Factors influencing temporal variation of scrapie incidence within a closed Suffolk sheep flock

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Several studies have shown that transmission of natural scrapie can occur vertically and horizontally, and that variations in scrapie incidence between and within infected flocks are mostly due to differences in the proportion of sheep with susceptible and resistant PRNP genotypes. This report presents the results of a 12-year period of scrapie monitoring in a closed flock of Suffolk sheep, in which only animals of the ARQ/ARQ genotype developed disease. Among a total of 120 of these, scrapie attack rates varied between birth cohorts from 62.5 % (5/8) to 100 % (9/9), and the incidence of clinical disease among infected sheep from 88.9 % (8/9) to 100 % (in five birth cohorts). Susceptible sheep born to scrapie-infected ewes showed a slightly higher risk of becoming infected (97.2 %), produced earlier biopsy-positive results (mean 354 days) and developed disease at a younger age (median 736 days) than those born to non-infected dams (80.3 %, 451 and 782 days, respectively). Taken together, this was interpreted as evidence of maternal transmission. However, it was also observed that, for the birth cohorts with the highest incidence of scrapie (90–100 %), sheep born to infected and non-infected dams had a similar risk of developing scrapie (97.1 and 95.3 %, respectively). Compared with moderate-attack-rate cohorts (62.5–66.7 %), high-incidence cohorts had greater numbers of susceptible lambs born to infected ewes, suggesting that increased rates of horizontal transmission in these cohorts could have been due to high levels of environmental contamination caused by infected placentas.

INTRODUCTION

Classical scrapie is a contagious disease naturally affecting sheep and goats, and can become endemic in affected flocks. Maintenance of infection within a flock relies on transmission of the infectious agent from infected to susceptible animals, and it has long been recognized that transmission dynamics are strongly modulated by the sheep genotype. Thus, it appears that only sheep of susceptible genotypes excrete sufficient infectivity to transmit infection to other sheep and maintain scrapie within the flock. In contrast, sheep of resistant genotypes, although exposed to the same or similar levels of infection, do not (or only rarely) develop clinical disease and appear not to be able to disseminate infection. This strong link between scrapie infection and PRNP genotype has formed the basis for programmes of scrapie control (reviewed by Dawson et al., 2008), which are based on the selection of sheep carrying the most resistant ARR allele [alanine136, arginine154, arginine171 of the ovine prion protein (PrP)] and the elimination of those carrying the most susceptible VRQ allele (valine136, arginine154, glutamine171).

Whilst genetic selection and compulsory eradication measures have apparently markedly reduced the incidence of scrapie in the UK, it is not without problems. These include (i) the unavailability of sheep of resistant PRNP genotypes in some breeds, (ii) the remote but not zero risk of ‘dormant’ infections being maintained by silent carriers, and (iii) the adaptation of the infectious agent to sheep of resistant genotypes, which could result in the re-emergence of scrapie outbreaks in the future. In some circumstances, therefore, control of infection by
reduction or elimination of environmental contamination may be a helpful alternative strategy. This can be achieved either by early detection of infected sheep by, for example, rectal biopsy (González et al., 2008) and their removal from the flock, or by the blockage of transmission from infected to uninfected sheep through management practices.

Numerous studies have shown that scrapie can be transmitted both vertically and horizontally, and both directly and indirectly. Embryo transfer experiments (Wang et al., 2001; Foster et al., 2006; Low et al., 2009) and examination for accumulation of disease-associated PrP (PrP\textsuperscript{d}) in tissues from fetuses of infected ewes (Tuo et al., 2001; Andreolotti et al., 2002) have indicated that direct vertical transmission is unlikely to occur in utero; this notion may need to be revisited in view of recent reports of detection of PrP\textsuperscript{res} (the protease-resistant form) in organs of ovine fetuses after \textit{in vitro} amplification (Garza et al., 2011). In any case, numerous studies have shown that the offspring from scrapie-affected ewes are more likely to develop scrapie than the progeny of non-infected dams. Whilst early studies (Dickinson et al., 1974; Hourrigan et al., 1979) did not take into account a confounding genetic effect, more recent ones (Hoinville, 1996; Elsen et al., 1999; Hoinville et al., 2010) have corrected for this possible error. After birth, lambs could acquire infection from their infected dams either through milk (Konold et al., 2008; Lacroux et al., 2008) or through other secretions, probably mostly saliva (Maddison et al., 2010), thanks to the close ewe–lamb contact.

Several investigations have pointed out that horizontal transmission of scrapie also occurs, so that, in an affected flock, the progeny of non-infected dams can also become infected and develop disease. Infectivity and/or PrP\textsuperscript{d} accumulation have been detected in placentas (Jeffrey et al., 2001; Andreolotti et al., 2002; Lacroux et al., 2007) and these are believed to be a potentially important source of environmental contamination, not just of lambing pens but also of pastures (Healy et al., 2004). Although direct evidence of prion excretion in faeces and urine is not available for natural sheep scrapie, the detection of PrP\textsuperscript{d} in the rectal mucosa (Espenes et al., 2006; González et al., 2006) and kidney (Sisó et al., 2008) of infected sheep strongly argues in favour of these excreta being infectious and possible sources of environmental contamination.

There are, therefore, several factors that can account for an increased or decreased rate of transmission of scrapie within infected flocks, which can explain the variations in the incidence and other aspects of the infection in flocks subjected to monitoring for extended periods of time (Elsen et al., 1999; Redman et al., 2002). Here, we analyse the dynamics of scrapie in a Suffolk sheep flock with long-term data in an attempt to provide further insights into the epidemiology of the infection.

**RESULTS**

**Attack rate (AR): differences between birth cohorts and effect of sex and maternal status**

Of the 132 QQ\textsubscript{171} sheep that populated the flock from 1 January 1998 to 31 December 2009 (see Supplementary Table S1, available in JGV Online), 12 were not included in the analysis as they either belonged to pre-1998 birth cohorts (\(n=6\)), died at a younger age than the earliest indication of infection in their respective birth cohorts (\(n=5\)) or presented with a threonine substitution at codon 112 (\(n=1\), born in 2006, culled at 715 days of age when the earliest indication of infection for this cohort was 700 days). Of the 120 QQ\textsubscript{171} sheep that fulfilled the established criteria, scrapie infection was detected in 101 and 19 were fully negative for PrP\textsuperscript{d} immunohistochemistry (IHC) at post-mortem (AR 84.2 %). Scrapie was not detected in any of the 75 sheep with PRNP genotypes other than QQ\textsubscript{171} or in the 37 of unknown genotype.

By birth cohort, ARs fluctuated between 62.5 % (5/8) for QQ\textsubscript{171} sheep born in 2005 and 100 % (9/9) for those born in 2003. To study the possible effect of various factors on the ARs, birth cohorts were divided into two distinct groups: high-AR cohorts (2000–2004) with ARs between 90.0 and 100 % and moderate-AR cohorts (1998, 1999, 2005 and 2006) with values ranging between 62.5 and 66.7 %. The median AR values of these two groups (95.0 and 65.4 %, respectively) were significantly different (\(P=0.016\); Mann–Whitney test). When birth cohorts were compared pair-wise, the only significant differences in AR (\(P<0.05\); Fisher’s exact test) were between the 2000 cohort (96.2 %) and the 1998 (64.0 %) and 2005 (62.5 %) cohorts, and also between the 1998 and 2002 (95.0 %) cohorts (Table 1).

There was some evidence to suggest that the differences in AR rate between birth cohorts were related to the number of clinical scrapie cases occurring during the corresponding housing periods (Table 1 and Fig. 1). Of a total of 60 scrapie cases that occurred indoors, 52 corresponded to the 2000–2004 cohorts [median seven, interquartile range (IQR) 4.5–18] and only eight to the moderate AR cohorts [1998–1999 and 2005–2006; median two, IQR 0–4]. There was, however, considerable individual variation among cohorts, so that such differences were not statistically significant in a Mann–Whitney test.

The probability of infection of susceptible QQ\textsubscript{171} sheep was influenced by the maternal scrapie status (Table 2), which was entirely genotype dependent (all scrapie-infected dams were QQ\textsubscript{171} and all non-infected dams were RQ\textsubscript{171}). Of a total of 107 susceptible lambs born to ewes with known scrapie status, 92 (86.0 %) went on to contract scrapie; this subpopulation was therefore representative in terms of AR of the whole 120 QQ\textsubscript{171} sheep analysed. The probability of scrapie infection was slightly but significantly higher [AR ratio 1.21, 95 % confidence interval (CI) 1.07–1.38; \(P=0.02\), Fisher’s exact test] for those whose dam was
infected (35/36, 97.2%) than for the progeny of scrapie-negative dams (57/71, 80.3%). This difference was not attributable to a shorter lifespan of the progeny of non-infected dams, which could have led to a lower probability of scrapie detection in such progeny; in fact, their lifespan was slightly longer (median 775.0 days, IQR 730–941) than that of the progeny of scrapie-infected dams (median 723.5 days, IQR 688–812; P = 0.07, Mann–Whitney test).

However, the influence of the maternal status was not apparent in the high-AR cohort group (2000–2004), in which the risk of scrapie for the progeny of positive dams (33/34, 97.1%) was the same (AR ratio 1.02, 95% CI 0.93–1.11; P = 1, Fisher’s exact test) as that for the offspring of scrapie-negative dams (41/43, 95.3%). It is noteworthy that the progeny of negative dams born in high-AR cohorts had a significantly higher probability of contracting scrapie (AR ratio 1.67, 95% CI 1.20–2.31; P < 0.001, Fisher’s exact test) than the offspring of scrapie-negative dams born in infected (35/36, 97.2%) than for the progeny of scrapie-negative dams (57/71, 80.3%). This difference was not attributable to a shorter lifespan of the progeny of non-infected dams, which could have led to a lower probability of scrapie detection in such progeny; in fact, their lifespan was slightly longer (median 775.0 days, IQR 730–941) than that of the progeny of scrapie-infected dams (median 723.5 days, IQR 688–812; P = 0.07, Mann–Whitney test).

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### Table 1. Scrapie data and influencing factors by birth cohort

<table>
<thead>
<tr>
<th>Year</th>
<th>Attack rate</th>
<th>Clinical incidence</th>
<th>Median ACEP</th>
<th>Scrapie indoors</th>
<th>Born to positive dam</th>
</tr>
</thead>
<tbody>
<tr>
<td>1998</td>
<td>162/25 (64%)</td>
<td>32/26 (96%)</td>
<td>784</td>
<td>48/100 (48%)</td>
<td>9/25 (36%)</td>
</tr>
<tr>
<td>1999</td>
<td>46/67 (68%)</td>
<td>35/26 (96%)</td>
<td>773</td>
<td>44/100 (44%)</td>
<td>7/15 (47%)</td>
</tr>
<tr>
<td>2000</td>
<td>12/12 (100%)</td>
<td>23/24 (96%)</td>
<td>1755</td>
<td>4/100 (4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>2001</td>
<td>10/10 (100%)</td>
<td>22/24 (92%)</td>
<td>120</td>
<td>0/100 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>2002</td>
<td>9/9 (100%)</td>
<td>4/4 (100%)</td>
<td>76</td>
<td>0/100 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>2003</td>
<td>9/9 (100%)</td>
<td>4/4 (100%)</td>
<td>756</td>
<td>0/100 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>2004</td>
<td>7/7 (100%)</td>
<td>4/4 (100%)</td>
<td>9/974 (8%)</td>
<td>0/100 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>2005</td>
<td>7/7 (100%)</td>
<td>4/4 (100%)</td>
<td>760</td>
<td>0/100 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>2006</td>
<td>5/5 (100%)</td>
<td>4/4 (100%)</td>
<td>768</td>
<td>0/100 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*Statistical analysis: cohorts with no letter in common are significantly different at the expressed confidence levels, either in terms of AR (Fisher’s exact test) or ACEP (Mann–Whitney test).

### Table 2. Influence of maternal scrapie status on the incidence of scrapie in the offspring in cohort groups of moderate (1998, 1999, 2005 and 2006) and high (2000–2004) ARs

<table>
<thead>
<tr>
<th>Dam status</th>
<th>Moderate AR</th>
<th>High AR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dam positive</td>
<td>2/2 (100%)</td>
<td>33/34 (97.1%)</td>
<td>35/36 (97.2%)</td>
</tr>
<tr>
<td>Dam negative</td>
<td>16/28 (57.1%)</td>
<td>41/43 (95.3%)</td>
<td>57/71 (80.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>18/30 (60.0%)</td>
<td>74/77 (96.1%)</td>
<td>92/107 (86.0%)</td>
</tr>
</tbody>
</table>
the cohorts of moderate AR (16/28, 57.1%; Table 2). This clearly indicated a significantly higher level of horizontal transmission in high-AR compared with moderate-AR cohorts, which appeared to be related to the number or proportion of QQ171 lambs born to scrapie-positive ewes. Whilst only two of 30 (6.7%) susceptible lambs within the four moderate-AR cohorts had been born to scrapie-positive dams (12 lambs were born to dams of unknown scrapie status), this proportion was 44.1% (34/77) within the five high-AR cohorts (2000–2004; one lamb born to a dam of unknown scrapie status; Table 1); these figures were significantly different in Fisher’s exact test ($P<0.001$).

No significant differences in overall ARs were observed between females (62/70, 88.6%) and males (39/50, 78.0%), and such similarity was maintained when the analysis was performed separately for the birth cohorts with high (2000–2004: females 97.7%, males 91.2%) and moderate (1998–1999 and 2005–2006: females 79.2%, males 57.1%) scrapie ARs.

**Clinical disease: incidence, age at clinical end point (ACEP) and clinical signs**

A total of 79 of the 101 QQ171 sheep in which scrapie was confirmed at post-mortem lived long enough to develop clinical disease. Clinical scrapie cases followed a rather seasonal pattern with almost three-quarters of them occurring in winter and spring (December–May: 58/79, 73.4%) and just over a quarter in summer and autumn (June–November: 21/79, 26.6%). With regard to the time of detection, most clinical cases (63/79, 79.7%) occurred when the flock was housed around lambing time. There were no significant differences in the incidence of clinical disease between birth cohorts, with values oscillating between 89 and 100% of infected sheep (Table 1).

The median age at which the 79 clinically affected sheep reached the clinical end point (ACEP) was 768 days (range 614–1938, IQR 720–896). Table 1 provides details of the median ACEP of clinically affected sheep in each birth cohort, which is illustrated in Fig. 1. Sheep of the 1999 cohort had the highest ACEP (median 1755 days, IQR 1132–1924), followed by those of the 2001 cohort (median 895 days, IQR 829–1243), whilst sheep of the 2002–2004 cohorts showed the lowest ACEP (medians between 716 and 736 days). Details of the statistical significance of the differences in ACEP (Mann–Whitney test) are given in Table 1. When comparing the two birth cohort groups established for the analysis of ARs, sheep of the high-AR group had a significantly shorter ACEP (median 761 days, IQR 717–845) than those of the moderate-AR group (1998, 1999 and 2005: median 822 days, IQR 732–1301; $P<0.05$, Mann–Whitney test). Similarly, the median ACEP of the progeny of infected dams (736 days, IQR 689–812, $n=30$) was significantly shorter ($P=0.01$) than that of the animals born to scrapie-negative ewes (782 days, IQR 732–942, $n=41$). Unlike the ARs, this effect was still significant within the cohorts with the highest ARs (2000–2004), in which the ACEP of sheep from positive dams (median 731 days, IQR 688–819, $n=28$) was significantly shorter ($P<0.05$) than that of the descendants of scrapie-negative ewes (median 774 days, IQR 733–884, $n=30$). The effect of the scrapie status of the dam on the AR and ACEP of the progeny is illustrated in Fig. 2.

A final analysis was performed to determine the effect of the temporal proximity of the birth of susceptible progeny and the development of clinical disease in their dams on the ACEP of those progeny. Although some weak correlation was found to indicate that the sooner the dam developed scrapie after lambing, the shorter the incubation period of the progeny, the correlation coefficient was low, with 14/30 of the observations falling outside the 95% CI (see Supplementary Fig. S1, available in JGV Online). Individual examples (see Supplementary Table S2, available in JGV Online) contributed to highlight this poor correlation, so that some sheep born to the same infected dam 1 or 2 years apart had a similar ACEP.

Details of the prevalence and severity of the different clinical signs of scrapie in this flock are provided in Supplementary Table S3 (available in JGV Online). Pruritus was observed in 97% of the affected sheep, although its severity was generally mild. Behavioural changes and dysphagia were observed less often and were also generally mild. Weight loss was the least commonly observed clinical sign, although it was severe in some sheep. The most relevant clinical sign was ataxia, which, in addition to being recorded in 77% of cases, was the only one that reached a score of severe in a substantial proportion of cases (48%) and accounted for their clinical end point.

**Biopsies**

Of the 63 QQ171 sheep from which biopsies were taken, eight were completely negative for PrP$^d$ at post-mortem and none of them had had a positive biopsy result. Of the 55 sheep confirmed as scrapie cases at post-mortem, 40 gave positive biopsy results and 15 were consistently negative, despite the fact that PrP$^d$ was detected by IHC in the corresponding relevant tissue, palatine tonsil and/or rectal mucosa taken at post-mortem from 13 of them. The time elapsed between the last negative biopsy and post-mortem examination of these sheep was very variable: for seven of them it was a mean of 632 days (95% CI 330–935), whilst for the other eight it was only 65 days (95% CI 29–101). Among the latter were the two sheep that were IHC PrP$^d$ negative in tonsil and rectal mucosa at post-mortem examination.

Among the 55 scrapie cases, the likelihood of a positive biopsy result was very similar for sheep of the moderate-AR cohorts (66.7%, $n=18$) compared with those of the high-AR cohorts (75.7%, $n=37$), as was the age at first positive biopsy (mean 527 and 481 days, respectively). When the effect of the dam scrapie status was analysed,
no clear differences ($P=0.1$) were observed in terms of likelihood of positive biopsy (63.0 and 87.5 % for negative and positive dam descendants, respectively), but significant differences were found in age at first positive biopsy. Thus, the scrapie dam descendants showed earlier ($P<0.05$; unpaired $t$-test) biopsy positivity (354 days, 95 % CI 292–416) than the offspring of scrapie-negative ewes (451 days, 95 % CI 380–521).

**DISCUSSION**

It has been known for a number of years that polymorphisms at codons 136, 154 and 171 of the PRNP gene modulate the susceptibility of sheep to scrapie and therefore the prevalence of infection and incidence of clinical disease in an infected flock. For those sheep breeds that can express valine at codon 136, the presence of this polymorphism results in moderate scrapie incidence, with ARs between 62.5 and 66.7 %, and another with high scrapie incidence, with ARs ≥90 %. Such differences were not attributable to the rates of maternal transmission (which can be equated to the risk of infection among susceptible sheep born to infected QQ171 dams), which approached 100 % ($35/36$) regardless of the birth cohort, but to variations in the probability of infection targeting between the two periods, and the overall conclusion is that, within this flock and for a 22-year period, sheep succumbing to scrapie were almost exclusively of the QQ171 genotype and, at least for the period 1998–2009, without additional polymorphisms.

However, temporal variations in the epidemiology of scrapie were observed in the flock even when the analysis was restricted to susceptible QQ171 lambs. Thus, the nine birth cohorts studied could be split into two groups: one with moderate scrapie incidence, with ARs between 62.5 and 66.7 %, and another with high scrapie incidence, with ARs ≥90 %. Such differences were not attributable to the rates of maternal transmission (which can be equated to the risk of infection among susceptible sheep born to infected QQ171 dams), which approached 100 % ($35/36$) regardless of the birth cohort, but to variations in the probability of infection targeting between the two periods, and the overall conclusion is that, within this flock and for a 22-year period, sheep succumbing to scrapie were almost exclusively of the QQ171 genotype and, at least for the period 1998–2009, without additional polymorphisms.

Fig. 2. Graphical representation of individual results amongst the 120 QQ171 sheep considered in the study. (i) Survival curves (percentage of survivors by age) of sheep that developed clinical scrapie born to infected dams (♀, $n=30$) or to negative dams (♂, $n=41$). Whilst only 1/30 sheep (3.3 %) born to a positive dam survived >32 months, 9/41 descendants (22.0 %) of negative dams lived >32 months. (ii) Scrapie-positive sheep at post-mortem, which died or were culled not having reached the ACEP, born to scrapie-infected (♀, $n=5$) or non-infected (♂, $n=16$) dams. Whilst all the former were <24 months, 6/16 of the latter were >28 months; had these sheep lived until the ACEP, their survival times would have been protracted. (iii) Scrapie-negative sheep at post-mortem, which died or were culled not having reached the ACEP, born to scrapie-infected (■) or non-infected (□) dams. Note that 8/14 of the latter were >30 months. (iv) Sheep whose dam status was unknown, which at post-mortem did not show evidence of scrapie (X), were pre-clinically infected (+) or developed clinical scrapie (♀). The results for (ii)–(iv) are to be read against the $x$-axis (age at post-mortem), the $y$-axis being irrelevant.
which can be correlated to the proportion of infected placenta shed at lambing. Although not systematically examined in this flock, such placentas were found to accumulate PrP^d by IHC and Western blotting when examinations were attempted (Hamilton et al., 2008), which is in agreement with previous reports of placental infectivity (Jeffrey et al., 2001; Andréoletti et al., 2002; Healy et al., 2004; Lacroux et al., 2007). Thus, whilst only two infectious placentas would have been shed in one of the four cohorts of moderate AR (2005 cohort), these would have been consistently contaminating the lambing environment of the five high-AR cohorts.

Evidence for an increased risk of scrapie transmission at lambing time has been found previously (Detwiler & Baylis, 2003; Touzeau et al., 2006) and can be attributed to a combination of factors that do not take place outside the peripartum period: maternal transmission, lateral transmission from placenta-contaminated environment and susceptibility of newborn lambs. The importance of maternal transmission has been highlighted on numerous occasions (Hoinville, 1996; Elsen et al., 1999; Hoinville et al., 2010) and agrees with the combination of different results from our study. On one hand, the overall relative risk of susceptible QQ171 sheep to developing scrapie was slightly but nonetheless significantly higher for those born to infected dams than for the offspring of non-infected ewes. On the other, even for those cohorts where no differences in AR rates were observed between the progeny of infected and non-infected dams (2000–2004), the ACEPs (which can be correlated to incubation periods) were significantly longer for sheep born to non-infected dams. Moreover, detection of PrP^d in tonsil or rectal biopsies was achieved at a younger age in sheep born to infected dams. All this suggests that susceptible lambs born to scrapie-infected ewes can either be infected in utero, as some recent evidence suggests (Garza et al., 2011), or are exposed to higher doses of infection after birth, perhaps at a slightly younger age. Thus, such lambs could become infected through ingestion of colostrum and milk (Konold et al., 2008; Lacroux et al., 2008) and would receive further infectious doses when starting to eat solid food in a contaminated environment. In contrast, lambs born to non-infected ewes would only be exposed to the last source of infection but still during the same lambing season. The exception would have been the 1999 cohort, in which six QQ171 lambs were born and only four developed scrapie, one – whose dam status was unknown – at 967 days of age and the other three – born to RQ171 dams – at >1700 days. These lambs were born in the absence of clinical cases during this and the previous lambing period (see Table 1) and, with the exception of two of unknown status, in the absence of infected placentas (the scrapie-infected sheep born in the previous 1998 cohort were not yet lambing). This relatively clean environment would have resulted in these lambs being exposed to very low levels of infection after birth, so that only one became infected at the time, whilst the other three would have become infected in subsequent lambing seasons.

The pathological phenotype of scrapie in this Suffolk flock has been described in detail elsewhere (Jeffrey et al., 2001; Begara-McGorum et al., 2002; González et al., 2002; Sisó et al., 2010). It is remarkable that, both in terms of vacuolation and PrP^d profiles in the brain and of relative involvement of brain and peripheral tissues, the disease phenotype has been constant throughout the epidemic, regardless of temporal variations in ARs, incidence of clinical disease, ACEP, maternal or horizontal source of infection, etc. Moreover, the minor differences in clinical presentation between animals do not appear to correspond with what basically are the same pathological changes. This again raises a question about the association between clinical disease and pathological changes in sheep scrapie.

To conclude, at least for this natural scrapie model, infected ewes giving birth to susceptible lambs were the key factor in maintaining an endemic situation, as they contributed to effective maternal and horizontal transmission of the infection.

**METHODS**

**Flock composition and evolution.** The flock was created in 1980 and registered with the Suffolk Sheep Society as the East of Scotland College of Agriculture (ESCA) flock. Its original purpose was to conduct research on genetic improvement of lean tissue growth rate (Hosie et al., 1997) and, after the phase of animal acquisition from different pedigree sources, the flock was closed in 1985 (with occasional exceptions as explained below). The first case of scrapie in the flock was confirmed in November 1990, and a further 107 cases were identified up to January 1996. During this period, several investigations related to scrapie were conducted within the flock, such as the relationship between scrapie occurrence and PRNP genotype (codons 136, 154 and 171 only; Hunter et al., 1997), the epidemiology of scrapie outbreaks in different years (Redman et al., 2002) and the transmission of scrapie by embryo transfer (Low et al., 2009).

With the exception of a few sheep that were of undetermined genotype or that carried histidine at codon 154 or 171, all sheep in the flock were AA136 and RR154 homozygotes. For this reason, genotypes in this report only refer to codon 171 (either Q or R). At the beginning of January 1998, the flock consisted of only 48 Suffolk sheep, of which only six were of the susceptible QQ171 PRNP genotype. However, provisions had been made the previous mating season to start increasing the number of genetically susceptible sheep to enable further scrapie investigations. Supplementary Table S1 provides a detailed account of the number of sheep of different genotypes present, born (weaned) and dead each year during the period 1998–2009, when the flock was finally depopulated. A total of 132 QQ171, 47 RQ171, 23 RR171 sheep and 42 sheep of other or of undetermined PRNP genotype were present at different times during this 12-year period. Genotypes other than the susceptible QQ171 were bred for and kept in the flock in order to maintain sustainable numbers.

In order to minimize inbreeding, two Suffolk rams were brought in from a New Zealand-derived, scrapie-free flock (Arthur Rickwood Sheep Unit, AVH, Weybridge, UK) in 2003. In view of new information on the association between additional polymorphisms in the ARQ allele and the susceptibility of sheep to scrapie (Laegreid et al., 2008; Vaccari et al., 2009) and to experimental bovine spongiform encephalopathy (Goldmann et al., 2006; Saunders et al., 2009), retrospective genotyping of the PRNP ORF (between codons 100 and 210) was carried out on 109 QQ171 sheep (samples could not...
be retrieved from the remaining 23 sheep). It was found that one of the two bought-in rams and six of its descendants were MT (methionine/threonine) heterozygotes at codon 112. None of these seven sheep was included in the study – the ram because it was not born in the flock and therefore could not be ascribed to any birth cohort, and five of the descendants because they died too young to be considered in the analysis (as explained below). The only remaining offspring (born in 2006) was also disregarded as indicated in Results. Therefore, the present study refers to ARQ/ARQ sheep without any other polymorphism.

**Flock management.** From 1998 to 2001, mating was completed by mid- to end of August, and sheep were housed by mid- to the end of October. From 2002 onwards, mating was completed by early to mid-November, and sheep were housed by the end of the month. Therefore, lambing took place in January for the first four cohorts and in March–April for the last six cohorts. Depending on the weather conditions and the lambing dates, sheep were returned to pasture between mid-April (first four cohorts) and mid-May/early June (last six cohorts). After weaning, male lambs were segregated from the female group and managed separately, both indoors and outdoors, except for the rams selected for breeding, which joined the female group for the duration of mating.

**Monitoring and diagnosis of scrapie.** Up to 1996, post-mortem diagnosis of scrapie was performed by histopathological detection of characteristic vacuolar lesions in the brain (Hunter et al., 1997). From 1998 to 2009, the period covered by this report, post-mortem identification of scrapie cases was achieved by IHC examination of brain, peripheral nervous, lymphoid and other tissues for the accumulation of PrP$^\text{Sc}$, the IHC protocol and PrP antibodies have been described in detail previously (González et al., 2002).

Monitoring for infection in the live sheep was achieved by IHC examination for PrP$^\text{Sc}$ in sequential tonsil and/or rectal mucosa biopsies, using methods reported previously (Jeffrey et al., 2001; González et al., 2008). Sheep from the 1998–2000 and 2002 cohorts were subjected to palatine tonsil biopsy only, those from the 2005 cohort to rectal biopsy only, and those from the 2003 and 2004 cohorts to both (most often simultaneously). Biopsies were not taken from the sheep of the 2001 birth cohort due to a foot-and-mouth outbreak in the UK, nor from the 2006 and 2007 cohorts as a decision to cull the flock had already been taken. In total, 221 tonsil and 63 rectal mucosa biopsy samples were examined from 63 susceptible QQ171 sheep. Forty-six of these sheep went on to develop clinical disease, confirmed as scrapie at post-mortem, nine were culled or died of intercurrent conditions while incubating the disease, and in the remaining eight no evidence of scrapie was found at post-mortem. Details of the biopsies taken by birth cohort and with regard to the scrapie status of the sheep and of their dams are provided as Supplementary Table S4 (available in JGV Online). The mean age of sheep at first biopsy was 156 days and, although there was some variation between cohorts, 80% of them were between 136 and 172 days old when initially examined; thereafter, sequential biopsies were obtained at a mean 138-day interval. Details are not given for biopsies taken from sheep of RR171 and RR171 genotypes, as these always gave negative results and scrapie was not identified at post-mortem examination in any of these sheep. In addition to biopsies, monitoring for clinical signs of scrapie was performed daily when the flock was housed, which varied from year to year depending on the lambing schedule, and weekly when the sheep were out at pasture. Visual inspection was performed and the response to different stimuli and body condition were assessed in order to detect signs of (i) locomotor dysfunction/ataxia, (ii) behavioural changes (from dullness to excitability), (iii) dysphagia (ptyalism to cud dropping), (iv) pruritus (rubbing, wool loss, scratch test) and (v) weight loss (body condition score from 1 to 5). These clinical anomalies were scored from 0 to 3 (see Supplementary Table S3), and a clinical end point was established when any of these five groups of signs was given a score of 3 or when a combined score of 8 was reached. At this point, sheep were killed and necropsies performed. Usually, sheep of the QQ171 genotype were allowed to develop clinical disease; in some instances, however, these sheep were culled for welfare reasons or to allow specific studies on pre-clinical infection, such as those reported by Siso et al. (2009), or they died from intercurrent conditions. Whilst these sheep were considered in the estimations of ARs as described below, they were not included in the analyses on clinical disease.

**Definitions and statistical analyses.** For the purpose of comparison between birth cohorts and of assessment of the effect of different factors, ARs, incidence of clinical disease, ages at clinical end point and ages at first positive biopsy were defined as follows for each cohort.

**AR.** The AR was defined as the number of IHC PrP$^\text{Sc}$-positive sheep at post-mortem/total number of QQ171 sheep, which at post-mortem were coeval or older than the youngest PrP$^\text{Sc}$-positive sheep of the same cohort, either at necropsy or by biopsy. Therefore, scrapie-negative QQ171 sheep that died younger than the earliest indication of infection in each cohort were not included in the denominator.

**ACEP.** As sheep in this flock were exposed to natural infection, the exact moment at which exposure to infection occurred could not be determined. Therefore, true incubation periods were unavailable and the expression ACEP was used to denote the time elapsed between birth and necropsy of clinically affected sheep.

**Incidence of clinical disease.** This was defined as the number of IHC-confirmed clinical cases/number of IHC-positive sheep that at post-mortem were older than the mean ACEP of the clinical cases for the corresponding cohort. Therefore, QQ171 sheep that died younger than the mean ACEP of each cohort were not included in the denominator.

**Age at first positive biopsy.** Because the intervals between consecutive biopsies, either tonsil or rectal, were not the same in all sheep, the age at which a biopsy first became positive was defined as the mid-point between the first positive specimen and the last negative sample. In the 19 sheep of birth cohorts 2003 and 2004, in which simultaneous tonsil and rectal biopsies were obtained, both samples first became positive at the same time (González et al., 2008); therefore, biopsy results are presented regardless of the tissue sample(s) examined. In addition, all biopsy data refer to ‘valid biopsies’, i.e. those containing at least five secondary lymphoid follicles; this figure was chosen in view of previous experience (Jeffrey et al., 2001; González et al., 2008).

To investigate the effects of the scrapie incidence in different birth cohorts and of the dam scrapie status on the proportion of positive biopsy samples and on the age at which the first positive samples were obtained, data were analysed by Fisher’s exact test and an unpaired $t$-test, respectively.

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