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"SPRING LEG DEFECT": a genetic cause is now indisputable!

FLASHBACK…Since the early 2000s, purebred sheep breeders have noticed the emergence of a new clinical entity in their herds. Affected animals present a hyperflexion of one or both hind limbs when moving at a slow pace. Breeders, amongst themselves, quickly started using the term "spring leg" to describe this defect since the hind limb of affected animals would lift off the ground abnormally as if the animal had a cramp. From an animal breeding point of view, affected animals do not seem to have poor growth performance. Breeders had therefore numerous questions: What is this defect? What causes it? Is the defect muscular or neurological? How is it transmitted? Is it genetic? Should affected animals be culled to prevent the spread of this problem in the sheep industry? But the most common question was: how can we detect this defect even before its onset? This paper answers many of these questions!

A PRELIMINARY PROJECT PROVIDING NEW HYPOTHESES. Since there was no information in the literature, CEPOQ in partnership with the *Société des éleveurs de moutons de race pure du Québec* (SEMRPQ), the Faculty of Veterinary Medicine (FVM) at the University of Montreal and the Canadian Centre for Swine Improvement (CCSI), carried out a preliminary study between 2009 and 2011[3]. This first study, which was carried out on Dorset lambs from the CEPOQ herd as well as different individuals contributed by breeders in Quebec (3 distinct breeds), showed that the defect was erroneously described as "cramping – spring leg" since no cramps had been detected by the team of veterinarians that carried out medical tests. A genealogical study on CEPOQ lambs suggested that a gene may be responsible for the defect since almost a quarter (22.2%) of lambs born from the same affected ram developed the problem. This observation suggested that the defect may be hereditary, transmitted by an affected ram to its progeny. This hypothesis could not be confirmed or validated even though several common ancestors were identified in pedigrees of affected individuals (at CEPOQ and in other herds in Quebec). In fact, the low number of individuals used in the preliminary study and the absence of genetic tests (DNA analyses) did not allow us to draw conclusions on this transmission factor. On the other hand, despite the numerous tests carried out on animals at the University of Montreal's FVM, it was not possible to identify specific lesions through necropsy. In light of all these completed analyses, the most probable hypothesis forwarded by veterinary neurologists was that the defect may be neurological. This hypothesis, just like the one that a genetic factor may explain the onset of the condition, could unfortunately not be confirmed in the preliminary project, which was exploratory. These hypotheses still needed validation...this paved the way for a new project which is presented in this paper.

« SPRING LEG » PROJECT 2012-2013. To validate the hypotheses that were brought forward by veterinary neurologists and geneticists, a larger project was carried out using funds received by the Canadian Agricultural Adaptation Program (CAAP). Access to new technologies, both in veterinary medicine and genomics, has allowed for more comprehensive work on various hypotheses raised in the preliminary study. The main objective of this study was to identify the causes of the defect through the validation of hypotheses raised in the preliminary project and to identify early detection methods of spring leg on live animals.

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METHODOLOGY. To adequately study the condition, two groups of lambs were produced following planned matings in the CEPOQ research herd. The CEPOQ herd is a closed herd since its creation in 1998, made up of purebred Dorset ewes.. The "spring leg" condition is quite common in this herd and several cases of the defect are reported every year. Animals from this herd were therefore ideal for this project. Initially, services were planned to produce a group of animals at a higher risk of developing the condition. The amount of risk was based on the presence of ancestors in the pedigree of selected CEPOQ rams and ewes, suspected being carriers of the defect as well as the presence of the defect in rams and ewes themselves (affected breeding stock). During the preliminary project, several common ancestors, which may have been carriers of one or more gene(s) responsible for the condition, were identified. With this information, it was possible to select ewes and rams having a greater chance of transmitting the gene(s) to their offspring. To maximize the number of breedings and obtain more variations with regards to genotypes, a maximum number of rams were used. In total, 20 rams were used to produce lambs from both experimental groups.

Planned matings produced a total of 150 lambs (72 males and 78 females) assigned to two experimental groups: AT RISK and at LOW RISK of developing the condition. It was not possible to have a control group with no risk of developing the condition. Since the CEPOQ herd hadn't introduced any new Dorset lines since its creation in 1998, the majority of animals were, in the least, distantly related to one of the problem ancestors. Table 1 presents the number of lambs produced within each experimental group.

Table 1. Number of male and female lambs allocated to each of the experimental groups based on different matings carried out during the project.

	AT RISK		**LOW RISK**	
	Males	**Females**	**Males**	**Females**
Affected rams	33	41	-	-
Affected rams and ewes	2	6	-	-
Unaffected rams*	2	4	32	28
Unaffected rams/affected ewes	3	0	-	-
TOTAL	**40**	**51**	**32**	**27**

*Unaffected rams in the AT RISK group had a very high probability of transmitting the defect to their progeny based on the presence of several problematic ancestors in their pedigree.

Following weaning, lambs were separated into their experimental groups, allocated by sex and weight to dry lots (10 to 12 lambs / pen). From that moment on, lambs were weighed on a weekly basis and subjected to a detailed observation of their gait (using video monitoring) to detect any signs of abnormal posture or gait that could lead to confirming the spring leg defect. Following weaning, lambs were monitored closely over a maximum period of 58 weeks.

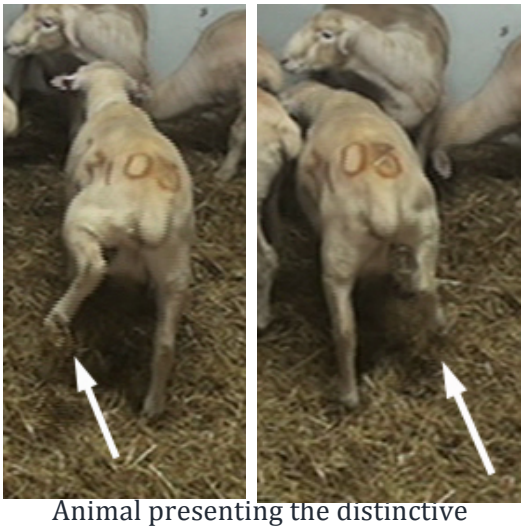
To proceed with genomic analyses, a DNA sample (blood or ear punch) was collected from all rams and ewes used in planned matings as well as from lambs produced by these breedings. All affected individuals present in the CEPOQ herd, which were not used as part of the project, were also sampled. Finally, since the project also required the collaboration of breeders, several purebred animals affected by the defect and from different farms across Quebec were sampled (2 distinct breeds). All samples were stored at -80°C and were afterwards carefully selected for genomic analyses. In total, 192 samples (105 normal individuals, 50 affected and 37 suspect animals) were selected and sent to the Delta Genomics laboratory located in Edmonton (Alberta). This laboratory extracted the DNA from samples and performed genomic analyses using the 600K ovine SNP chip

(Single Nucleotide Polymorphism-SNP). SNP chips have several thousand DNA sequences fixed on a slide and allow for the identification of different alleles at a precise location. Results of genomic analyses were afterwards interpreted and analyzed by geneticists at the Canadian Centre for Swine Improvement. The main objective of genomic analyses was to determine if a defect detected as a DNA polymorphism could be found in the genome of affected individuals. In order to complete genetic analyses, all genealogical information on monitored and sampled animals as part of the project were used to perform a pedigree analysis.

In order to validate the hypothesis of a neurological cause, twelve lambs (6 affected and 6 normal) as well as 4 adult rams severely affected by the defect, were sent to the Faculty of Veterinary Medicine at the University of Montreal for a series of tests. Upon their arrival, blood samples were collected by veterinary neurologists to perform biochemical and hematological tests. Different tests were afterwards carried out on anesthetized animals including a cytological exam (urine analysis), different electro-diagnostic measures (EMG, *Nerve conduction velocity, Somatosensory evoked responses, BAER),* a computed tomography scan of the whole body with iodine contrast, magnetic resonance imaging of the spine and finally an analysis of cerebrospinal fluid. Following all these tests and sampling, animals were euthanized. Following death, muscle and peripheral nerve biopsies were collected prior to a full necropsy and examination of the spinal cord.

RESULTS.

Identification of early signs associated with the spring leg defect. To detect the spring leg defect, special attention was paid to the movement of hind limbs in animals that were freely moving around in pens. As early as the first observations, an abnormal gait was observed in several animals. Thus, we defined different statuses: NORMAL, AFFECTED, SUSPECT or VERY SUSPECT. Suspect and very suspect animals had a jerky gait, which was distinct from normal animals (unaffected individuals that never developed the defect during the project). In normal and healthy animals, the gait is smooth and hind limbs are placed gently on the ground in an even, normal stride movement. At the end of each stride, limbs are placed in a cross (see Figure 1). However, when suspect animals move around, the hind limb is raised off the ground, dropped heavily in a half-stride and placed in parallel to the opposite hind limb (hind limbs never cross at the end of a stride, but are always placed one right next to the other). "Step-stop" or "lift-stop" was the characteristic term given to this type of movement. In very suspect animals, the stride of the animal looked even slow and maintained.



Animal presenting the distinctive hyperflexion of the spring leg defect.

Only individuals showing the distinctive hyperflexion of hind limbs could be confirmed as affected. As soon as an animal's gait was described as suspect or very suspect, this observation was noted. Thus, during the project, except for one animal that was affected at weaning, all individuals that developed the condition had "suspect" or "very suspect" gait observations prior to being confirmed as affected by the defect. The delay between the observation of a suspect gait and confirmation of the condition in the animal varied between individuals, but was on average 2 to 3 months following the onset of the first signs of an abnormal gait.

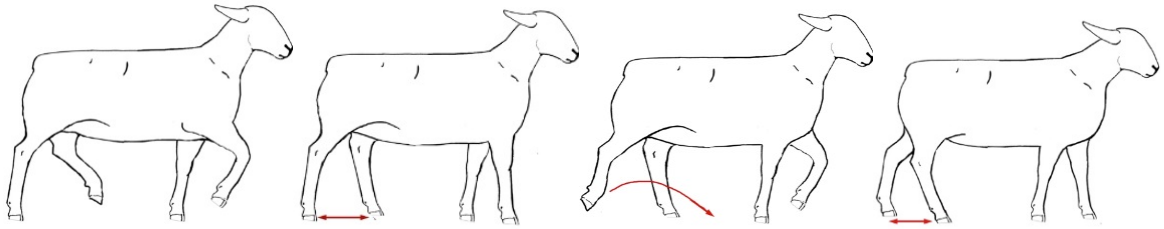


Figure 1. Sketches of an animal presenting a smooth and normal gait when walking.

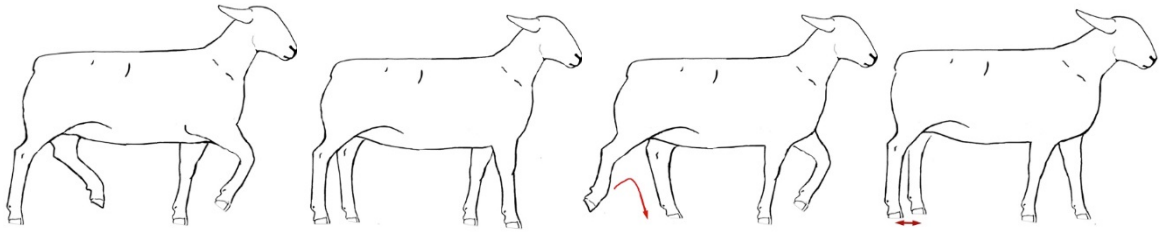


Figure 2. Sketches of an animal with a suspect and jerky gait when walking (step-stop or lift-stop).

Aside from the abnormal gait detected in suspect animals, individuals in the process of developing the condition moved little in pens, standing still longer than other unaffected subjects. These animals adopted a distinctive posture different from healthy animals. Their hind limbs were placed towards the back, away from the body, they had hollow backs and their weight shifted on to the fore limbs. In several cases, animals moved their fore limbs without their hind limbs following the stride. All these new observations constitute new signs that could help breeders detecting the condition at an early age in "at risk" individuals.

Frequency of affected individuals and pedigree analysis. In total, following planned matings, a total of 23 lambs developed the spring leg defect during the observation period. It should be noted that two males initially in the "low risk" group developed the defect during the project. It is possible that these two males inherited the defect from an ancestor that was not previously identified as problematic during planned matings. A slightly higher number of males were affected than females (14 males vs. 9 females). Moreover, the onset of clinical signs related to the condition does not appear to be sex related since the defect appeared in the same age ranges in males and females. On average, animals developed the defect at about 200 days of age, but it varied considerably between individuals (confirmation of the defect occurred at 65 to 258 days of age). Table 2 shows the frequency of affected individuals during the project.

Table 2. Number and frequency of affected, suspect, very suspect or normal individuals based on their initial experimental groups.

STATUS	AT RISK		LOW RISK	
	No	Frequency	No	Frequency
AFFECTED	21	23.1 %	2	3.4 %
SUSPECT	16	17.6 %	9	15.2 %
VERY SUSPECT	14	15.4 %	2	3.4 %
NORMAL	40	43.9 %	46	78.0 %
TOTAL	**91**		**59**	

The frequency of affected individuals was almost similar to the frequency observed in the preliminary project, where almost a quarter (22.2 %) of lambs sired by an affected ram developed the spring leg defect. It is also interesting to note that the number of animals with a perfectly normal gait neared 80% in the low risk group whereas in the at risk group, less than 45 % of individuals had a perfectly normal gait. The contrast in results between both experimental groups confirms the hypothesis of genetic transmission of the defect, but does not provide more information about the defect's precise determinism (one or more genes, recessive or dominant). Since parents of different statuses were used in the matings, it was relevant to assess the frequency of affected individuals from these breedings (Table 3).

Table 3. Number of lambs and frequency of affected subjects in the AT RISK group as a function of the status of their parents (rams and ewes affected or unaffected by the defect).

STATUS	Affected ram*		Affected ram and ewe		Normal ram		Normal ram and ewe	
	No	%	No	%	No	%	No	%
AFFECTED	15	20.3 %	4	50.0 %	0	0.0 %	2	66.7 %
SUSPECT	14	18.9 %	1	12.5 %	1	16.7 %	0	0.0 %
VERY SUSPECT	11	14.9 %	2	25.0 %	1	16.7 %	0	0.0 %
NORMAL	34	45.9 %	1	12.5 %	4	66.6 %	1	33.3 %
TOTAL	**74**		**8**		**6**		**3**	

** When the status of the ewe was not listed, they were normal.*

Although the number of lambs is variable between parents of different statuses, the risk of a lamb developing the condition is clearly greater when one or both parents are affected by the condition. Indeed, no lambs born from unaffected rams and ewes developed the defect even if they were in the "at risk" group. These data show that ewes affected by the spring leg defect should not be kept for breeding since they could also contribute to the spread of the defect. Results observed in the sheepfold, notably with regards to individuals confirmed as affected, supported the hypothesis that this defect may be hereditary. Pedigree analyses performed on all genealogies confirmed the existence of a transmission pattern for the spring leg defect, which would appear to be linked to a gene. The defect is therefore transmitted genetically.

New observation: effect of stress on the development of the defect. During the project, a new observation was made at CEPOQ and at the University of Montreal's FVM. Both research teams noticed that following an important source of stress (shearing, hoof trimming, transportation), several lambs suddenly developed the condition or, if they were already affected, the severity of their condition worsened. Besides, in the CEPOQ herd, in parallel to the lambs monitored during the project, two females that were never previously confirmed as affected developed the defect on the day following a difficult lambing. The hypothesis that stress could speed up the onset or increase in severity of the condition constitutes a new discovery and could be used by breeders who suspect that this defect may exist in their herds. The exposure to a stressor could potentially help breeders detect the defect at an early age in "at risk" animals.

Conformation and spring leg. Just like in the preliminary project, no conformation trait was linked to the spring leg defect since affected animals just like healthy ones could have similar conformation flaws or qualities. In the CEPOQ herd, several lambs monitored in both experimental groups (at risk and low risk groups) had splayfooted hind limbs. Splayfooted animals have naturally an unsightly

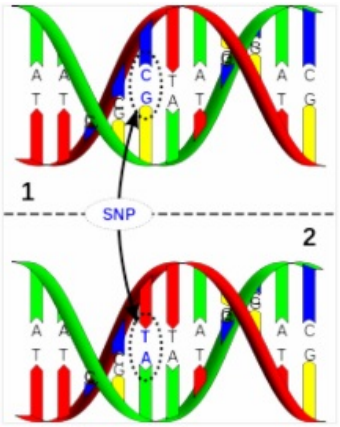
gait caused by their flawed conformation. Even if we could describe their gait as not very smooth, this defect of the hind limbs should not be confused with typical signs of spring leg. In other words, to confirm the spring leg status in splayfooted animals, they have to present the distinctive hyperflexion of the hind limbs associated with spring leg.

Clinical test results. Even though significant results were obtained in magnetic resonance and imaging to discriminate healthy animals from affected ones, other tests were not as conclusive. Thus, the series of tests performed by the FVM team at University of Montreal did not, for the moment, specify the neurological location of the lesion. For that matter, since veterinary neurologists raised the hypothesis that unaffected animals could actually be clinically healthy animals that haven't yet developed the defect (no null risk in the transmission of the defect in CEPOQ individuals) and that only significant differences were visible in affected animals, it seems unlikely that these tests can help breeders and veterinarians to detect the defect prior to onset of the first clinical signs. To date, results from these analyses suggest instead that only magnetic resonance imaging could possibly confirm this problem in already affected individuals.

Genomic analysis results. Association analyses carried out as part of the genomic study identified three SNP markers significantly associated with spring leg in sheep. The significant SNPs are located on chromosome 24, at positions 1341130, 1343567 and 1358660. With the goal of exploring the relationship between the spring leg defect and the frequency of significant SNP markers in healthy and affected animals, the frequencies of the three significant SNPs on chromosome 24 were calculated across different genotypes and alleles. The following table presents different genotypes where polymorphisms were significantly associated to the spring leg defect.

Genomic analysis: Three SNP markers significantly associated with the spring leg defect.

A SNP or single nucleotide polymorphism (pronounced "snip"), is a variation in the DNA sequence (A, T, C or G) at one nucleotide between two individuals of the same species and at the same location on a chromosome.



DNA molecule 1 differs from 2 by a single nucleotide (C/T polymorphism). In this case, there are two alleles CG and TA.

Table 4: Frequency of the two significant haplotypes (AGG and GAA) on chromosome 24 in sick and healthy animals.

Genotype[1]	AGG/AGG	AGG/GAA	GAA/GAA
Healthy	23	60	17
Affected	3	13	25

[1]Only significant genotypes (AGG and GAA) are shown.

Table 4 shows that a very low number of affected animals are carriers of the AGG/AGG genotype compared to healthy animals. Based on these frequencies, it seems possible to select against the GAA allele to reduce the incidence of spring leg. The numerous completed tests show that SNPs associated with spring leg might be linked to other genes located in the same area of the genome.

Thus, to study the possible relationship between observed mutations in animals with spring leg and other genes located in close proximity of these mutations, a functional analysis was carried out. This analysis found that the three studied mutations on chromosome 24 were located close to 34 genes. Since information on

gene functionality is very limited in sheep, human orthologues (same genes in another species) were studied. One of the genes, NDUFB10, was located close to the significant SNP markers. This gene is involved in different processes that cause diseases such as Parkinson's, Alzheimer's and Huntington's disease. Parkinson's disease is a degenerative disorder of the central nervous system, in which the most obvious symptoms are movement-related. Studies have shown that the NDUFB10 gene is also associated with Huntington's disease, which is a neurodegenerative genetic disorder that affects muscle coordination. Considering the effects of NDUFB10 gene in humans, it is also possible that a variation on this locus could be associated with the spring leg defect in sheep but it is also possible that many genes may be involved in causing the defect, just like in the case of Parkinson's disease.

Results suggest that the spring leg defect could be associated with several SNPs across the genome. Some of the SNPs are promising and could be used in selecting against spring leg, but it is necessary to continue genotyping more animals to estimate marker effects in a larger population and perform validation tests. It is also recommended to explore in detail the regions of the genome that contain significant SNPs as a means of understanding the process involved in causing the spring leg defect and to identify one or more causative mutations leading to the defect. A simple validation test could consist of looking at the frequency of the three significant SNPs on an independent dataset of healthy animals completely independent from CEPOQ animals. This would help to accurately confirm the alleles linked to the spring leg defect. In the medium term, if validation confirms these findings, a simple, affordable DNA test based on the three significant SNPs could be developed since SNP genotyping currently only costs a few cents per marker. Alternatively, if sheep breeders adopt SNP genotyping (with 50K or 600K chips) for their breeding animals, they could select against the risk of spring leg while benefiting from all the other markers on the chip to select for other traits of interest (growth, reproduction, etc.).

IN CONCLUSION ... Carrying out this project enabled to reach the objectives that were targeted at the start of the project. Through leading edge technologies, it was possible to address different hypotheses that were raised. We now know that there is a genetic component explaining this problem, which explains the apparent mode of transmission of spring leg in sheep herds. Collection of DNA samples and genomic analyses are keys to reaching this new objective.

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