cyclic ewes (%)</th>
<th>Chi-Square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lines</td>
<td>Total</td>
<td>Lines</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>H</td>
<td>L</td>
</tr>
<tr>
<td>1995</td>
<td>71</td>
<td>69</td>
<td>140</td>
</tr>
<tr>
<td>1996</td>
<td>100</td>
<td>94</td>
<td>194</td>
</tr>
<tr>
<td>1997</td>
<td>100</td>
<td>101</td>
<td>201</td>
</tr>
<tr>
<td>1998</td>
<td>100</td>
<td>101</td>
<td>201</td>
</tr>
<tr>
<td>1999</td>
<td>76</td>
<td>81</td>
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</tr>
<tr>
<td>2000</td>
<td>84</td>
<td>90</td>
<td>174</td>
</tr>
<tr>
<td>2001</td>
<td>82</td>
<td>104</td>
<td>186</td>
</tr>
<tr>
<td>2002</td>
<td>70</td>
<td>103</td>
<td>173</td>
</tr>
<tr>
<td>2003</td>
<td>64</td>
<td>114</td>
<td>178</td>
</tr>
<tr>
<td>2004</td>
<td>63</td>
<td>126</td>
<td>189</td>
</tr>
<tr>
<td>2005</td>
<td>74</td>
<td>135</td>
<td>209</td>
</tr>
<tr>
<td>2006</td>
<td>89</td>
<td>121</td>
<td>210</td>
</tr>
<tr>
<td>2007</td>
<td>77</td>
<td>78</td>
<td>155</td>
</tr>
<tr>
<td>2008</td>
<td>74</td>
<td>79</td>
<td>153</td>
</tr>
<tr>
<td>2009</td>
<td>92</td>
<td>85</td>
<td>177</td>
</tr>
<tr>
<td>Total</td>
<td>1216</td>
<td>1481</td>
<td>2697</td>
</tr>
</tbody>
</table>

Table 3: Number of MA ewes and spontaneous cyclicity (%) in early spring of the MA ewes in selected lines L and H by age.

<table>
<thead>
<tr>
<th>Age</th>
<th>Ewes number</th>
<th>Cyclic ewes (%)</th>
<th>Chi-Square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lines</td>
<td>Total</td>
<td>Lines</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>H</td>
<td>L</td>
</tr>
<tr>
<td>1.5</td>
<td>235</td>
<td>312</td>
<td>547</td>
</tr>
<tr>
<td>2.5</td>
<td>215</td>
<td>257</td>
<td>472</td>
</tr>
<tr>
<td>3.5</td>
<td>195</td>
<td>225</td>
<td>420</td>
</tr>
<tr>
<td>4.5</td>
<td>179</td>
<td>202</td>
<td>381</td>
</tr>
<tr>
<td>5.5</td>
<td>152</td>
<td>179</td>
<td>331</td>
</tr>
<tr>
<td>6.5</td>
<td>112</td>
<td>145</td>
<td>257</td>
</tr>
<tr>
<td>7.5</td>
<td>80</td>
<td>98</td>
<td>178</td>
</tr>
<tr>
<td>8.5</td>
<td>43</td>
<td>57</td>
<td>100</td>
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<tr>
<td>9.5</td>
<td>4</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>10.5</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>1216</td>
<td>1481</td>
<td>2697</td>
</tr>
</tbody>
</table>
\[ h_t^2 = h^2 z^2 / p(1-p) \]

where \( z \) is the value of the density of the underlying normal distribution at the threshold point \( p \), corresponding to the mean percentage of ewes in ovulatory activity.

### III- Results and discussion

#### 1- Descriptive statistics and sources of variation

The number of ewes present in each line from 1995 to 2009 is given in table 2. At the onset of the selection process (i.e., 1997), the groups L and H were constituted of 100 ewes each. Since 1998, year of the first generation of selection, on average, the line L was constituted of approximately 80 ewes and the line H was constituted of 100 ewes.

The variation of spring spontaneous cyclicity of the MA ewes from 1995 through 2009 are described in table 2 for both divergent lines L and H. Logistic model showed that the divergent lines presented a large and significant difference for cyclicity throughout the experiment (Line effect, \( p < 0.0001 \)) and a significant effect of the year of measurement on the percentage of cyclic ewes (Year of measurement effect, \( p < 0.0001 \)). The performances of cyclicity reported in 1995, 1996 and 1997 widely differed between both lines L and H. On average over the three years, 2.1% of the founder ewes of the L line presented a spontaneous ovulatory activity (SOA) against 68.3% for the H line. These animals correspond to the founders of the divergent lines H and L and were used to establish the genetic values at the origin of the selection of the G0 ewes. They were chosen as the extremes of the overall genetic value distribution and therefore naturally widely differed for cyclicity performances after repartition in the L and H groups. On average over the 1998-2009 period, 27.8% of the ewes of the L line presented a SOA against 52.1% for the H line. A significant interaction between line and year of measurement was found (\( p < 0.0001 \)). For each year, the percentage of ewes with ovulatory activity in early spring was always higher for the H line than for the L line but with a positive difference varying with year of measurement and ranging from 4.6% (2005) to 37.7% (2000). Pair wise comparisons indicated that the line difference was not significant in 2005. Such results confirm the repeatability of this trait. However, the between line difference has dramatically decreased last two years and the percentage of ewes with ovulatory activity in early spring reached very low levels in 2009 in both lines: 2.2%
Table 4: Number of MA ewes and spontaneous cyclicity (%) in early spring of the MA ewes in selected lines L and H by Live weight of ewes just before the mating period.

<table>
<thead>
<tr>
<th>Live weight before mating</th>
<th>Ewes number</th>
<th>Cyclic ewes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lines</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>H</td>
</tr>
<tr>
<td>&lt;41</td>
<td>243</td>
<td>310</td>
</tr>
<tr>
<td>[41,44]</td>
<td>227</td>
<td>305</td>
</tr>
<tr>
<td>[45,48]</td>
<td>268</td>
<td>339</td>
</tr>
<tr>
<td>[49,54]</td>
<td>318</td>
<td>344</td>
</tr>
<tr>
<td>&gt;=55</td>
<td>160</td>
<td>183</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>1216</td>
<td>1481</td>
</tr>
</tbody>
</table>
and 12.9% in the L and H line respectively. These values are lower than the percentage of MA ewes with SOA in spring (i.e., 27.9%) reported in the experimental flock present at the experimental farm Le Merle from 1995 to 1997 (Hanocq et al., 1999) and also lower that the 29% of the Chios breed ewes exhibiting spontaneous out-of-season ovulatory activity in early May (Avdi et al., 2003).

A significant effect of age on the percentage of cyclic ewes was also found (Age effect, p <0.0001) without significant interaction with line. An increase of about 24.5% in the ovulatory activity was observed in 1.5- to 8.5-year-old ewes in both L and H lines (Table 3). Classes of age 9.5 and 10.5 were not discussed due to the low number of ewes in these classes. The maximum percentage of cyclic ewes was found at the age of 8.5 years in the L line (i.e., 37.2%) and 6.5 years in the H line (i.e., 71.0%). After 6.5 years of age, a slight decrease was found in the H line. The present results were consistent with the BLUP estimates for the effect of age of ewes on spring ovulatory activity previously reported in MA ewes (Hanocq et al., 1999). Hanocq et al. (1999) reported in non selected MA ewes an increase of about 8-9% in the ovulatory activity for 2.5 to 4.5-year-old ewes. Interestingly, higher increases in the ovulatory activity for 2.5 to 4.5-year-old ewes were found in our study in the L and H selected lines (i.e., 17.4% and 12.7% respectively). The youngest females included in the analysis were 18 months old. Thus performance of reproduction was independent of any influence of puberty. Adult ewes (with at least one previous lambing) and hoggets were consequently analysed together. A confounding effect can occurred between age and physiological status effects on ovulatory activity. Physiological status effect could be tested by distinguishing young ewes that never lambed before, the ewes that lambed before but not in the previous season; and the ewes that lambed for the first, second, third, fourth, fifth, sixth and seventh time in the previous season.

A significant effect of the ewe’s live weight on the spontaneous out-of-season ovulatory activity (Live weight effect, p <0.0001) was found without interaction with the line effect. Percentage of cyclic ewes in spring increased with the increase in the live weight of ewes in both lines (Table 4). A difference of 18.8% and 34.7% in SOA was observed between extreme classes in L and H lines, respectively. Similar effect of live weight on SOA was previously reported in Chios and MA breeds showing that heavier ewes had in general higher rate of spontaneous ovulatory activity (Hanocq et al., 1999; Avdi et al., 2003). In non selected MA ewes, BLUP estimates effect of live weight just before the mating period on SOA showed an almost null effect below 49kg and a marked positive effect for higher weight (Hanocq et al., 1999). They reported a lower difference in SOA (i.e., 9%) between extreme
classes than differences found in the present study. Age and live weight were strongly correlated factors. Interaction between age and live weight was statistically significant and will further investigated. Joint effect of age and live weight on SOA in both lines will be investigated by fitting BLUP estimates for this joint effect using a linear animal model.

Other sources of variation in SOA might be investigated since many different records are available such as the lambing date of ewes in the previous autumn, the number of lambs suckled, the litter size. Post-partum interval since the previous lambing has been reported to significantly affect SOA in the Chios breed (Avdi et al., 2003). The highest rates of SOA were measured in the ewes that had the longest periods between lambing and testing and the lowest for those that had the most recently lambed. Even if no significant effect of the lambing date in the previous autumn on SOA has been reported in non selected MA ewes (Hanocq et al., 1999), this might be tested in the divergent selected lines L and H. It should be noted that all ewes in the present study, whether they had exhibited spontaneous out of season ovulatory activity or not in the previous season, were exposed to the ram at the same time. Non-seasonal breeders were thus not used in an early mating. Therefore, no confounding between spontaneous out-of-season ovulatory activity and date of previous lambing should be expected in the present study.

2- Genetic analysis

Genetic analyses are currently in process and will not be presented and discussed here. Nevertheless, previous results about spontaneous out-of-season ovulatory activity obtained using similar approaches (i.e., linear animal model or threshold sire model) are discussed.

The genetic profile of spontaneous out-of-season ovulatory activity has been determined in the Chios and Mérinos d’Arles (MA) breeds. Heritability estimates of 0.216 and 0.291 were found in the Chios breed using linear and threshold models, respectively (Avdi et al., 2003). Similar results have been reported in the MA breed with heritability estimates of 0.20 and 0.37 obtained with a linear and a threshold model, respectively (Hanocq et al., 1999). Heritability was higher with the threshold sire model than with the linear model. This is an expected result in accordance with the theoretical comparison of both methods and the categorical nature of the trait studied.

Simulation studies indicated that efficiency of selection was usually unchanged when using a linear method of evaluation or the threshold method. Furthermore, in both studies, estimate with the threshold model was similar to the expected heritability value obtained by Robertson and Lerner formula (Robertson and Lerner, 1949). Concerning repeatability estimate, the
linear model repeatability was 0.23 and 0.30 in the Chios and MA breeds, respectively. These values were quite close than to the heritability estimate indicating that the source of the estimated repeatability was mainly genetic. These results were consistent with the high number of ewes that exhibited SOA in successive years.

Heritability estimates were quite high and supported the efficiency of a selection for SOA. Heritability and repeatability values that will be estimate in the divergent lines L and H using the models described in the present study will be compared to these previous results described above.

Heritability estimates of other seasonal reproduction traits such as for the date of onset, the date of cessation, or the duration of the breeding season have been found between 0.20 and 0.35 (Fahmy, 1982; Quirke and Hanrahan, 1985). These values were higher than those obtained for fertility trait (almost always below 0.10) (for review, see Notter, 2008). Fertility trait may be considered as a more complex trait than the spontaneous out-of-season ovulatory activity. Fertile ewes in spring gather those which are already cycling and present spontaneous ovulatory activity (SOA) with those which are on their seasonal anoestrous but respond to a male effect. Both events imply different physiological and genetic mechanisms.

Relationships between spontaneous out-of-season ovulatory activity and reproductive parameters corresponding to the following mating period (fertility, prolificacy, litter size...) will be investigated. Estimates of genetics relationships between characteristics of seasonality and reproductive parameters are quite scarce. No genetic relationships or positive genetic correlation between the date of onset of the breeding season and litter size have been reported (Purser, 1972 cited by Hanocq et al., 1999; Owen et al., 1986). Negative genetic correlations between spontaneous out-of-season ovulatory activity in spring and prolificacy have also been reported (Dzabirski and Notter, 1989).

In the present study, the spring ovulatory activity just before mating was used as criteria to measure the out-of-season breeding ability of ewes. One can ask whether the spring ovulatory activity recorded corresponds to the onset, the middle or the end of the breeding season, a sporadic ovulatory activity or a continuous activity of aseasonal ewes. Therefore, even with later or earlier tests, there is a risk to select either ewes with only a sporadic activity preceded or followed by anoestrus period or ewes with a complete shift breeding season without any increase in the duration. Since the objective is to select truly aseasonal ewes, measurement of the ovulatory activity during the full out-of-season breeding period appears necessary. This is proposed in the project of QTL mapping for SOA described in the first part.
of the manuscript in which a more detailed analysis of the spontaneous ovulatory activity of ewes throughout the year is planned.

**Conclusion**

These very preliminary phenotypic results show the possibility and efficiency of a selection for spontaneous spring ovulatory activity in the MA breed and probably other sheep breeds. The high proportion of ewes cycling out-of-season presents the opportunity to better manage the breeding period and reproduction of the flock. However, results also show the difficulty to select for this trait and efforts that have to be done to attempt to increase the divergence between lines. In addition, SOA measurement is technically difficult and only feasible in experimental farm. Therefore, genomic approach and more particularly QTL detection for seasonality appears necessary to develop future efficient selection for seasonality. Moreover, further physiological and genetic studies on the mechanisms controlling seasonal breeding would help to investigate the consequences of such selection and might improve efficiency of selection.
References


