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Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov

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Summary. Information from a scrapie epidemic in a closed INRA Romanov flock is presented. Performances, pedigree, histopathological diagnoses and *PrP* genotypes were recorded from the beginning of the outbreak (in 1993). Between 1st of April, 1993 and 1st of May, 1997, 1015 animals were exposed to scrapie, and 304 died from this disease. A major influence of the polymorphisms at codons 136, 154 and 171 is shown, $A_{136}H_{154}Q_{171}$ allele carriers proving to be nearly as resistant as $A_{136}R_{154}R_{171}$ carriers. A possible relationship between gastrointestinal parasitism and scrapie is discussed. There is evidence of maternal transmission, with a risk ratio for artificially fed lambs of 67 percent of the risk of lambs fed by their mother. Our results strongly suggest that resistant animals were not healthy carriers or at least were less infectious when comparing risk for lambs born to healthy dams either of resistant (risk = 0.431) or of susceptible (risk = 1.000) genotype.

Introduction

Scrapie is a fatal neurodegenerative disease of sheep and goats belonging to the group of transmissible, subacute, spongiform encephalopathies (TSE) such as the bovine spongiform encephalopathy (BSE) in cattle and the Creutzfeldt-Jakob disease (CJD) in humans.

TSEs are characterised by the accumulation of an abnormal form of a host-encoded protein, PrP, in the central nervous system of affected individuals.

It has been known for a long time that resistance/susceptibility of sheep to scrapie is largely under genetic control. An essential contribution to this knowledge was made by the experimental transmission works started in the UK in 1938 ([6], for an historical review). Sheep lines were selected on their scrapie response following injection of standardised infection sources, such as ⟪SSBP/1⟫ – Sheep Scrapie Brain Pool n° 1-made of pool of brains from scrapie animals.

Polymorphisms of *PrP* (the gene encoding PrP) were rapidly found to be associated with differences in artificially induced susceptibility (NPU Cheviot animals injected with the SSBP/1 isolate). These polymorphisms are located at codons 136, 154 and 171.

All possible combinations of the 3 codons 136 (A/V), 154 (R/H) and 171 (Q/R/H) were not found. The wild type allele is probably ARQ (simplified notation for $A_{136}R_{154}Q_{171}$) and 4 mutated alleles have been detected so far (VRQ, AHQ, ARH, ARR), each of them deriving from the wild allele by a single codon variation. The general picture is that scrapie susceptibility is conferred by the VRQ gene and resistance by ARR and AHQ genes, with an intermediate situation for ARQ and ARH. Extreme alleles VRQ, AHQ and ARR are dominant over intermediate ones, with a reduced susceptibility in heterozygous VRQ/ARQ and VRQ/ARH as compared to VRQ/VRQ. A few Suffolk [23, 24] were the only exceptions found to this rule. Dominance relation between resistant (ARR, AHQ) and susceptible (VRQ) alleles are variable, but in most cases, VRQ/ARR and VRQ/AHQ are resistant. An excellent review of genetics of scrapie susceptibility was given by [19].

It has been known for a long time that the scrapie agent itself is polymorphic, and artificial infection has proved that interactions exist between host genotypes and scrapie strains. Multiflock studies mix all these variables and results may be difficult to interpret. As suggested previously [23], single flock case-control studies tend to give less complex results, and should be preferred when possible.

A drawback of most previous analyses is the lack of statistical analyses accounting for the effect of phenomena such as time at birth in relation to the first scrapie event in the flock(s), birth rank, dam scrapie status etc. These nuisance effects should be accounted for in the evaluation of *PrP* genotype effects on scrapie susceptibility. Survival analysis [5] gives a consistent framework and has been successfully used for exploring the genetics of disease resistance (e.g. [32]).

The present paper analyses a scrapie outbreak which started in 1993 in a single closed flock of Romanov (INRA Langlade farm), previously considered as scrapie free. Since the first observations made on a limited number of cases [4], it has been possible to get the *PrP* genotype of nearly all the animals living on the farm at the start of the epidemic, and of all reproducers born later. All clinical suspicions of scrapie were confirmed by histopathological detection of spongiform changes of brain. Our description includes survival analyses which allowed for evaluation of *PrP* genotype, maternal transmission and other effects.

Materials and methods

Animals

The studied flock was managed by INRA on the ⟪ Langlade farm ⟫, near Toulouse, France. The flock, created in 1971, was genetically closed from 1979 up to 1996. A few Berrichon du Cher rams from other farms were nevertheless used during this period as terminal sires for meat production but none of their progeny had been kept for reproduction. All the animals were carefully identified and the pedigree and production information have been recorded by INRA staff since creation of the flock. Before 1996, most of the animals belonged to the Romanov breed. Romanov is a prolific breed, with a mean litter size of 3.1 in adults, ranging between 1 and 6. The general rule was to leave no more than 2 lambs to be fed by their dam, extra lambs, in large litter size, being given artificial milk for 5–6 weeks, after 24 h spent with the mother in an isolated lambing compartment.

The Langlade flock was used for various scientific purposes including the evaluation of genetic variability of ovulation rate and embryonic mortality using laparoscopy and the evaluation of genetic variability of resistance to a parasite, *Teladorsagia circumcincta*. Detailed information on these experiments are given elsewhere [3, 15, 30].

In 1991, the experiment aiming at measuring genetic susceptibility to *Teladorsagia*, concerned two batches of lambs born in October: the first, 116 lambs (50 males, 66 females), received 3 doses of 20 000 L3 between 6 and 10 months of age, the second, 111 males, was sent to another farm where animals grazed an artificially infested pasture, for a period of 5 months. In March 1993, 18 rams were selected in the first group, 17 in the second group and all were gathered in Langlade for reproduction purposes. From the 66 females challenged in Langlade, 44 were kept for reproduction at 1 year of age.

The first signs of scrapie appeared in April 1993 in both batches of the sub-flock of animals assigned to parasite resistance studies (P flock), with the first scrapie-animals being found in the main flock (NP flock) in August 1993. Following these observations, it was decided 1) to send all suspect animals (on the basis of clinical symptoms) to the Veterinary School in Toulouse for histopathological diagnosis 2) to bleed all live animals and extract their DNA for genetic studies.

In this paper, we analyze the data corresponding to all Romanov animals alive on the Langlade farm between the 1st of April, 1993 and the 1st of May, 1997, and whose *PrP* genotype has been determined. From the 1528 animals satisfying these criteria, 1015 were classified as ⟪ exposed ⟫ to scrapie, i.e. with a lifespan longer than 1 year, the youngest age at scrapie (some died between the 1st of April, 1993 and the 1st of May, 1997, while others were still alive on the 1st of May, 1997).

Scrapie diagnosis and histopathology

The first symptoms (mainly pruritus and lack of gait coordination) of scrapie were reported by the people in charge of animal husbandry. Within 2 weeks after detection of clinical signs, the animals were sent to the Veterinary School in Toulouse for a final diagnosis using histopathology of the brain. After intravenous pentorbital injection and death by bleeding, the brain was immediately removed and stored in a 10% formalin-buffered solution for 3 weeks. Five samples for each brain (pons, obex, cerebellum, thalamus, cerebral cortex) were dehydrated, embedded in paraffin, sliced into 4 μm thick sections and stained using a classical hematoxylin-eosin method. Diagnostic criteria of neuronal vacuolization, neuronal loss and astrocytosis were recorded. In our analyses, individuals were classified as “scrapie” if the histopathological diagnosis was positive, “suspect” when the clinical signs were positive without positive histology (animals still alive, analysis not done or uncertain conclusions),

"healthy" when the animals are still alive without clinical signs, or died without clinical or histological signs.

PrP *genotyping*

Genotype at the *PrP* locus was described for the three codons for which polymorphisms were known or suspected to be linked to scrapie sensitivity: 136, 154 and 171. Analyses were performed using RFLP-PCR techniques and aimed at detecting the following variations: codon 136: Alanine/Valine, codon 154 : Arginine/Histidine, Codon 171: Arginine/Glutamine. A total of 1207 individuals were genotyped.

DNA was purified from peripheral blood leukocytes using the alkaline lysis method. Samples were amplified in 15 μl reaction mixtures containing 25 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X100, 1.5 mM MgCl, 200 μM of each dNTP, 10 pmol of PrP1-PrP2, PrP2-PrP5, and 0.5 unit of Taq polymerase (Promega).

Two reactions were performed PCR conditions for both reactions were performed using an MJ research thermocycler as follows: cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 55 °C, and 1 min elongation at 72 °C were repeated 30 times.

Codon 171 was typed with primer PrP1: 5′AAGTGTACTACAGACCAGTTGATC3′ and PrP2: 5′GCACATTTGCTCCACCACTCGC3′, the modified primer PrP1 near to the substitution A/G create [16, 28] an artificial restriction site BclI for Q and H allele.

The other reaction with primer PrP5: 5′GGAGCTGCTGCAGCTGGAGC3′ and PrP2: 5′GCACATTTGCTCCACCACTCGC3′ enabled the genotyping of codon 136 and 154 using BspHI digestion [25].

Genotypes of individuals which had not yet been typed were reconstructed when possible from their ancestral or progeny information using a specific iterative algorithm (coded in FORTRAN, available on request). This allowed for genotyping of 102 additional individuals. From the 1309 individuals with a *PrP* genotype, 1299 had a full record for the variation factors listed below and 944 were classified ⟨⟨exposed⟩⟩ to the disease

Statistical analyses

Basic analyses (χ^2 tests analysis of variance) were performed using procedures from the SAS library.

Survival analyses were performed considering the age at scrapie diagnosis (t) as the survival measurement. These analyses are based on modelling of the hazard function $\lambda(t) = \lim_{\delta \to 0} \text{Prob}(t < T < t+\delta \mid t < T)/\delta$ which is the probability of scrapie diagnosis at age t, given that the individual did not show scrapie symptoms before this age t. Beyond a general trend, this probability depends on various factors. The model chosen for the hazard function is the proportional hazards model, for which $\lambda(t, z_i)$ is the product of a time-dependent baseline function, $\lambda_0(t)$, which describes the mean risk as an age function, and a stress-dependent term, $e^{z_i'\beta}$, which represents how a vector of covariates z_i influences scrapie rate, independently of time. The risk associated with a given level (1) of a given factor (k) is expressed as a risk ratio ($e^{\beta_{kl}}/e^{\beta_{km}}$), choosing as a reference the level (m) for the considered effect with the maximum number of scrapie cases. The baseline hazard function was left completely arbitrary, therefore defining what is known as a Cox model [5]. For each model studied, likelihood ratio tests of β effects were processed sequentially (by introducing effects with decreasing significance levels in the model) and marginally (each effect being tested with all others already included). All calculations were performed using ⟨⟨The Survival Kit⟩⟩ [9], a software package for the analysis of survival data.

Sources of variation

Various recorded factors were supposed to have a possible influence on susceptibility:

- calendar time, grouped in ***quarters (Q)***. Quarters are treated as time-dependent covariates in the Cox model, i.e., the hazard for a particular animal changes at the beginning of each new quarter. This factor describes the evolution of the level of exposure during the epidemic. Twenty periods were included, the first describing any time before 1993, the 2nd the first 1993 quarter, and so on.
- ***year of birth (YB)***. Fifteen years are considered, the first 8 (1983 to 1990) being grouped in a single level. This factor was substituted in some analyses for the time-dependent quarter effect, as an alternative description of the level of exposure. In this case, a distinct baseline hazard function of the Cox model defined for each year of birth (stratification by year of birth).
- ***flock (FL)***, distinguishing those animals which were artificially infected with *T. circumcincta* (P flock) and the main flock (NP flock).
- ***Genotype (GI)***. Ten PrP genotypes were considered (Table 2).
- ***Sex (SX)***. From the 1299 animals with PrP genotype, 327 were males and 972 females. The general policy for the Langlade flock was to replace all rams after 1 year of reproduction. Thus rams are globally less exposed to scrapie than ewes.
- ***Birth rank (BR)***. Birth rank varied between 1 and 6 (6, 18, 33, 32, 9 and 1 pcent).
- ***Rearing type (RT)***. Most of the animals were fed by their mother (n = 942), 357 received artificial milk after dam's colostrum ingestion.
- ***Mode of birth/rearing (MO)*** is a combination of BR and RT. Values of 1, 2 and 3 were given to lambs fed by their dam, 4 to lambs artificially fed (n = 357). MO = 1 for lambs born single (n = 76), MO = 2 for those born in multiple litters but left alone with their mother (n = 89), MO = 3 for lambs born and fed along with their siblings by the dam (n = 768).
- ***Dam PrP genotype (GD)***. The different possibilities were grouped into 3 classes: susceptible (ARQ/ARQ, ARQ/VRQ or VRQ/VRQ, see below), resistant (other genotypes) and unknown.
- ***Dam scrapie status (SD)***. For most of the animals (SD = 1, n = 1150) the dam did not show scrapie, largely because they died before the outbreak. The group of animals with scrapie dams (SD = 2) included 149 individuals.

Results

General presentation of survival analyses

Different models were studied, differing in the way dam information and birth/rearing mode were included. The dam information considered was its scrapie status in model 1, its *PrP* genotype in model 3, their interaction in models 4, 5 and 6. Concerning birth and rearing modes, we examined the effects of BR and RT in model 1, of RT only in models 2, 3 and 4, of MO in model 5. For each of the models studied, Table 1 shows a measurement of the proportion of explained variation (Maddala's r^2, [33]), and the significance level of each included effect, with its rank in the sequential χ^2. These results will be given in detail in the following sections.

History of the epidemic

From the 1528 recorded animals, 304 were classified as scrapie and 48 as suspect. The evolution of the epidemic is shown in Fig. 1. After a starting phase, the

Table 1. Models studied in the survival analyses. Total explained variation and level of significance of each factor (P values) (rank of factors in the sequential analyses)

Model	1	2	3	4	5	6
Maddala r^2	.5297	.5266	.5291	.5299	.5306	.3251
Q	.0000 (1)	.0000 (1)	.0000 (1)	.0000 (1)	.0000 (1)	
YB × FL						.0000(1)
GI	.0000 (2)	.0000 (2)	.0000 (2)	.0000 (2)	.0000 (2)	.0000 (2)
FL	.0002 (3)	.0002 (3)	.0002 (3)	.0002 (3)	.0002 (3)	
RT	.0104 (4)	.0104 (4)	.0100 (4)	.0100 (4)		.0060 (3)
MO					.0507 (4)	
SX	.0860 (5)	.0860 (5)	.0843 (5)	.0843 (5)	.0944 (6)	.1791 (4)
GI × FL	.9866 (6)					
BR	.6446 (7)					
SD	.1604 (8)					
GD			.0655 (6)			
SD × GD				.0966 (6)	.0785 (5)	.0656 (5)

Q Quarters ; *YB* year of birth; *FL* flock; *GI* genotype; *SX* sex; *BR* birth rank; *RT* rearing type; *MO* mode of birth/rearing; *GD* dam PrP genotype; *SD* dam scrapie status

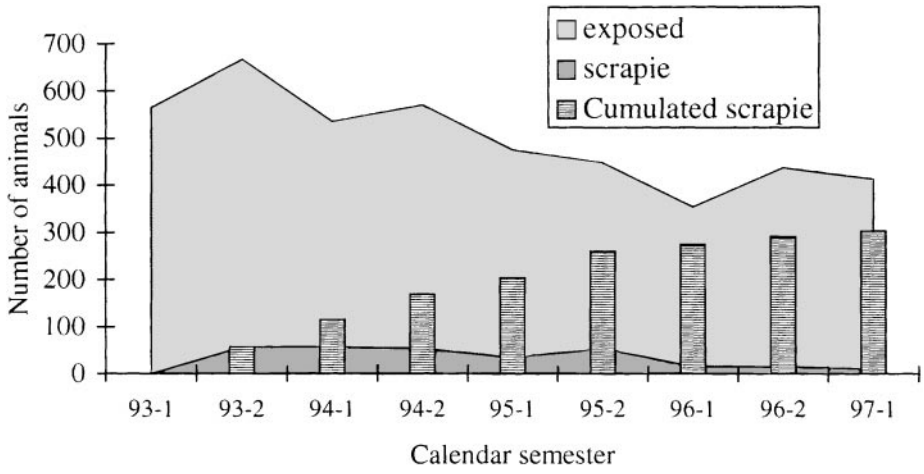

Fig. 1. Evolution of the number of exposed and scrapie animals

epidemic killed about 50 animals per semester between 1993 and 1995. As shown above, the decrease in the number of cases in 1996–97 is due to the reduction of both total flock size and the proportion of susceptible sheep. The number of scrapie cases is detailed by birth cohort in Fig. 2. Note that numbers and not frequencies are represented: observed maxima depend both on the total size and susceptibility of cohorts. Two groups of mortality curves are clearly displayed. The first group includes the parasite flock and animals born after 1991. These animals were rapidly infected with the disease, between 1 to 2 years of age, with

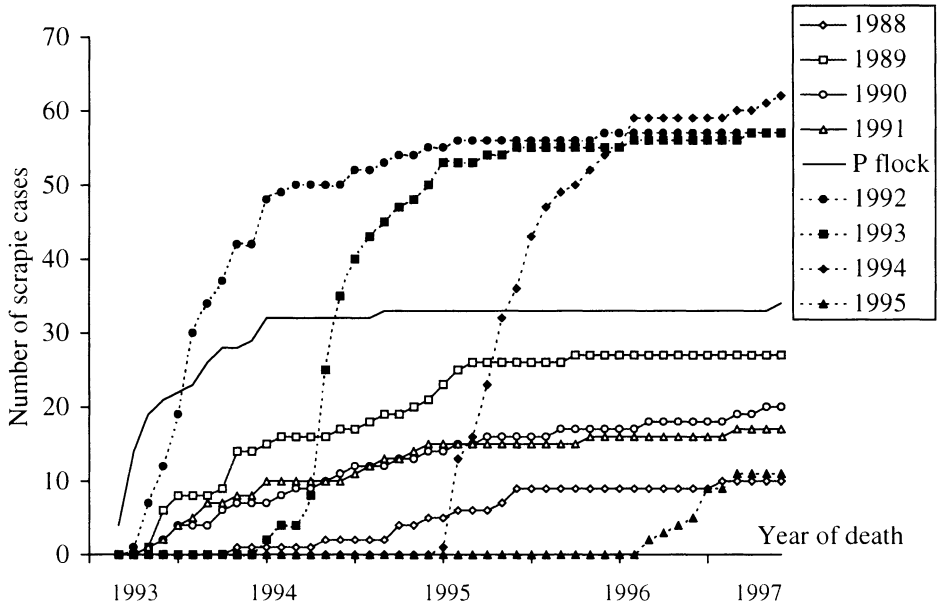

Fig. 2. Mortality due to scrapie per year of birth for all exposed (*) animals [(*) animals alive between the 1st of April, 1993 and the 1st of May, 1997, with a lifespan longer than 1 year and which had their PrP genotype determined]

Table 2. PrP genotype in the three classes of exposed animals

Status	PrP genotype										Total
	ARR ARR	ARR AHQ	AHQ AHQ	ARQ ARR	ARQ AHQ	ARR VRQ	AHQ VRQ	ARQ ARQ	ARQ VRQ	VRQ VRQ	
Scrapie	0	0	0	0	1	0	0	77	142	56	276
Suspect	0	0	1	0	1	3	3	5	12	3	28
Healthy	47	37	10	96	89	75	51	102	118	15	640
Total	47	37	11	96	91	78	54	184	272	74	944

PrP genotype is codons 136/154/171 information

a lag of 1 years between each cohort. The initial mortality rate is highest for the P flock. The second group includes all animals born before October 1991. In this group, the number of scrapie cases increased slowly, from 1993 up to 1997. The higher mortality in the 1989 cohort should be noted.

PrP genotypes and susceptibility

Four alleles were found in the Langlade flock, which will be noted in a simplified way VRQ, ARQ, AHQ and ARR. Table 2 gives the *PrP* genotype frequencies in the exposed group, depending on the classification (scrapie, suspect, healthy). Both ARR and AHQ alleles gave a nearly dominant resistance to the scrapie agent

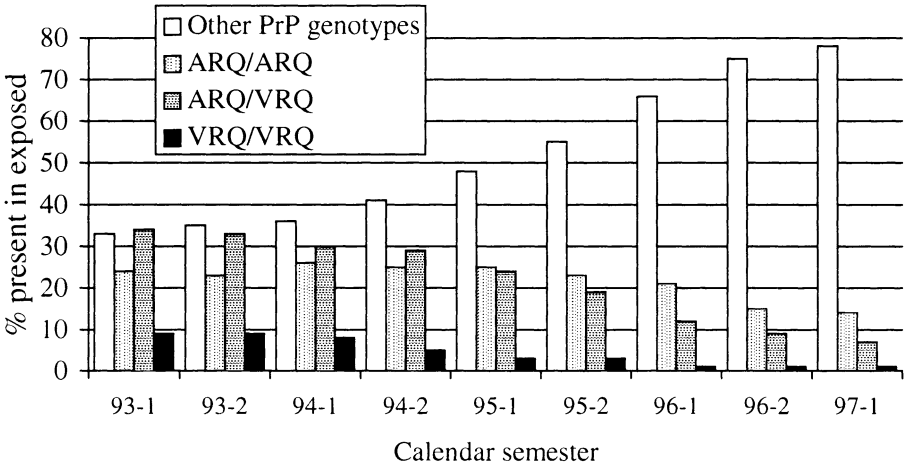

Fig. 3. Evolution of PrP genotype frequencies

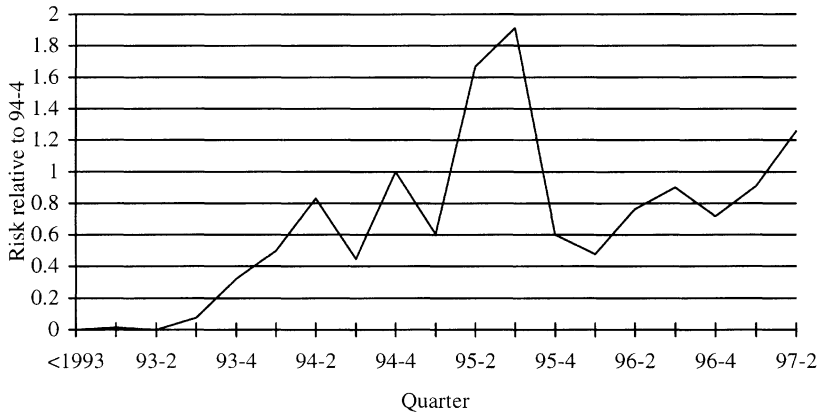

Fig. 4. Effect of Quarters in model 1 of survival analysis (relative scrapie risk during a given quarter as compared to 94-4)

strain(s) of Langlade (only 1 ARQ/AHQ scrapie, 405 healthy). ARQ and VRQ were associated with susceptibility, and behave in a co-dominant way, with 76 percent of scrapie in the VRQ/VRQ group, 52 percent in the ARQ/VRQ and 42 percent in the ARQ/ARQ.

Mostly due to natural selection against the susceptible genotype, the distribution of the different genotypes changed drastically (Fig. 3). When expressed relative to the number of animals, both exposed and susceptible, the proportions of scrapie diseased animals were rather stable when comparing semesters (.12, .16, .16, .13, .27, .13, .13, .09 from the 2nd half of 1993 to 1st of 1997), and not significantly different ($P < 0.41$) with the notable exception of the end of 1995. In the survival analyses, the quarter effect was always highly significant (and the main factor explaining total variation). The singularity of 1995 appears clearly in these analyses (Fig. 4).

Table 3. Risk ratios for the PrP genotype (survival analysis with models 2 and 6, see Table 1)

Genotype	Model 2	Model 6
ARR-ARR	.000	.000
ARR-AHQ	.000	.000
AHQ-AHQ	.000	.000
ARQ-ARR	.000	.000
ARQ-AHQ	.006	.005
	(0.001–0.041)	(0.001–0.033)
VRQ-ARR	.000	.000
VRQ-AHQ	.000	.000
ARQ-ARQ	.465	.520
	(0.350–0.618)	(0.391–0.694)
ARQ-VRQ	1.000	1.000
VRQ-VRQ	1.782	1.654
	(1.282–2.476)	(1.189–2.737)

PrP genotype is codons 136/154/171 information

Table 4. Risk ratios for the different cohorts (survival analysis with model 6)

Cohort	Risk ratio
1991	0.080
	(0.043–0.149)
P flock	0.541
	(0.324–0.904)
1992	0.392
	(0.264–0.583)
1993	1.133
	(0.775–1.656)
1994	1.000
1995	1.079
	(0.506–2.299)

The risk ratios associated with *PrP* genotypes were very similar in all models (Table 3 for models 2 and 6), showing no possible confusion between the genotype and the year of birth (YB) or period (Q). The risk for VRQ/VRQ is three times the risk for ARQ/ARQ animals, with ARQ/VRQ being intermediate. In a simple variance analysis, using model 6, adjusted age at scrapie diagnosis was significantly, 91 and 141 days lower, in VRQ/VRQ as compared to ARQ/VRQ and ARQ/ARQ, respectively (general mean of the population: 877 days), the only ARQ/AHQ scrapie animal being observed 300 days after the VRQ/VRQ mean.

Influence of parasitism

The first scrapie animals were observed in the P flock. Globally, the proportions of scrapie diseased animals were 77 (n = 13), 88 (n = 15) and 100 (n = 8) percent in the P flock vs. 39 (n = 171), 51 (n = 257) and 73 (n = 66) percent in the NP flock in ARQ/ARQ, ARQ/VRQ and VRQ/VRQ respectively. Adjusted age at scrapie was 37 days lower in the P flock as compared to the NP flock 1992 cohort born 3 months later.

The risk ratio for animals in the P flock is very high in all survival analysis models including the quarter effect (about 3 times the risk for NP flock animals): for the same period of time, the risk is much higher for individuals in the P flock. However, this P flock had no contemporaries is the NP flock, and therefore there was a confusion between birth date and experimental status (P or NP). The risk ratios for the different cohorts, estimated for model 6, are given in Table 4. The risk for the P flock is higher than the risks for the 2 cohorts born just before and after, but it is lower than the risk for cohorts born later. The high incidence of scrapie in later years (in particular 1995) is thus explained by a higher pathogenic pressure which compensated the decrease in mean genetic susceptibility.

Influence of birth rank and rearing mode

Birth rank was significant at the 1 percent level when considering the incidence of scrapie without adjustment, with a much lower proportion of cases in single born lambs (16 percent, n = 57 vs. 31 percent; n = 951). However, when considering simultaneously birth rank and rearing type in survival analyses (model 1), birth rank was no longer found to be significant.

In contrast, rearing type (material vs. artificial) was significant at the 1 percent level in all survival analyses, whether or not birth rank was considered. The risk was much lower for lambs fed artificially (between 0.655 and 0.711 when the combined effect MO is not included). With model 6, corrected age at scrapie diagnosis was 100 days earlier in maternally fed lambs.

Risk ratios for the MO variable combine the effect of both the rearing type and the birth rank. As compared to lambs fed by their dam (MO = 3, n = 768, risk = 1.000), i.e. the more frequent situation, the relative protection given by artificial milk was confirmed (n = 357, risk = 0.667, $p(\chi^2) < 0.005$). However, lambs fed alone by their dam had a similar risk to artificially fed lambs, as the difference was not significant (MO = 1, n = 76, risk = 0.694, $p(\chi^2) = 0.352$, MO = 2, n = 89, risk = 0.634, $p(\chi^2) = 0.214$).

On the whole these results suggest maternal transmission of scrapie and an absence of risk due to artificial feeding. The maternal transmission could be due either to the maternal milk or to extra contact between lamb and dam during the nursing period.

Influence of the dam status

Lambs born to dams which became ill with scrapie seemed to have a higher chance of developing the illness. This was not only due to susceptible PrP allele trans-

Table 5. Proportion of scrapie depending of dam status

Dam status	Progeny genotype		
	ARQ/ARQ	ARQ/VRQ	VRQ/VRQ
Healthy	48% (n = 23)	55% (n = 32)	58% (n = 23)
Scrapie	61% (n = 81)	78% (n = 96)	100% (n = 24)
$P(\chi^2)$	0.281	0.021	0.001

Table 6. Proportion of scrapie depending on dam status x genotype (model 4) (P value when comparing a level of SD × GD to healthy, susceptible dams)

SD × GD	n	Risk ratio	$P(\chi^2)$
Healthy, resistant	69	0.431 (0.205–0.909)	0.027
Healthy, susceptible	111	1.000	
Scrapie, susceptible	110	1.101 (0.764–1.589)	0.605
Non exposed	282	0.995 (0.679–1.458)	0.981
Unknown	64	0.960 (0.668–1.381)	0.827

mission, as shown in Table 5. The figures corresponding to VRQ/VRQ progeny are particularly clear, with all lambs from scrapie dams dying, while only half of the lambs from healthy dams became ill from scrapie. Suprisingly, the dam status was not significant ($p = 0.160$, model 1) in the survival analysis. This may be due to the limited number of cases observed. However, when considering dam genotypes (model 3), the risk (which is corrected for the lamb genotype) is lower for lambs born from resistant dams than for lambs from susceptible dams (risk ratio 0.620 vs. 1.00).

Survival analyses revealed another phenomenon: when considering dam status x dam genotype interaction, the risk is higher for susceptible dams, even if they did not show scrapie signs (Table 6). This results strongly suggests that resistant animals were not healthy carriers or at least were less infectious. The absence of change in the relative risk for different individual genotypes (model 2 vs. 3 or 4) proves that there was no confusion between dam and lamb genotype effects. The high risk ratio for non-exposed and unknown dams (i.e. who died before the scrapie outbreak) suggests that most of them were susceptible, in accordance with Fig. 3.

Influence of sex

In all our analyses, males appeared to be less susceptible to scrapie than females (16% scrapie, n=149 vs. 32% scrapie, n = 866; $p < 0.001$). As rams were replaced much faster in the flock, this observation may simply reflect the lower exposure of males to the disease. However, survival analyses, which account for this difference in length of time at risk, confirmed a slight effect of sex on susceptibility (risk 0.611 vs. 1.00 for male vs. females in model 2), consistent with the observation of proportions of animals developing scrapie within one semester of life (16% scrapie vs. 26%, n = 469, $p(\chi^2) = 0.051$ for animals between 1.5 and 2 years of age).

Discussion

Our observations concerning the effect of *PrP* genotype on scrapie susceptibility confirm to some extent, on a large scale, the results already published by others about different breeds, and probably different scrapie strains [1, 2, 7, 17–23, 25, 26, 34]. In particular, the VRQ allele was found to give a high susceptibility to natural scrapie, with 76 percent of scrapie diseased animals in our case, and the ARR allele, a high resistance, with no ARR carriers developing scrapie in our flock.

We found ARQ to be codominant to VRQ and not protective against scrapie, in contradiction with observations in Cheviot [18–20], Ile de France [26] or Flemish and Swifter [2], but in accordance with observations in Swaledale [17, 22], Texel [1] or Lacaune [4, 25]. This susceptibility of ARQ to the disease could be related to its very high incidence in our flock (more than 300 killed in 3 years), suggesting a threshold effect, ARQ/ARQ animals becoming scrapie above a certain level of exposure.

Allele AHQ behave very similarly to ARR in the Romanov breed, with only 1 heterozygous AHQ/ARQ being affected by scrapie. The protective role of AHQ was suggested in some other breeds on the basis of fewer observations [1, 4, 20, 23, 25].

Selecting ARR or AHQ carriers appears to be a very attracive way of improving resistance to natural scrapie. However, as noted by others, it must be emphasized that 1) other scrapie strains may attack these apparently protected animals (e.g. [24]), 2) they may be healthy carriers diffusing the pathogenic agent. These points should be addressed in the future before selecting for PrP in large populations. Our finding that animals from healthy dams had significantly lower risk if their dam was resistant rather than susceptible, is a positive argument against the infectivity of healthy genetically resistant carriers.

The Langlade flock was considered to be scrapie free before the outbreak. The origin of the epidemic has still not been identified. Ovulation rates were currently measured on the flock with a laparoscope which was also used in other farms. Although no iatrogenic CJD has been described through laparoscopy in humans, this means of contamination cannot be excluded in sheep (see [29], for a review).

A large proportion of lambs were given artificial milk. This milk is traditionally made partially from cattle products, including tallow. The geographic origins of these products varies and the hypothesis of a contamination with BSE could not be excluded. However, our survival analyses revealed a lower risk for lambs fed artificially, as compared to maternally fed animals, suggesting that transmission through artificial milk is not likely.

The first scrapie cases were found in the sub-flock submitted to experimental parasite infection, and survival analyses showed that this group had a higher risk than others. Unfortunately, no control allowed for discriminating between a possible direct effect of parasitism and simple time coincidence. Moreover, it could be that subclinical scrapie was present in the flock but was not observed prior to the first registered cases. As already suggested by [4, 10, 27], infection with *T. circumcincta* may be a cofactor of scrapie, facilitating the penetration of the agent through lesions due to larvae in the gastrointestinal tract. Another hypothesis could be that the nematodes may themselves be vectors of the disease. This hypothesis was tested in the past without success with intra-cerebral inoculation of the parasites [11].

Maternal transmission was often taken as an hypothesis for scrapie. In particular, infectivity of placenta had been demonstrated (e.g. [7, 8, 14, 30]). Possible direct transmission to the offspring via the embryo or the uterus has also been suggested by [13], but the protocol was criticised [12]. In other experiments transmission via embryo transfer could not be demonstrated [12]. More globally, experiments proving maternal transmission were very critically reexamined [31], with authors arguing that most of the familial transmission may be explained by *PrP*.

Our observations clearly support maternal transmission, with very high differences in the number of scrapie cases, within *PrP* genotype, depending on the dam status (scrapie/healthy). Due to insufficient information, it was however not possible to quantify the effect of the delay between lamb birth and dam scrapie death on the risk for the lamb.

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