No Evidence of Uptake or Propagation of Reindeer CWD Prions in Environmentally Exposed Sheep

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Research Article

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Abstract

Background: Chronic wasting disease (CWD) is a prion disease of cervids. In 2016, CWD was discovered for the first time in reindeer. The affected population was situated in Nordfjella mountain region in Norway. In an attempt to eradicate the disease, all reindeer in the region were culled during winter 2017-18. Because many sheep have their summer pasture in Nordfjella, concern exists about the potential cross-species transmission of CWD to sheep. In this study, global positioning system (GPS) data from sheep and reindeer from the relevant time frame were analyzed to determine spatial overlap. Samples of gut-associated lymphoid tissue (GALT) from 503 lambs and sheep having grazed in the region were analyzed for the presence of prions. The rectoanal mucosa-associated lymphoid tissue (RAMALT) from all animals and ileal Peyer's patch (IPP) from 37 of them, were examined by histology, immunohistochemistry (IHC), and enzyme-linked immunosorbent assay (ELISA).

Results: GPS data showed a direct overlap in area use between an infected reindeer and some of the sampled sheep. Generally, the sampled sheep herding areas were used intensively by reindeer before culling. No prions were detected in the GALT of sheep. The mean lymphoid follicle number per sample was 22.6 for RAMALT and 37.5 for IPP.

Conclusion: Despite evidence of close reindeer to sheep interaction and spatial overlap in Nordfjella, prions were not detected in the GALT of sheep.. We document that the easily accessible RAMALT tissue at the mucocutaneous border is well suitable for slaughterhouse screening of prions in sheep.

Introduction

Prion diseases are neurodegenerative, fatal diseases in animals and humans that occur as transmissible, familial, or sporadic forms. While Creutzfeldt-Jakob disease in humans and bovine spongiform encephalopathy (BSE) in cattle are transmissible through alimentary uptake of infectious material, chronic wasting disease (CWD) in cervids, and scrapie in sheep and goats are the only known prion diseases that are contagious between individuals. The causative agent is composed of a misfolded form (PrP^{Sc}, or prions) of the host's normal cellular prion protein (PrP^{C}). During infection, PrP^{Sc} interacts with PrP^{C} and induces profound alterations in the three-dimensional shape, transforming the protein into PrP^{Sc}. The newly formed PrP^{Sc} can then template misfolding of further PrP^{C} molecules, thus propagating the prion agent [1]. PrP^{Sc} accumulates in the central nervous system (CNS) and for some of the diseases, in peripheral organs and is the pathognomonic marker for prion disease [1, 2].

CWD is the only prion disease observed in natural outbreaks among wild animals and was first described in mule deer (*Odocoileus hemionus*) in Colorado in the 1960s [3]. Since then, the disease has been discovered among free-ranging and farmed deer in at least 26 states in the USA, and in three Canadian provinces, affecting white-tailed deer (*Odocoileus virginianus*), elk *(Cervus elaphus nelsoni*) [4], and rarely moose (*Alces alces*) [5], in addition to mule deer. The disease has also been found in South Korea, traced back to the importation of elk from Canada [6]. In 2016, CWD was diagnosed in a Norwegian wild reindeer

in the Nordfjella mountain region [7]. This was the first time the disease was found in Europe, and the first natural case observed in reindeer.

Nordfjella is a mountainous area in South Norway (Fig. 1a) with lush grassland pastures in valleys, surrounded by high-alpine lichen-rich areas with summer snow ideal for reindeer to avoid insects during mid-summer. Following the CWD outbreak, the authorities defined a “Nordfjella management area” which consists of six municipalities in two counties. Within the management area, there are two important subareas: zone 1, the area of the CWD outbreak, and zone 2, where a small population of wild reindeer still live (Fig. 1a). These two zones are mainly separated by a road and geographical obstacles. In addition, a potential crossing area has been fenced to prevent the movement of reindeer between the two zones [8]. Since the affected population was partly confined, it was decided to cull the entire population of around 2400 animals in zone 1, to prevent spreading of the disease to the adjacent wild reindeer in the south (zone 2), semi-domesticated reindeer in the north, and other cervid species in the surrounding valleys. All animals from zone 1 were tested for CWD and 19 of these were positive (0.8%) with adult males being 2.7 times more likely to be positive than adult females [9].

Concurrently with the outbreak of CWD, other cases of CWD in old moose have been observed in Norway (eight), Finland (two) and Sweden (four). There has not been established any epidemiological connection between the outbreak in reindeer and the cases in moose, and further investigations, including prion strain characterization in bioassays, have established that the diseases were caused by distinct CWD strains, all different from those characterized from North America [10]. In addition, a single case of CWD has been detected in a 16-year-old female red deer (*Cervus elaphus*) in Norway, the pathological and molecular features suggest that this represents an additional unique CWD strain [11]. Due to the old age of the affected animals, the absence of detectable prions in peripheral lymphoid tissues, and differences in molecular characteristics, the types of CWD observed in moose and the case in red deer are currently considered to represent less contagious, most likely sporadic forms of CWD, like atypical BSE in cattle and atypical/Nor98-scrapie in sheep and goats [12–14]. In 2020, a new case of contagious CWD was discovered in a wild reindeer in the Hardanger mountain plateau south of Nordfjella [15]. The laboratory examinations hitherto performed, have not revealed disease characteristics different from the cases in Nordfjella, suggesting that they are epidemiologically related. However, as of September 2021, no additional cases have been found among 1948 examined reindeer shot in 2020 and 2021 from this area.

Nordfjella is also a high-quality, nearly predator-free grazing area for sheep, used by both local and distant farms. It is divided into 16 grazing areas, of which 12 are fully or partially located within zone 1. During the summer of 2017, approximately 70000 sheep were released into the area, and 52000 of these grazed in zone 1. The density of sheep within the region varies, with some areas such as Fødalen being relatively small and having a density close to 100 sheep/km2 [16] (Fig. 1B). Traditionally, and increasingly the last few decades, sheep farmers in Nordfjella have used mineral licks as a management tool to limit the movement of sheep into less accessible mountain areas. Surveillance data from Nordfjella and other regions [16] show that mineral licks also attract reindeer, as well as other cervids, leading to considerable indirect interactions between these species. After the detection of CWD in

Nordfjella, 684 mineral licks were recorded in the area, of which 278 were in zone 1 [16]. In the initial phase of the disease management operation, 170 of these were identified as potential “hot-spots” for transmission of CWD from wild reindeer to other cervids, mainly red deer [16]. These hot-spot mineral licks were fenced off and closed permanently, whereas the remaining mineral licks were fenced, but equipped with openings that should allow sheep to pass, but not cervids (Fig. 2). This construction did not work as intended, since both reindeer and red deer crawled through the openings, gaining access to the mineral lick, sometimes side-by-side with sheep. It has been demonstrated that CWD can be transmitted between cervids directly and indirectly [17] and it is likely that both mineral licks [18] and water [19] can serve as environmental reservoirs for CWD prions and facilitate their transmission.

The close interaction between reindeer and sheep in Nordfjella has raised concerns regarding the potential interspecies transmission between them, an event that would have far-reaching social and economic implications. Propagation of CWD prions in sheep that return to farms far away from Nordfjella could provide a way of spreading the disease that would be difficult to discover and control. Sheep with a long incubation period could potentially serve as an additional source of environmental contamination of prions, undermining the efforts made to eradicate CWD. It is therefore important to identify and characterize the degree of contact and overlap between sheep and reindeer, and test sheep that have been grazing in areas frequently used by reindeer from the affected population for possible uptake and accumulation of prions in gut-associated lymphoid tissues (GALT).

Definite diagnosis of scrapie in sheep is based on detection of prions in lymphoid tissue or CNS by immunohistochemistry, ELISA, or Western Blot tests [20]. In subclinical cases, prions in the brain are sparse or not detectable [21], while there can be a significant accumulation of prions in the peripheral lymphoid system [22, 23]. After oral exposure, uptake and early accumulation of prions take place in the GALT, draining lymph nodes [24, 25], and the enteric nervous system (ENS) in both scrapie and CWD [26, 27]. In naturally infected and highly susceptible lambs, scrapie prions were detected in the ileal Peyer’s patch (IPP) and mesenteric lymph node as early as at the age of two months [43]. Another study of natural scrapie infection described the accumulation of prions in the IPP of three-month-old lambs carrying other, less susceptible prion protein (PrP) genotypes [21]. Thus, early detection of prions in subclinical animals is possible by examination of lymphoid tissues which are accessible by minimally invasive procedures. Such lymphoid tissues were traditionally the 3rd eyelid [28] and tonsils [29], but the rectoanal mucosa-associated lymphoid tissue (RAMALT) has proven to be more suitable for early detection of prion diseases [30, 31]. RAMALT is found just proximal to the mucocutaneous border of the anal opening and is rich in lymphoid follicles [32]. Its distal and easily accessible location makes it an attractive sampling area for the diagnosis of prion disease in live animals [33, 34] as well as post-mortem [30].

This study aimed to examine the overlap in area usage between sheep and reindeer in Nordfjella, and to elucidate whether potential environmental prion exposure of sheep could lead to uptake and accumulation of the infectious agent in their GALT.

Results

Histology and immunohistochemistry

We examined lymphoid follicles that are found immediately proximal to the rectoanal junction (RAMALT), and in a subset, the IPP (Fig. 3), and the ENS included in these sections. In total, 503 RAMALT samples comprising 12746 lymphoid follicles were analyzed. Of these, 28 samples were from adult sheep that had grazed one or more seasons in the Lærdal area of Nordfjella, and 475 were from Fødalen, of which the majority were lambs (Fig. 1B). None of the samples showed immunolabeling indicating the presence of prions when stained with the F99/97.6.1 antibody.

The mean number of lymphoid follicles in the RAMALT samples was 22.6 (95 % CI 21.2–23.9). There were 82 (16.3 %) samples that contained less than six lymphoid follicles and 19 (3.7 %) samples contained no lymphoid follicles (Table 1). All the IPP samples were rich in lymphoid follicles, the mean number was 37.8 (95 % CI 33.5–42). The number of lymphoid follicles per sample was significantly higher in IPP compared with RAMALT ($p < 0.01$), (Fig. 4).

Table 1
Comparison of the number of lymphoid follicles (LF) between rectum (RAMALT) and ileal Peyer's patch (IPP).

Sample type	N=	Mean LF	Median LF	Samples containing < 6 LF (%)	Samples without LF (%)
RAMALT	503	22.6	21	82 (16.3%)	19 (3.7%)
IPP	37	37.5	36	0	0
N = number of samples					

The total area of SSE covering the section was subjectively estimated as being below or above 50 %, as an indicator for the location of sampling with relation to the rectoanal junction [34]. The majority of the samples (308) contained SSE which covered less than 50 % of the tissue (SSE1), and the mean follicle number was 25.5 (95 % CI 23.7–27.2). In 189 samples, there was no SSE present (SSE0) indicating a more proximal sample site from the mucocutaneous junction, and the mean number of follicles was 18.1 (95 % CI 16.1–20.2). The difference in follicle numbers between SSE0 and SSE1 groups was significant ($P < 0.01$). In only 6 samples, more than 50 % of the tissue was covered with SSE (SSE2) indicating a more caudal sampling site, and the mean number of follicles was 13.5 (95 % CI 2.3, 24.7). There was no statistical significance when comparing group SSE2 to group SSE0 or SSE1. The results for sampling site and follicle counts are summarized in Fig. 4 and Table 2.

Table 2
Number of lymphoid follicles (LF) and amount of stratified squamous epithelium (SSE) in RAMALT sections

	N=	Mean LF	Median LF	Number of samples with < 6 LF (%)
SSE 0	189	18.1	17	52 (27.5 %)
SSE 1	308	25.5	24	28 (9 %)
SSE 2	6	13.5	15	2 (33 %)
Totals	503	22.6	21	82 (16.3 %)
N – number of samples. SSE 0 – No SSE present. SSE 1 - <50 % SSE. SSE 2 - >50 % SSE.				

ELISA

PrP^{Sc} ELISA was performed on RAMALT samples parallel and neighboring to the histological samples. In total, 515 samples were analyzed with ELISA compared to 503 sections that were histologically and immunohistologically evaluated. The discrepancy was a result of the unreadable marking of 12 of the histological samples. The values of all samples were lower than the cutoff value and considered negative.

Overlap in reindeer and sheep area use

We identified overlap between reindeer and sheep at all spatial scales investigated: a) historical use of the diseased reindeer population in Lærdal and Fødalen sheep herding units b) overlap between a CWD infected reindeer individual that was GPS tracked during spring 2017 and six sampled ewes, and c) spatiotemporal overlap in utilization of local resources (Figure 1B-D). The two sheep herding units that were sampled had relatively high to very high densities of sheep (Figure 1B) and were both intensively used by reindeer in Nordfjella zone 1 in the period before they were culled (Figure 1C). Sheep and reindeer both visited highly attractive mineral lick sites and were potentially exposed to environmental disease transmission in form of saliva, urine, and feces from former visitors as exemplified in Figure 2.

Discussion

The present study was performed as a response to the recent outbreak of CWD among reindeer in Norway. This outbreak has raised concerns about the possible spread of CWD to both wildlife and to the high number of sheep that graze in the region of the affected reindeer population. This concern still exists despite the effective depopulation of the reindeer in Nordfjella zone 1 [16] since prions have been shown to maintain infectivity in the environment for a long period [38–40], and can therefore potentially be taken up by other species even after the removal of the affected population.

In general, the barrier for interspecies transmission of prions is high [41]. The potential interspecies transmission of CWD from reindeer to sheep will mainly depend on the amount of prions available in the environment [42], to which degree sheep ingest contaminated material (by licking on contaminated surfaces or ingesting contaminated food, soil, or water) and the ability of prions to infect and propagate in the new host [18, 43]. Little is known about the shedding of prions from Norwegian reindeer infected with CWD, but studies of several cervid species in North America have shown that prions are shed in saliva, urine [44], feces [45], and antler velvet [46]. The distribution of prions in peripheral tissues in the Norwegian reindeer cases [7] suggests that shedding of infected materials from these animals occurs in the same way as in cervids with North American CWD. Environmental contamination of prions will therefore depend on the number of infected animals and their shedding capacity, but other factors such as climate and soil characteristics [47–49] will inflict on the availability and persistence of prions in the environment.

In Nordfjella, there has been extensive use of mineral licks intended for sheep [16], but these have also been used by reindeer and other cervids as documented by us (Fig. 2) and others [16]. These mineral lick sites are regarded as high-risk spots for environmental prion contamination and potential cross-species transmission [18, 50, 51]. On the other hand, we know that at the time of stamping out, the prevalence of the disease was less than 1 %, and in approximately half of the infected animals, prions were only found in lymphoid tissues (personal communication S. Benestad), indicating that the individuals probably not had shed prions for a long time and possibly only in small amounts relative to an animal in a later stage [9].

GPS data show that the now eradicated reindeer herd utilized a large area, including the high-density sheep grazing area Fødalen, where about 3200 sheep and lambs grazed during the summer of 2018 (Fig. 1B). We examined 475 of them (approximately 15 %), and prions were not detected in GALT samples from these animals. The other sampling area was Lærdal, which has a lower sheep density (Fig. 1B) but higher reindeer usage than Fødalen (Fig. 1C). From there, we sampled 28 adult sheep that had used the same pasture area for several seasons. GPS data from six of these sheep confirmed considerable overlap of area use with a CWD positive reindeer (Fig. 1D), and the affected reindeer herd in general. Neither of the sheep had evidence of prions in their GALT. To increase the confidence in the negative immunohistochemical results, all RAMALT samples were additionally tested with a validated ELISA rapid test [52]. All samples were negative using this commercial kit, reported to have nearly 100 % specificity when retropharyngeal lymph nodes were analyzed in both cervids and sheep [53, 54], meaning that the likelihood of false negative test results are low.

Transmission of North American mule deer CWD prions via the intracerebral route has been successfully performed for genetically susceptible sheep, but the number of animals and different PrP genotypes were limited [55]. The second passage of the disease in sheep showed the ability of the prions to adapt to the new host and increase the attack rate [56]. In a conference paper, sheep were reported to be infected when challenged intracerebrally with elk CWD, but not after oral inoculation [57]. The difficulties to overcome the species barrier were also shown by intracerebral inoculations of transgenic ovinized mice with white-

tailed deer CWD and cervidized mice, with classical scrapie, both of which showed very low attack rates [59]. However, several recent papers report differences between Norwegian and North American CWD isolates [11, 59, 60]. Norwegian reindeer CWD has a remarkable low attack rate in bank voles [10] but transmits efficiently into transgenic and gene-targeted cervidized mice, albeit with longer incubation times than North American moose CWD [59]. An *in vitro* protein misfolding cyclic amplification (PMCA) study suggests that Norwegian reindeer CWD has a lower species barrier than North American isolates towards sheep, cattle, hamsters, and mice (all transgenic mice substrates), but a higher species barrier towards humans (substrate from humanized mice) compared to North American isolates [60].

The majority of sheep investigated for prion uptake in this study were lambs born during March/April and sampled in September the same year. Generally, oral uptake of prions and further propagation of the disease is more efficient in young lambs compared to adult sheep [61, 62]. Thus, lambs are suitable indicators of oral uptake of environmental prions able to infect sheep, although older sheep that have grazed in the same area for several seasons could be better for demonstrating propagation and aggregation of prions in tissues. Sheep of all age groups investigated in the present study were included due to their history of close temporospatial contact with the population of infected reindeer and because tissues from these animals were accessible from the abattoir.

Choosing tissue type to screen a large number of animals for prion disease should be based on knowledge of the disease progress, including uptake and propagation of prions, in the species of interest. Scrapie in sheep and CWD in cervids have similar pathogenesis with an early accumulation of prions in peripheral tissues and later involvement of the CNS [23, 63]. It is therefore hypothesized that if sheep are able to take up and propagate CWD prions, the pattern of dissemination will be similar between these species. The early accumulation of prions in GALT, including the lymphoid follicle-rich RAMALT [30] and the easy accessibility of the tissue, makes it ideal for sampling and screening purposes [34, 43]. The technique is relatively easy to perform on live animals [30] and RAMALT is also well suited for postmortem sampling [30, 31] of a large number of animals in a short time, as was done on fresh slaughterhouse material in the present study. Other lymphoid tissues such as the medial retropharyngeal lymph node, the third eyelid, distal jejunal lymph node, and the IPP have also proven to be tissues with high diagnostic value [22, 28, 43]. These tissues, however, were less accessible and would have required a longer time to sample in this particular abattoir, which made them less suitable for mass sampling. The IPP in sheep is shown to undergo involution early in life [6], which makes it less reliable as sampling tissue as the animal gets older, but since some studies suggest that accumulation of prions occurs earlier in the IPP than in other lymphoid organs [43], we included IPP from 37 of the lambs in the study.

The histological analysis of over 500 slides confirmed that RAMALT is well suited for post-mortem screening of a large number of sheep. Some publications refer to 6 or more lymphoid follicles in a sample as “sufficient”, while samples with 5 or fewer follicles are considered as “insufficient” [33, 65]. When using these criteria in our work, 83.7 % of the samples are considered “sufficient”. However, even samples with less than 6 lymphoid follicles can contain detectable prions. In sheep with scrapie, the probability of false-negative results based on the number of lymphoid follicles in the sample has been calculated [66].

These calculations are intended to predict the probability of an individual scrapie-positive sheep within a herd to escape detection. Our work, on the contrary, is designed to detect any evidence of prions in sheep that spend time in a certain geographical region, and not necessary to locate an individual animal with prion disease. Thus, sample volume seems to be more important than individual lymphoid follicle count for our type of study. Since we have sampled approximately 15 % of all animals that grazed in Fødalen during the summer of 2018 and examined nearly 13000 lymphoid follicles from these animals, we are confident that the likelihood of false-negative results in our study is low.

The lymphoid aggregates in the rectum area are most abundant in the circumference starting from the mucocutaneous junction and approximately 1 cm proximally [30, 67]. Thus, sampling should be directed to that region. We demonstrated that using the mucocutaneous junction as a guideline is beneficial to achieve high-quality samples. We further showed that when the mucocutaneous junction was missing from the sample due to sampling from a more proximal location of the rectum (designated as SSE0), the mean number of lymphoid follicles was significantly lower than the mean number of lymphoid follicles counted in samples that included the mucocutaneous junction. However, when the sample was covered by more than 50 % SSE, the number of lymphoid follicles seemed to be declining. In our sample pool, only 6 samples fell into this category, a number that was too low to show statistical significance (Fig. 4A).

The samples from the IPP contained a higher mean number of lymphoid follicles than the RAMALT samples. In addition, the sample with the lowest number of follicles in the IPP group contained 9 follicles, while for the RAMALT group, 3.7 % of the samples contained no follicles at all (Fig. 4B, Table 1). This shows that the IPP is a reliable tissue for screening individual young lambs, and although the IPP undergoes involution and the number of lymphoid follicles gradually declines with age, some also remain in adulthood [68]. The number of RAMALT follicles decreases as well with age, but the process is much slower [32]. This favors RAMALT as a reliable tissue for screening different age groups.

Conclusion

Despite evidence of close interaction and spatial overlap between reindeer and sheep in Nordfjella zone 1, we did not find any evidence of uptake and propagation of prions by sheep that were very likely exposed to environmental CWD prions. We conclude that RAMALT is a suitable tissue for postmortem mass sampling, and although it yields on average fewer lymphoid follicles than the IPP per sample, the ease and speed of sampling makes it a better choice. We also showed that using the mucocutaneous junction as a guideline for sampling increases the mean number of lymphoid follicles in the sample, and hence reduces the probability of false-negative results. This study was not aimed to determine whether CWD is transmissible to sheep. For that, long-term inoculation studies are needed. Still, the absence of prions from sheep RAMALT and IPP indicates that CWD prion uptake and propagation by sheep under the environmental conditions that exist in Nordfjella, is not prevalent.

Methods

Animals

The majority of the material was sampled from 475 sheep belonging to the Fødalen sheep management area, slaughtered at the abattoir Nortura Gol on September 2nd, 2018. Most of the animals were lambs born in 2018 and had grazed one summer in the Fødalen valley in Nordfjella zone 1 (Figure 1B). A subset of the samples was from adult sheep that had grazed at least one summer season in the area. Fødalen has the highest density of sheep in Nordfjella during summer grazing (Figure 1B), and the area was also frequently used by the CWD-affected wild reindeer population (Figure 1C). In addition, 9 adult sheep were sampled at Nortura Førde in March 2020, and 19 adult sheep were sampled at the same abattoir in October 2020. These sheep had grazed in the Lærdal sheep management area of Nordfjella zone 1 for one or more summers while reindeer were still present (Figure 1B). Lærdal is not as densely populated by sheep during summers as Fødalen but was intensively used by reindeer before the culling (Figure 1C). The management of the abattoirs had given their consent to perform the sampling.

Sampling procedure

Immediately following slaughter, the terminal part of rectum was removed by means of a circular incision around the anus as a standard slaughtering procedure. To sample the follicle-rich lymphoid tissue RAMALT, a rectangular area of mucosa from the mucocutaneous junction and approximately 2 cm cranially was collected [31,56]. The tissue samples were divided horizontally into two halves. One half was fixed in 4% formaldehyde solution, and the other half was frozen and stored at -70 °C. A section of the ileum containing IPP was taken from 37 of the animals that were slaughter in Nortura Gol and fixed in 4% formaldehyde solution only.

Histology

The rectum samples were orientated perpendicular to the surface in a longitudinal direction. All formaldehyde-fixed samples were dehydrated in graded ethanol and embedded in paraffin. Three µm sections were placed on glass slides, deparaffinized, and stained with hematoxylin and eosin for histological analysis. For each sample, the number of lymphoid follicles was counted. In rectum samples, the presence or absence of cutaneous stratified squamous epithelium (SSE) was noted, and if present, the amount of SSE was judged subjectively. The scoring for SSE was as follows: 0, no SSE present; 1, < 50 % of the sample was covered with SSE; 2, > 50 % of the sample was covered with SSE [33].

Immunohistochemistry

Paraffin-embedded samples were sectioned at three µm and placed on positively charged glass slides (Superfrost Plus®, Menzel-Gläser, Thermo Scientific, Oslo, Norway). The samples were deparaffinized with xylene and graded ethanol in decreasing concentrations and then immersed in 98 % formic acid for

15 minutes prior to incubation in a citrate buffer bath (pH 6) for 15 minutes at 121 °C. Following cooling, endogenous peroxidase activity was inhibited by 3 % H_2O_2 diluted in methanol for 15 minutes, and non-specific binding sites were blocked with 1:50 goat N-serum in 5 % bovine serum albumin (BSA) for 20 minutes. The samples were then incubated with the primary PrP antibody F99/97.6.1 (VMRD, Pullman, Washington, USA) for 60 minutes at room temperature. The remaining procedure was performed using a commercial kit (Envison+ K4005, Agilent Dako, California, USA). The secondary antibody conjugated to horseradish peroxidase (HRP) was applied for 30 minutes, and signals were visualized by incubating the slides in 3-amino-9-ethylcarbazole (AEC) for 10 minutes. Counterstaining was performed with hematoxylin for 1 minute. Each run included one scrapie-positive control and one negative control, in addition to a methodological control in which the primary antibody was substituted with 1 % BSA.

Enzyme-linked immunosorbent assay (ELISA)

All RAMALT tissues were stored at -70 °C prior to analysis. ELISA analysis was performed using a commercial kit validated for scrapie and BSE detection in the brain, spleen, and lymphoid tissue (HerdChek* Bovine Spongiform Encephalopathy-Scrapie Antigen Test Kit, IDEXX Laboratories, Inc., Westbrook, ME, USA). For tissue homogenization, approximately 300 mg of RAMALT tissue was cut into small pieces and placed in a grinding tube provided in the test kit. The samples were then subjected to 4 cycles of 30 seconds each at 7500 rpm using a ribolyser (FastPrep fp120 cell disruptor; Thermo Savant, USA). The samples were allowed to cool for 5 minutes between each cycle. The remaining procedure was performed according to the manufacturer's instructions. Briefly, 100 µl of the sample homogenate was added to a well in a dilution plate along with 50 µl diluent and mixed gently. 100 µl of the diluted samples were pipetted into wells on the antigen capture plate and incubated at room temperature for 2 hours. The wells were then washed 6 times with the wash solution provided in the kit, a conditioner buffer was added to each well and the plate was incubated for 10 additional minutes at room temperature. After washing the wells 3 times, a conjugate was added, and the plate was incubated for 60 minutes at room temperature. The wells were then washed again 5 times, and 100 µl substrate was added, after which the plate was incubated for 15 minutes at room temperature. Finally, an acid stop solution was added, and the optical density of the samples was read at 450 nm and 620 nm using an automated 96-well spectrophotometer (Thermo Scientific Multiskan go, ThermoFisher, USA). The cut-off values were calculated based on the mean optical density of two negative controls added to 0,150. Samples with a value above the calculated cut-off were deemed positive, while samples with a value under the cut-off were deemed negative.

Overlap in reindeer and sheep area use

Reindeer location data was collected from 29 reindeer (24 females, five males) outfitted with global positioning system (GPS) collars (Vectronics GPS+ and GPS PRO LIGHT) on various points of time from 2007 until the Nordfjella zone 1 herd was culled in 2018. The animals were chemically immobilized by

use of a dart gun from a helicopter during winter, following standard procedures at the Norwegian Institute for Nature Research. The use of animals for research was approved by the Norwegian Animal Research Authority (application identity numbers (FOTS id) 2375, 3993, 6052 and 8558). The GPS data was subsampled to one location every 3 hours.

The intensity of reindeer area use was estimated using fixed kernel utility distribution (UD) with a bivariate normal kernel and 0.4*h as the smoothing parameter, using library adehabitatHR [37] in program R (https://www.R-project.org/). The smoothing parameter was selected using a method proposed by Kie [37], sequentially reducing the reference bandwidth 'h' with 0.1 increments (0.9*h, 0.8*h, etc.) until balancing a close fit to original GPS locations and avoiding fragmentation.

Sheep location data were collected by farmers outfitting ewes with GPS collars (Findmy Model1) to ease and improve herding. To evaluate overlap in area usage between abattoir sampled sheep (6 sheep in summer 2017 – 2020, corresponding to 10 grazing seasons) and a GPS collared reindeer with verified CWD (data collected in the period 29 March 2017 – 21 June 2017), individual seasonal home ranges were estimated using 100 % Minimum Convex Polygons (MCPs). Sheep and wildlife utilization of mineral lick sites were monitored using motion-triggered camera traps (Recognyx XP9 UltraFire and Browning Strike Force HD).

Statistical analysis

For statistical analysis of histological findings in RAMALT and IPP slides, a one-way ANOVA with Tukey post hoc test and t-test were performed using GraphPad Prism version 6.07 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). Microsoft Excel (v.16.0.13801.20442) was used for descriptive statistics.

Abbreviations

CWD: chronic wasting disease

GPS: global positioning system

GALT: gut-associated lymphoid tissue

RAMALT: rectoanal mucosal associated lymphoid tissue

ENS: enteric nervous system

IPP: ileal Peyer's patch

PrP: prion protein

IHC: immunohistochemistry

ELISA: enzyme-linked immunosorbent assay

BSE: bovine spongiform encephalopathy

PrP^{Sc}: misfolded prion protein

PrP^{C}: cellular prion protein

CNS: central nervous system

SSE: stratified squamous epithelium

BSA: bovine serum albumin

HRP: horseradish peroxidase

AEC: 3-amino-9-ethylcarbazole

UD: utility distribution

MCP: minimum convex polygons

PMCA: protein misfolding cyclic amplification

Declarations

Ethical approval

The darting and immobilization of reindeer for installing GPS collars was approved by the Norwegian Animal Research Authority (application identity numbers (FOTS id) 2375, 3993, 6052 and 8558)

Consent for publication

Not applicable

Data availability

The datasets analyzed in the present study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Author's contribution

CE, MAT, AE, BY, and ØS designed the study. GRR analyzed and plotted the sheep and reindeer's areal use. EH, AM. SB and LT performed the laboratory procedures. EH read the slides, performed the statistical analysis, and drafted the manuscript. CE participated in the interpretation of data and drafting of the manuscript. All authors have read and approved the final manuscript.

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Figures

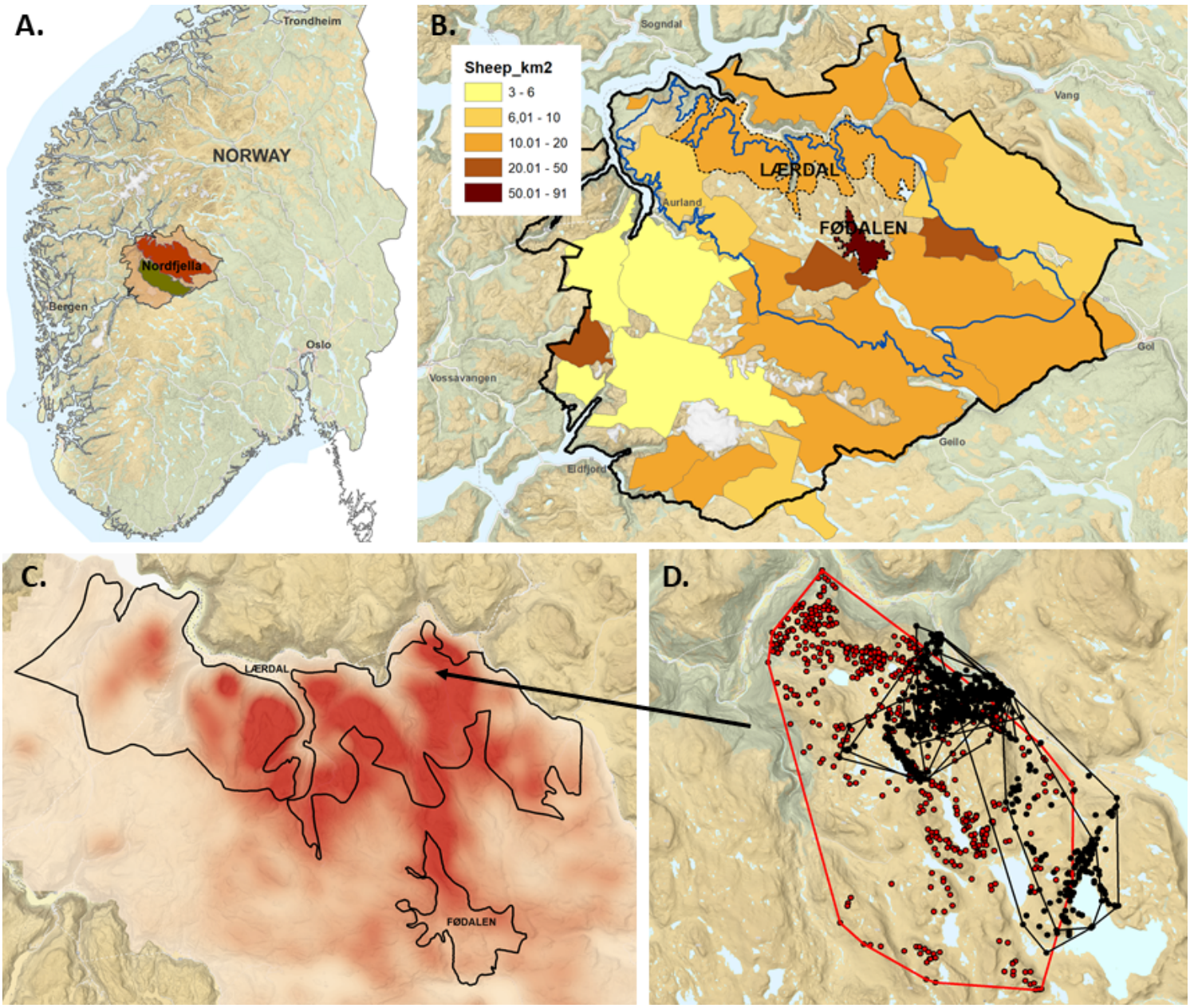

Figure 1

Nordfjella management area, sheep and reindeer density and movements A. Location of Nordfjella management area in Norway. Nordfjella wild reindeer zone 1 is marked in red and zone 2 is marked in green. B. Density of sheep released within the herding units of Nordfjella management area in the spring of 2017. Zone 1 is outlined in blue. The sheep herding units Fødalen and Lærdal where the sampled sheep were grazing are marked with dotted black lines (Data source: Organisert beitebruk/NIBIO,

https://kilden.nibio.no). C. Estimated reindeer area use in Nordfjella zone 1 based on GPS locations from marked reindeer (26 females and 5 males) during 2007-2018 The more intense red color, the more intense reindeer area use. Sheep grazing areas Lærdal and Fødalen are marked with solid black lines. D. GPS locations of adult female sheep (n=6) from the Lærdal herding unit included in the study (black dots and lines) and a CWD-positive reindeer that was marked on the 29th of March and culled the 21st of June 2017 (red dots and lines) showing overlapping area use.

Figure 2

Sheep and reindeer visiting the same mineral lick site in Nordfjella 2018. The two pictures were taken by a camera trap four hours apart. At the mineral lick sites, the animals lick on the salt stone and other surfaces, ingest soil, and graze. They are consequently exposed to saliva, urine, and feces of previous visitors. The fences were supposed to keep reindeer and other cervids outside the salt lick site, while allowing sheep to crawl through the 40 x 53,5 cm large openings. This did not work as presumed, and the openings were closed later the same year.

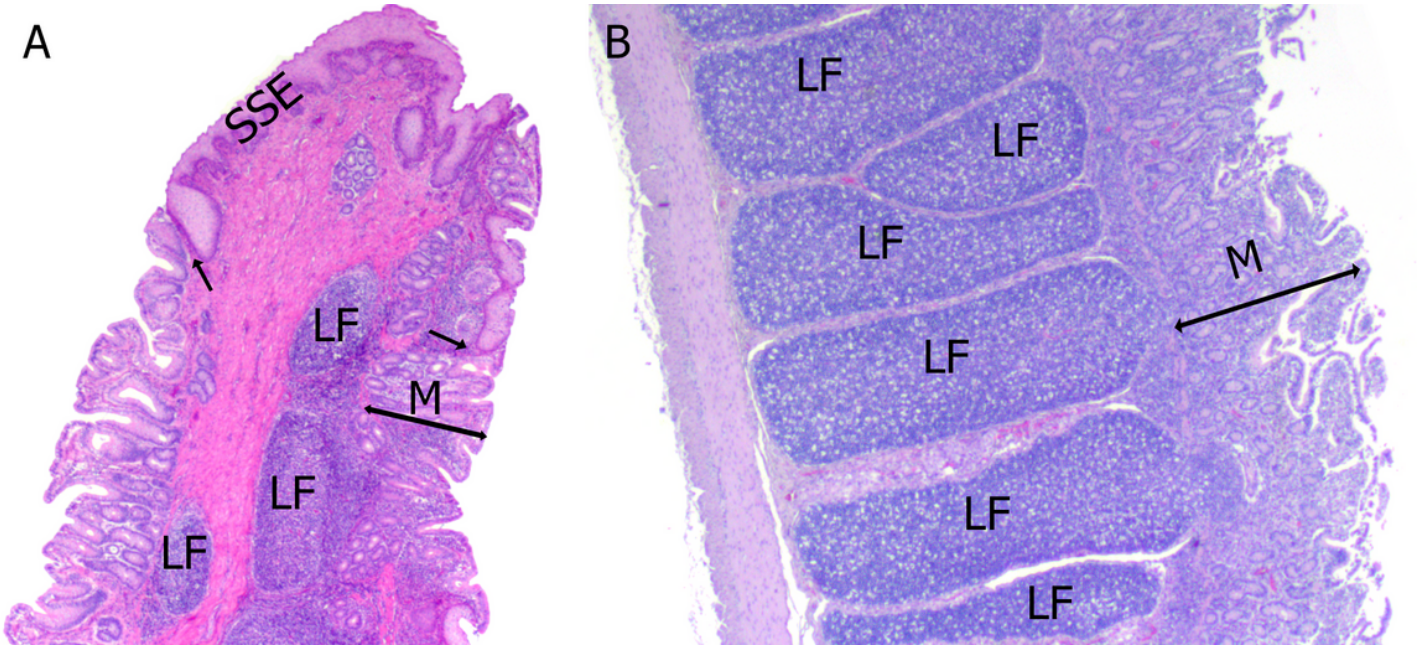

Figure 3

Representative histological sections of sampled gut-associated lymphoid tissue. A. Part of RAMALT section that contains less than 50 % stratified squamous epithelium (SSE). Lymphoid follicles (LF) are found next to the mucocutaneous junction (arrows) B. In the ileal Peyer's patch, the lymphoid follicles (LF) are more elongated and densely packed. M - mucosa. Hematoxylin and eosin, magnification 25 X.

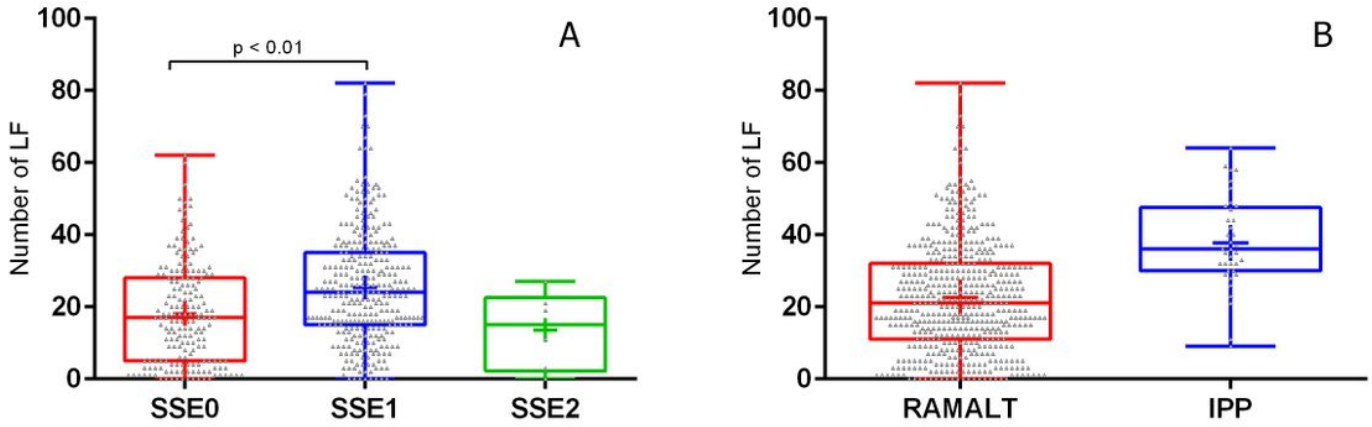

Figure 4

Distribution of lymphoid follicles in the RAMALT and IPP A. Distribution of lymphoid follicles in the different scoring groups based on the amount of stratified squamous epithelium (SSE) present in RAMALT sections. The number of follicles is significantly different between SSE 0 and SSE 1. B. Distribution of lymphoid follicles in the RAMALT and IPP. SSE 0 – No SSE present. SSE 1 - <50% SSE. SSE 2 - >50% SSE. Whiskers represent the minimum and maximum number of lymphoid follicles. The box represents the 2nd and 3rd quartiles. The middle bar is median, + is mean. The dots represent individual samples.