Veterinary Microbiology 304 (2025) 110478

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Pathological and serological insights into *Lagovirus* diseases dynamics in the European brown hare (*Lepus europaeus*): A nine-year longitudinal study

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ARTICLE INFO

Keywords:
European brown hare syndrome
Epidemics
Host-shift
Mortality
Population
Rabbit haemorrhagic disease
Wildlife disease surveillance

ABSTRACT

The European brown hare syndrome virus (EBHSV; GII.1) and rabbit haemorrhagic disease virus 2 (RHDV2; GI.2) are pathogenic lagoviruses affecting the European brown hare (*Lepus europaeus*). EBHSV/GII.1 causes periodic epidemics, while RHDV2/GI.2 infections emerge from spillover events in areas where hares are sympatric with European rabbits (*Oryctolagus cuniculus*). In the northeast of the Iberian Peninsula, the overlap of these species provides a unique opportunity to investigate how the epidemiology of these viruses correlates with disease course. We analysed the presence of lagoviruses in 113 European brown hare carcasses recovered in Catalonia (NE Spain) between 2015 and 2024. Animals were necropsied, and tissue and serum samples were collected for histopathology, virological investigation, and serology. Sera from hunted hares apparently healthy (n = 89, 2015–2023) were also included in the study. PCR on liver samples (n = 58) and virological ELISA on positive sera (n = 52) confirmed 28 EBHSV/GII.1 and 24 RHDV2/GI.2 cases. After the first EBHSV/GII.1 detection in 2016, antibody titres decreased progressively until 2020–2021, coinciding with an outbreak. No conclusive seropositivity for RHDV2/GI.2 was observed during the study. Pathology revealed more acute lesions in RHDV2/GI.2-infected hares compared to EBHSV/GII.1. These lesions, resulting in sudden death due to a deficient immune response, may explain this distinct epidemiological scenario. Despite a decade of circulation, RHDV2/GI.2 has not fully adapted to hares. However, ongoing monitoring is essential, as mutations or recombination events could increase its epizootic potential. The co-circulation of both lagoviruses, combined with other co-factors, might jeopardise the viability of European brown hare populations at the southern limit of their range.

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https://doi.org/10.1016/j.vetmic.2025.110478
Received 31 December 2024; Received in revised form 10 March 2025; Accepted 12 March 2025
Available online 22 March 2025

1. Introduction

The European brown hare syndrome virus (EBHSV; *Lagovirus europaeus*/GII.1) and rabbit haemorrhagic disease virus 2 (RHDV2; *Lagovirus europaeus*/GI.2), hereafter called by both the common name and the genotype name (Le Pendu et al. 2017), are two small, non-enveloped RNA viruses belonging to the genus *Lagovirus*, within the family *Caliciviridae*, causing the European brown hare syndrome (EBHS) and rabbit haemorrhagic disease (RHD), respectively (Capucci et al., 2019).

EBHSV/GII.1, since first described in Sweden in 1980 (Gavier and Mörner, 1989; Lavazza and Vecchi, 1989), has been responsible for epidemics in the European brown hare (*Lepus europaeus*) in several European countries where now it is considered endemic (Duff and Gavier-Widén, 2012). This viral infection causes severe necrotising hepatitis and is fatal to 40–70 % of infected hares when introduced into a naïve population (Cammi et al., 2003; Duff and Gavier-Widén, 2012). The virus also affects other leporid species living in proximity to European brown hare populations, including the mountain hare (*Lepus timidus*), the Italian hare (*Lepus corsicanus*) and the eastern cottontail (*Sylvilagus floridanus*) (Gavier-Widén and Mörner, 1993; Lavazza et al., 2015; Domanico et al., 2023). However, transmission to European rabbits (*Oryctolagus cuniculus*) has neither been observed in the wild nor experimentally demonstrated (Lavazza et al., 1996).

Disease occurrence is density-dependent, being influenced by population size, distribution, immune status and availability of new susceptible recruits (Sokos et al., 2018). In places where the density of hares is low, outbreaks with high mortality may cyclically emerge, coinciding with population renewal and a high number of susceptible individuals. By contrast, in high-density areas, mortality may be lower due to an increased likelihood of hares developing protective herd immunity (Paci et al., 2011; Chiari et al., 2014; Salvioli et al., 2017). The disease has not been observed in leverets younger than 40–50 days. Leverets aged between two and three months may become infected but typically do not develop clinical symptoms (Paci et al., 2011). The non-pathogenic hare calicivirus (HaCV/GII.2), a lagovirus genetically related to EBHSV/-GII.1, has also been described in the European brown hare in Europe and Australia. This virus causes a subclinical infection in the small intestine without inducing noticeable clinical signs or significant pathological lesions (Mahar et al., 2019; Droillard et al., 2020; Cavadini et al., 2021).

RHDV2/GI.2 emerged in 2010 in European wild rabbit populations in France (Le Gall-Reculé et al., 2011; Le Gall-Reculé et al., 2013), and subsequently spread rapidly across the globe (Rouco et al., 2019). This novel virus is now considered endemic in Europe and has displaced the classical strains (RHDV/GI.1, firstly described in China in 1984 (Liu et al., 1984)), almost worldwide (Le Gall-Reculé et al., 2013; Calvete et al., 2014; Dalton et al., 2014; Lopes et al., 2014; Mahar et al., 2018). Interestingly, a notable characteristic of RHDV2/GI.2 is its high recombination rate — a mechanism inherent to all caliciviruses — that maintains the same capsid protein while having different donors for the non-structural proteins (Lopes et al., 2015; Abrantes et al., 2020). The capsid protein determines the virus' ability to infect and induce fatal disease in several species within the *Lepus* genus (Mahar et al., 2021; Asin et al., 2024), including the European brown hare (Hall et al. 2017; Le Gall-Reculé et al. 2017; Velarde et al. 2017; Cavadini et al., 2024).

Like EBHSV/GII.1 and RHDV/GI.1, RHDV2/GI.2 primarily affects the liver of its host, causing fulminant hepatitis (Neimanis et al., 2018b). Lesions caused by RHDV2/GI.2 may be indistinguishable from EBHSV/GII.1 infection in hares if laboratory confirmation and typing are not performed (Velarde et al., 2017; Neimanis et al., 2018a). Data regarding the epidemiology of RHDV2/GI.2 on *Lepus* species is limited, as well as the consequences on hare populations (Byrne et al., 2022). The most accepted hypothesis suggests that infection may occur through spillover events from infected rabbits coexisting in the same habitat (Hall et al., 2017; Le Gall-Reculé et al., 2017; Velarde et al., 2017; Mahar et al., 2018; Neimanis et al., 2018a), although transmission between hares cannot be excluded (Le Gall-Reculé et al., 2017; Neimanis et al., 2018a).

The European brown hare is a lagomorph with a broad distribution across Eurasia, serving as a primary prey for numerous carnivores and raptors (Viviano et al., 2021). Moreover, it is one of the most relevant small game species in its historical distribution range (Tsokana et al., 2020). Over the past few decades, a generalised progressive population decline has been observed across Europe (Edwards et al., 2000; Hacklander and Schai-Braun, 2019). This decrease could be attributed to various causes, including agricultural intensification, predation and hunting pressure, climate change or transmissible infectious diseases (Smith et al., 2005; Duff and Gavier-Widén, 2012; Lavazza and Cooke, 2018; Schai-Braun et al., 2019). In the north-eastern Iberian Peninsula, where rabbits and hares cohabit (Gortazar et al., 2009; Ruiz-Olmo and Camps, 2023), both diseases have been described in the European brown hare, with the first RHDV2/GI.2 infection cases observed in February 2014 (Velarde et al., 2017; Almeida et al., 2024).

The biological reasons behind the epidemiological differences between pathogenic lagoviruses in the European brown hare remain unclear. Many uncertainties still exist regarding RHDV2/GI.2-host interactions that could influence viral fitness in this species (Byrne et al., 2022), which likely requires experimental infections to address (Cavadini et al., 2024). Regardless, considering hares as spillover hosts for RHDV2/GI.2, it is possible that these differences could be attributed to varying susceptibility to each virus infection, measured by the severity and duration of the disease. In fact, in many infectious diseases, high virulence can result from a host shift (Longdon et al., 2015). In this study, we leveraged the concurrent presence of both lagoviruses in the European brown hare population of Catalonia (north-eastern Spain) to explore this hypothesis. Our main objectives were to compare EBHSV/GII.1 and RHDV2/GI.2 epidemiology through a wide serosurvey in the same hare population and to investigate whether pathological differences could explain the distinct disease dynamics of these viruses.

2. Materials and methods

2.1. Necropsy, sampling and histopathology

Between October 2015 and March 2024, a total of 113 carcasses of free-ranging European brown hares opportunistically found dead in the field by hunters and Rural Agents (forestry rangers of the Catalan government) were transported to the Veterinary Faculty of the Autonomous University of Barcelona (UAB), Spain. This was part of the passive surveillance program for game species in Catalonia. Hares were confirmed as European brown hares based on phenotypic morphometric characteristics (Palacios, 1989) and sexed (Female=64; Male=48; Not determined=1), weighed and aged (Adults=77; Juveniles=36) by using radium-ulna ossification criterion (Broekhuizen and Maaskamp, 1981). A systematic necropsy was performed for each animal. Liver samples (n = 113) were retrieved and preserved at −20 ºC, and blood from the heart or thoracic fluid (n = 110) was collected into sterile tubes. When the carcass decomposition status allowed, samples of brain (n = 40), lung (n = 43), trachea (n = 45), heart (n = 43), liver (n = 44), spleen (n = 40) and kidney (n = 40) were collected and fixed in 4 % neutral-buffered formalin. Subsequently, tissue samples were trimmed and embedded in paraffin, and 3–4 μm thick sections were stained with Mayer's haematoxylin and eosin for microscopic evaluation.

Additionally, blood samples collected from 89 apparently healthy European brown hares shot by hunters during regular game activities between December 2015 and March 2023 were also included in the study. These animals were examined in the field and identified as European brown hares based on the geographical distribution area where they were harvested (Gortazar et al. 2009). They were sexed (Female=36; Male=35; Not determined=18) and, when possible, classified into two age categories (Adults=49; Juveniles=9; Not determined=31) using morphological and sexual maturity characteristics. However, since hunters did the classification, details — particularly

about age — were not always recorded. Blood samples collected from hunted and found dead individuals were centrifuged at 3500 g for 15 min to separate the serum, which was then stored at −20 ºC for future analysis.

2.2. Serological and virological surveillance for Lagovirus detection

Serum samples frozen at −20 ºC (n = 199) were sent to the WOAH Reference laboratory for RHD at Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER) (Brescia, Italy) where they were tested in two cELISAs to detect specific antibodies to EBHSV/GII.1 and RHDV2/GI.2. The two methods are identical with the exceptions of the specific immunological reagents towards EBHSV/GII.1 and RHDV2/GI.2 (World Organisation for Animal Health, 2024). Briefly, a hare anti-EBHSV/GII.1 or a rabbit anti-RHDV2/GI.2 hyperimmune serum was adsorbed to the ELISA plates (Nunc Maxisorb) in standard carbonate buffer. Then, each serum tested at four dilutions starting from 1/10, 1/40, 1/160 and 1/640, was incubated with the specific virus used at a dilution giving 1.0–1.2 OD492. Finally, a specific anti-EBHSV/GII.1 or anti-RHDV2/GI.2 HRP-conjugated MAb semi-quantified the virus bound to the solid phase. A serum sample was considered negative if its OD value at the 1/10 dilution was higher than 85 % of the OD value at the same dilution of the negative control serum. As samples were coming from carcasses, serum was considered doubtful (inconclusive result) if its OD value at the 1/10 dilution was equal to or higher than 40 % of the OD value at the same dilution of the negative control serum. A serum sample was considered positive if its OD value at the 1/10 dilution was lower than 40 % of the OD value at the same dilution of the negative control serum. The titre of a positive serum sample corresponded to the dilution causing a 40–60 % reduction of the OD value of the negative control serum. Since the cELISA serological test also works as a virological test, the virus (antigen) concentration used is the limiting factor in the reaction to achieve the highest possible analytical sensitivity. Consequently, when a blood or serum sample from an animal with acute RHD or EBHS is analysed using cELISA, the large amount of virus in the sample adds to the antigen in the reaction, often causing the OD to exceed 2.0. Therefore, all blood or serum samples that showed an OD value greater than 1.4 at the initial 1/10 dilution (n = 52) in cELISA were subsequently subjected to a virological sandwich ELISA to confirm or rule out the presence of the virus (World Organisation for Animal Health, 2024).

2.3. Lagovirus molecular confirmation

Liver samples from animals showing lesions consistent with a *Lagovirus* infection or found dead during the EBHSV/GII.1 outbreak from December 2020 to September 2021 (n = 58) (Almeida et al., 2024) were sent to the Research Centre in Biodiversity and Genetic Resources (CIBIO-InBIO) (Porto, Portugal) to confirm *Lagovirus* infection by molecular analyses. A portion of the liver (approximately 30 mg) was homogenised for each sample using a rotor-stator homogeniser (Mixer Mill MM400, Retsch) at 30 Hz for 7 min. Total RNA was extracted with the GeneJET RNA Purification kit (Thermo Scientific). Viral cDNA was then synthesised using the NZY first-strand cDNA synthesis kit (Nzytech) according to the manufacturer's instructions.

EBHSV/GII.1 and RHDV2/GI.2 screening was performed with two PCRs for each genogroup, as a further confirmation of the presence of lagoviruses. The PCRs were carried out using the Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific), two pmol of each primer, one µL of cDNA and ultra-pure water for a final PCR volume of 10 µL. Cycling conditions consisted of 98ºC for three min, followed by 40 cycles of 30 sec at 98ºC, 30 sec at the annealing temperature and extension at 72 ºC (extension times indicated below). A final extension of 5 min at 72 ºC terminated the reaction. For EBHSV/GII.1, the primers used were 5′-ATGGAGGGTAAGCCWCGGGCTGA-3′ (nucleotide positions 5275–5297) and 5′-GACATAGGAATATCCAGTGGTGGC-3′ (nucleotide positions 6979–7002) with an annealing temperature of 54ºC and 1 min. 30 sec. of extension and primers 5′-GACAGACCTCATTGACGTG-3′ (nucleotide positions 6903–6921) and 5′-CAAAYCGCTAGGCGTTACTC-3′ (nucleotide positions 7330–7349), with annealing temperature of 54ºC and 30 sec. of extension. For RHDV2/GI.2, the primers used were 5′-GTGAAAGTTATGGCGGCTATGTCG-3′ (nucleotide positions 1–24) and 5′-GCCACATTTGTCACATGTCTCCAG-3′ (nucleotide positions 178–201), with annealing temperature of 52ºC and 20 s of extension, and primers 5′-CAGCGGGCACTGCTACCACAGCATC-3′ (nucleotide positions 5342–5366) and 5′-CCAGCCCAACCAGCYTACAT-3′ (nucleotide positions 5629–5648), with annealing temperature of 55 ºC and 15 s of extension. After purification, positive results were confirmed by Sanger sequencing on an automatic sequencer ABI PRISM 3500xL Genetic Analyzer (PE Applied Biosystems).

2.4. Data analyses

Differences between EBHS and RHD macroscopic and microscopic lesions were tested using a multiple chi-squared test of independence. We prepared a contingency table to display the frequency counts and computed the chi-squared statistic. Significance was initially set at $\alpha = 0.05$ but later corrected using a Bonferroni correction to control type 1 error rate. Additive analysis was conducted using the package "mgcv" 1.9–1 version (Wood, 2010) of the R statistical software 4.4.2 version (R Core Team, 2023).

3. Results

A total of 202 European brown hares were analysed for this study. Among them, 52 individuals were diagnosed with a lagovirus infection (EBHSV/GII.1, n = 28; RHDV2/GI.2, n = 24, with no co-infections detected). Specifically, 50 out of 58 hares tested positive through molecular PCR analyses, with 42 yielding positive results in the virological sandwich ELISA performed on serum samples. Additionally, two sera samples from hunted hares, for which tissue samples were not collected, tested positive for RHDV2/GI.2 using the virological sandwich ELISA (Supplementary table S1).

Between 2015 and 2024, RHDV2/GI.2 cases in European brown hares were registered annually, except for 2023, with a cumulative total of 24 cases. In contrast, EBHSV/GII.1 was initially documented as a single case in 2016 and later as an epidemic outbreak between 2020 and 2021 (n = 27) (Fig. 1). Seasonal distribution was similar for both diseases, with most cases occurring during the cold seasons, from October to March (n = 17 for EBHSV/GII.1; n = 21 for RHDV2/GI.2), whereas in spring and summer, *Lagovirus* occurrence was lower (EBHSV/GII.1, n = 11; RHDV2/GI.2, n = 13). Regarding geographic distribution, EBHSV/GII.1 cases were homogenously observed in the northern half of the region, whereas RHDV2/GI.2 cases were found in central, western and north-eastern Catalonia (Fig. 1).

Data from the serological survey on European brown hares are summarised in Fig. 2. Doubtful and negative results were grouped, as the quantity and quality of the sera obtained from blood samples collected in the field or obtained as surrogates from carcasses (heart clot/thoracic fluid) are often suboptimal, leading to an increased proportion of uncertain results. Additionally, confirmed lagovirus infection cases were excluded from data analysis and seroprevalence calculations. EBHSV/GII.1 seroprevalence remained relatively stable, with peaks in 2015 (n = 25, 84 %) and 2021 (n = 12, 66.7 %) (Fig. 2, left panel). The lowest seroprevalences were recorded in 2019 (n = 9, 0 %), and 2020 (n = 12, 33.3 %) (Fig. 2, left panel). The results showed slight variations when examining EBHSV/GII.1 antibody titres. Although titres were generally low, ranging between 1/10 (26 %) and 1/20 (31 %) (Fig. 2, right panel), a first peak was observed in 2016–2017 (Fig. 2, left panel). Following a period of decline, titres peaked again in 2020–2021, just after the large observed outbreak. During this year, most sera showed titres between 1/40 and 1/80, with occasional cases reaching higher levels of 1/160, 1/

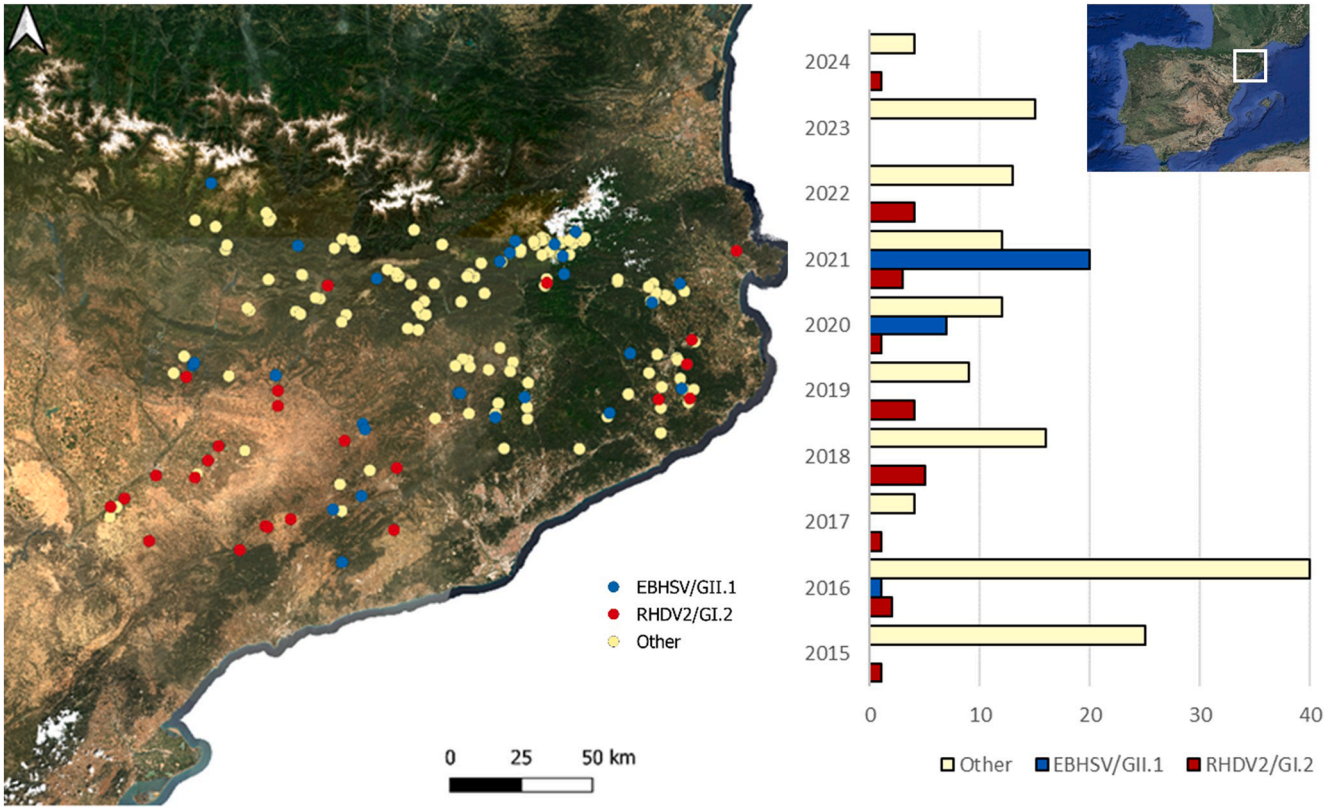

Fig. 1. Geographical (left) and temporal (right) distribution of the 52 *Lagovirus*-positive cases, caused by either European brown hare syndrome virus (EBHSV/GII.1; in blue) or rabbit haemorrhagic disease virus 2 (RHDV2/ GI.2; in red). Yellow bars and dots indicate other hares found dead with a diagnosis different from *Lagovirus* disease or apparently healthy hunted individuals with no gross lesions observed.

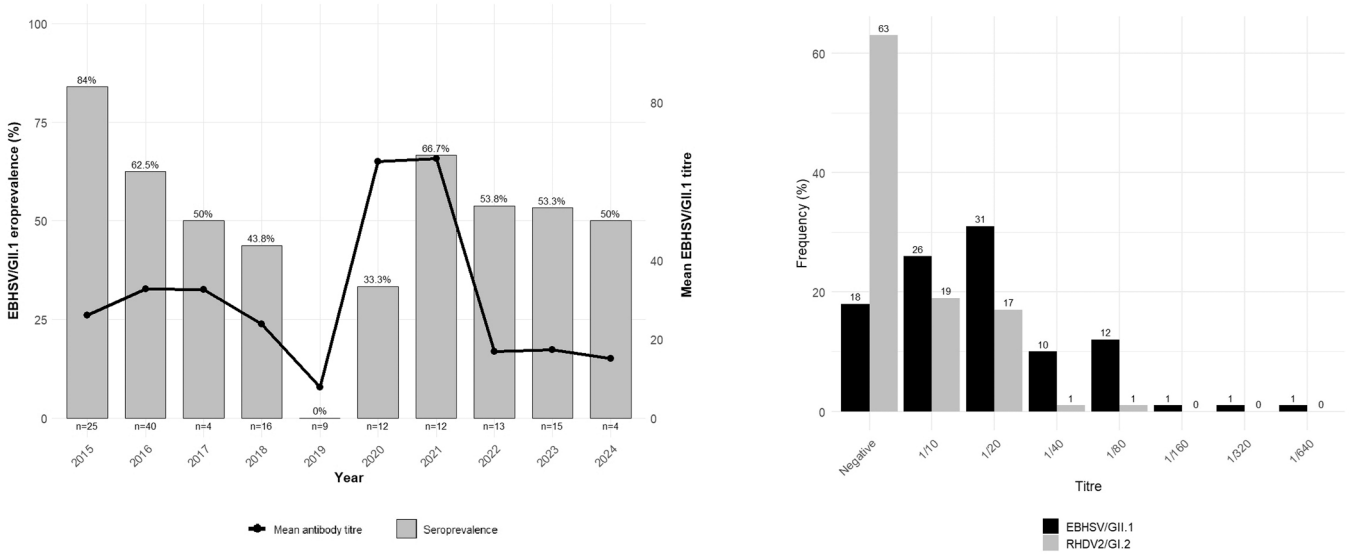

Fig. 2. Seroprevalence (left Y axis) and mean antibody titre (right Y axis) for European brown hare syndrome virus (EBHSV/GII.1; left) and antibody titres frequencies (%) against both lagoviruses, EBHSV/GII.1 and rabbit haemorrhagic disease virus (RHDV2/GI.2), in the study area between 2015 and 2024 (right). Seropositive individuals include those with an antibody titre of 1/20 or higher.

320 or 1/640 (Fig. 2, right panel).

Concerning RHDV2/GI.2, seroprevalence and titre distribution were like EBHSV/GII.1 serology but with more negative/doubtful cases (Fig. 2, right panel). Only one sample (1 %) showed titres > 1/80. To better understand the serological data and infer the possible presence of RHDV2/GI.2 antibodies while discarding cross-reactivity, we used the approach proposed by Velarde et al. (2017) and also followed in other studies (Strive et al., 2020; Faehndrich et al., 2023), calculating the ratio (Rt) between the EBHSV/GII.1 and RHDV2/GI.2 titres. Most of the serum samples had titres consistently higher for the respective specific viral antigen (Rt > 2), except for 18 cases, in which the ratio was 1 (n = 16) or 0.5 (n = 2). Thus, none of the tested serum samples could be

definitively classified as positive for RHDV2/GI.2 antibodies, except in those cases with Rt = 1 or 0.5. In such instances, the results are inconclusive; thus, infection cannot be ruled out.

Of the 52 *Lagovirus*-infected cases, 49 were suitable for macroscopic and microscopic post-mortem evaluation. Lesions consistent with *Lagovirus* syndrome were observed in 47 European brown hares previously confirmed as molecularly positive (EBHSV/GII.1, n = 25; RHDV2/GI.2, n = 22). In the remaining two EBHSV/GII.1-infected individuals without consistent lesions of *Lagovirus* infection, the cause of death was trauma. The macroscopic and microscopic pathological features of both diseases, EBHS and RHD, are detailed in Tables 1 and 2 and Figs. 3 and 4. In most cases, the anatomopathological alterations were generally similar for both diseases. Hares typically exhibited pale livers with a reticular pattern, indicative of extensive zonal hepatocellular loss. Microscopically, this was characterised by periportal to massive (i.e., in the entire lobules) coagulative necrosis/apoptosis, occasional sinusoidal haemorrhages, portal mononuclear inflammatory infiltrate, small multifocal areas of lytic necrosis, activated Kupffer cells, and sporadic mitochondrial mineralisation of the hepatocytes. Splenomegaly, associated with congestion and fibrinoid deposition in the red pulp, respiratory congestion and oedema, and renal tubular injury were other pathologic findings observed. Interestingly, in both diseases, two hares showed microthrombi in the glomerular capillaries. Despite these similarities, the statistical analysis revealed some distinctions upon in-depth comparison (Tables 1 and 2).

On external examination, EBHSV/GII.1-infected hares showed poorer body condition, as evidenced by diminished peri-renal and subcutaneous fat storage, than those infected with RHDV2/GI.2 (respectively 10/28 and 1/20; p = 0.03, but p = 0.05 after Bonferroni correction). Nasal epistaxis and conjunctival and respiratory congestion were more pronounced in RHDV2/GI.2 cases, but only the former was statistically significant (20/20 and 14/28 respectively; $p = 0.01$). Conversely, subcutaneous jaundice, although not consistently present, was more frequently observed in EBHSV/GII.1-infected individuals than in those with RHDV2/GI.2 (11/28 and 1/20 respectively; $p = 0.02$).

Histopathologically, the hepatocellular necrosis pattern was always massive in RHDV2/GI.2 cases (19/19) with absent to minimal mononuclear inflammatory reaction in portal areas (RHDV2/GI.2 =14/14; EBHSV/GII:1 = 9/15; $p = 0.02$). On the other hand, in some EBHSV/GII.1-infected hares, the distribution of the hepatic cell damage was more restricted to periportal to midzonal areas (EBHSV/GII.1 =7/22; RHDV2/GI.2 =0/14; $p = 0.01$), with a more marked inflammatory reaction composed of mononuclear and some polymorphonuclear cells (EBHSV/GII.1 =6/15; RHDV2/GI.2 =0/14; $p = 0.03$), and an extensive degree of lipid-type hepatocytic vacuolisation (EBHSV/GII.1 =11/22; RHDV2/GI.1 =0/15, $p = 0.01$). In kidneys, multifocal degeneration of the renal tubular epithelium, frequently associated with haemoglobin casts (haemoglobinuria), was also more regularly and significantly found in EBHSV2/GII.1 infection cases rather than RHDV2/GI.2 (15/18 and 6/14 respectively; $p = 0.04$, but $p = 0.05$ after Bonferroni correction).

4. Discussion

This study provides new insights into the epidemiology and pathology of lagoviruses in the European brown hare population of the Iberian Peninsula, enhancing our understanding of the associated viral diseases. Furthermore, the epidemiology of RHDV2/GI.2 in the European brown hare was investigated using an integrative approach that combined molecular diagnosis, serology and pathology.

The temporal distribution of the positive cases revealed two different epidemiological scenarios: a constant incidence of RHDV2/GI.2 and a periodic outbreak-like pattern of EBHSV/GII.1. In fact, while EBHSV/GII.1 seropositivity dynamics of the populations in the study area suggest a relatively stable disease circulation, fluctuation in antibody titres aligns with the temporal distribution of cases, providing a clearer understanding of what occurs in the field. EBHSV/GII.1 titres were generally low (<1/40). Still, two peaks of higher titres, preceded by low titres in prior years, were registered in 2016–2017 and, especially in 2020–2021, indicating recent infection (Scicluna et al., 1994; Drews et al., 2011). These findings suggest that EBHSV/GII.1 was likely present and circulating silently in the hare population throughout the observed period, manifesting a cyclical outbreak pattern as detected through passive health monitoring. This epidemiological pattern is directly related to hare population density (Paci et al. 2011; Chiari et al. 2014; Salvioli et al. 2017), particularly in areas with low mean densities such as Catalonia, where values range from 0.5 to 3 hares/km^2 (Ruiz-Olmo and Camps, 2023).

In contrast, RHDV2/GI.2 infection in the European brown hare may follow epidemiological dynamics that are distinct and potentially opposite to EBHSV/GII.1. The observed RHDV2/GI.2 antibody titres are likely attributed to cross-reactivity (EBHSV/GII.1 previously infected hares) or, in case of low titres, to non-specific factors such as sample quality or, perhaps, infections with non-pathogenic caliciviruses, as it may occur with RHDV/GI.1 (McPhee et al., 2009; Velarde et al., 2017). Based on these findings, RHDV2/GI.2 seropositivity in the hare population appears to be either minimal or absent. This suggests that RHDV2/GI.2 does not circulate endemically in hares and that ongoing infections likely result from spillover events from the rabbit population, as previously proposed (Hall et al., 2017; Le Gall-Reculé et al., 2017; Velarde et al., 2017), causing a high case fatality rate with few seropositive survivors. Although formal spatial analyses have not been conducted, the geographical distribution of lagomorph species in Catalonia suggests a strong correlation between RHDV2/GI.2 cases in hares

Table 1
Summary of the gross pathology lesions in European brown hares infected either with European brown hare syndrome virus (EBHSV/GII.1), or rabbit haemorrhagic disease virus 2 (RHDV2/GI.2). The number of cases assessed for each category varies due to tissue availability and degree of post-mortem artefact.

Organ/Tissue	Gross Pathology	n/Total		χ^2	*p-value*	Bonferroni adjusted *p-value*
		GII.1	GI.2			
Body condition	Poor (absence of fat)	10/28	1/20	4.61	0.03*	0.05
	Fair	13/28	8/20	-	-	-
	Good or very good	5/28	11/20	5.67	0.02*	0.02
External						
Nose	Nasal Epistaxis	14/28	20/20	11.80	0.0006*	0.01
Eye conjunctiva	Congestion/haemorrhage	12/28	14/20	2.45	0.11	-
Internal						
Subcutis	Icterus	11/28	1/20	5.60	0.02*	0.02
Trachea	Mucosal congestion	21/28	19/20	2.07	0.15	-
Lung	Congestion/haemorrhage	12/28	19/20	0.31	0.57	-
Liver	Pale	22/28	20/20	3.13	0.08	-
	Reticular Pattern	19/28	18/20	2.11	0.15	
Spleen	Enlarged	22/28	13/20	0.51	0.48	-

* Statistically significant

Table 2
Summary of the histological lesions in European brown hares infected either with European brown hare syndrome virus (EBHSV/GII.1), or rabbit haemorrhagic disease virus 2 (RHDV2/GI.2). The number of cases assessed for each category varies due to tissue availability and degree of post-mortem artefact.

Organ/Tissue	Histopathology	n/Total		χ^2	*p-value*	Bonferroni adjusted *p-value*
		GII.1	GI.2			
Trachea	Mucosal congestion	20/25	20/20	2.70	0.10	-
Lung	Oedema/Congestion	22/24	19/19	0.31	0.56	-
	Haemorrhages	6/15	2/6	1.60e−31	1	-
Liver	Necrosis pattern					
	Massive	15/22	19/19	3.30	0.07	-
	Periportal	7/22	0/19	5.22	0.02*	0.01
	Lytic necrosis areas	22/22	15/15	-	-	-
	Inflammation					
	Minimum	9/15	14/14	4.83	0.03*	0.02
	Mild-moderate	6/15	0/14	4.83	0.03*	0.02
	Vacuolar degeneration	11/22	0/15	8.41	0.004*	0.01
	Mineralisation	9/23	14/21	2.32	0.12	-
Spleen	Lymphoid depletion	19/20	8/9	4.58e−31	1	-
	Lymphocytolysis	12/19	2/8	1.93	0.16	-
	Fibrinoid deposits	12/20	5/9	1.44e−31	1	-
Kidney	Tubular degeneration	15/18	6/14	4.07	0.04*	0.05
	Haemoglobin casts	16/23	6/17	3.36	0.07	-
	DIC	2/21	2/15	4.58e−31	1	-

DIC, disseminated
* Statistically significant

and agricultural areas in western, central, and eastern Catalonia, where rabbit populations are abundant (Fig. 1) (Ruiz-Olmo and Camps, 2023). This sympatric context between species likely increases the probability of infection for hares in these regions.

Historically, RHDV/GI and EBHSV/GII genogroups were regarded as "rabbit" and "hare" viruses, respectively (Green et al., 2000). The emergence of RHDV2/GI.2 (Le Gall-Reculé et al., 2011) followed by spillover events leading to infection and mortality in various hare species, including the European brown hare (Hall et al., 2017; Le Gall-Reculé et al., 2017; Velarde et al., 2017), has blurred the pathological distinction between the two diseases, as both lagoviruses can now infect hares. Our results suggest that hares infected with RHDV2/GI.2 may consistently experience a more acute clinical form of *Lagovirus* syndrome, leading to more rapid death. In contrast, hares infected with EBHSV/GII.1 usually exhibited a more prolonged disease course. This is firstly observed in the body condition of hares. Even if nutritional status may depend on factors such as sex, age, or breeding period (Bustos et al., 1998), EBHSV/GII.1-infected hares were consistently in poorer body condition compared to those infected with RHDV2/GI.2.

The hallmark lesion of *Lagovirus* diseases, periportal to massive coagulative hepatocellular necrosis (Duff and Gavier-Widén, 2012), present differently in the two infections. In RHDV2/GI.2, necrosis tends to be massive, whereas in EBHSV/GII.1, hepatocellular damage may occasionally be confined to periportal areas. In the literature, the massive necrosis pattern in EBHS has often been associated with the acute form of the disease, typically affecting individuals with weaker immune system (Zanni et al., 1993; Gavier-Widén, 1994; Drews et al., 2011). However, most EBHSV/GII.1-infected hares also exhibited changes indicative of longer disease progression, such as vacuolar hepatocyte degeneration, a slightly heightened inflammatory response, and subcutaneous jaundice. These findings suggest a less aggressive clinical form and a more prolonged disease course, despite the ultimately lethal outcome (Gavier-Widén, 1994; Syrjälä et al., 2005; Frölich and Lavazza, 2008).

Renal degeneration was significantly more pronounced in EBHSV/GII.1 than in RHDV2/GI.2 infections. Despite tubular degeneration being commonly reported, little is known about the specific renal pathology associated with lagoviruses infection in hares (Poli et al., 1991; Frölich et al., 2001; Velarde et al., 2017). In rabbits infected with RHDV/GI.1, renal degeneration appears to be linked to the progression of liver failure (Alonso et al., 1998; Chen et al., 2008), a correlation also well-established in human medicine (Moore et al., 2013). Chen et al. (2008) and Plassiart et al. (1992) observed initial renal tubular lesions in rabbits experimentally infected with RHDV only after 30- and 54-hours post-infection, respectively, while others succumbed to the disease before these changes could develop. Extrapolating cautiously to hares, these findings agree with longer clinical progression in EBHSV/GII.1 infection rather than in RHDV2/GI.2 infection.

The pathological presentation of these infections may partially explain the striking differences in the epidemiology of the two lagoviruses. Acute and subacute EBHSV/GII.1 cases were identified through passive surveillance, along with evidence of individuals surviving the infection, as indicated by antibody detection in their sera. In contrast, no evidence was found of hares surviving RHDV2/GI.2 infection. This may also result from the rapid clinical progression of the disease, which prevents animals from mounting an effective immune response. Notably, little is known about the immune response of hares to RHDV2/GI.2 infection. By comparison, in rabbits infected with RHDV/GI.1 via oral infection transmission, IgM and IgA responses begin approximately 72 hours post-infection, while IgG production starts around 5–7 days post-infection (Cooke et al., 2000; Müller et al., 2021). A similar timeline has been observed in rabbits vaccinated or passively immunised against RHDV2/GI.2 (Müller et al., 2019; Hall et al., 2021). Therefore, it may be hypothesised that RHDV2/GI.2 infection in the European brown hare is sporadic, as they are not well-adapted hosts for this virus and typically have a limited chance to survive once infected. However, the factors predisposing some hares to infection and disease while others remain uninfected are yet to be disclosed. These may include variables such as infection dose and infection pressure (e.g., exposure to high or repeated doses of virions). In the United States, RHDV2/GI.2 outbreaks are affecting large numbers of individuals from *Lepus* species, as well as from *Sylvilagus* and other leporids (Ringenberg et al., 2024). There are still many gaps in knowledge regarding transmission dynamics, but interactions with domestic rabbits may continue to be the potential source of infection (Asin et al., 2022). Variations in susceptibility among *Lepus* species may also contribute to large-scale outbreaks, as observed in the Sardinian Cape hare (*Lepus capensis mediterraneus*) (Puggioni et al., 2013). Understanding the mechanisms underlying susceptibility, transmission and influencing factors, potentially linked to the virus's genetic structure (Cavadini et al., 2024), will require further investigation.

Sampling was partially carried out through passive surveillance, which is often subjected to bias such as underreporting, delayed detection, or limited geographic coverage (Ryser-Degiorgis, 2013; Tomaselli,

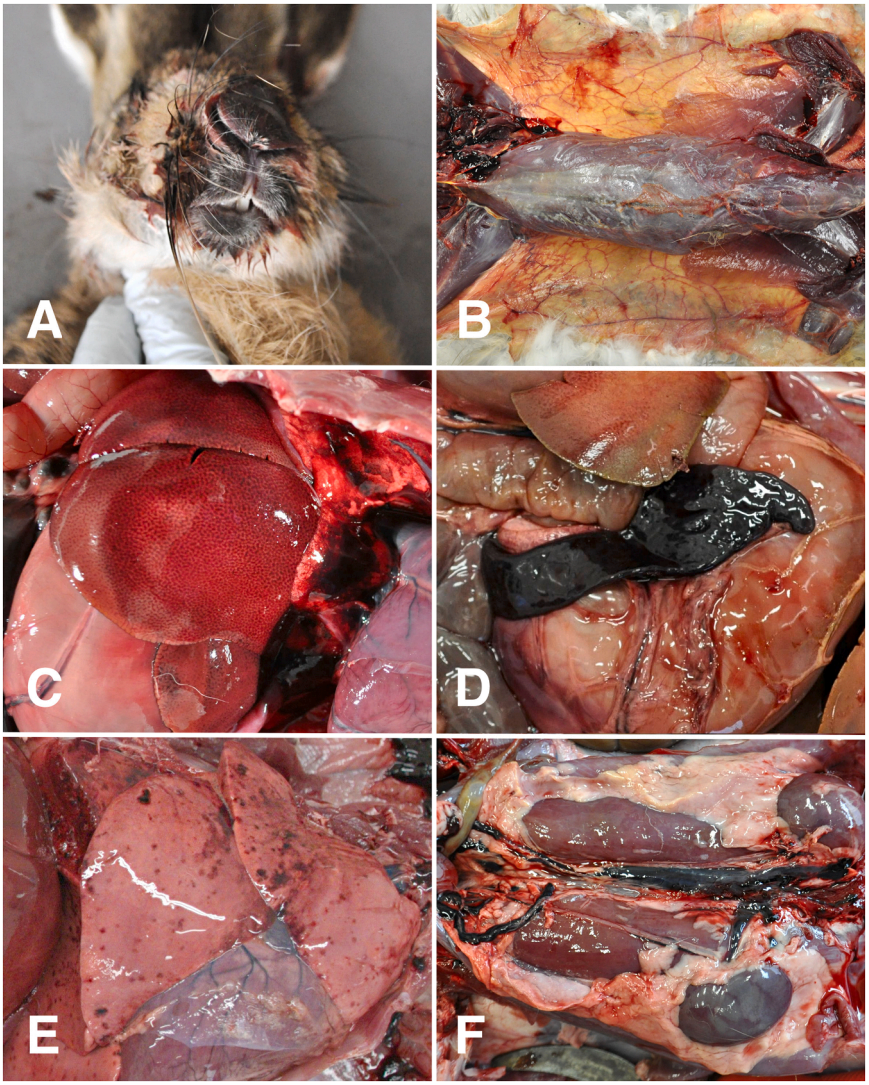

Fig. 3. European brown hare (*Lepus europaeus*), various animals. A) Bilateral nasal epistaxis in an individual infected with rabbit haemorrhagic disease virus 2 (RHDV2/GI.2). B) Icterus of the subcutaneous tissue in a hare with European brown hare syndrome (EBHS) caused by EBHSV/GII.1. C) Liver pallor with diffuse reticular pattern and multifocal lung haemorrhages, features observed in an EBHS-affected hare but also visible in cases infected with RHDV2/GI.2. D) Marked splenomegaly in a hare affected by EBHS. E) Multifocal haemorrhages in the lungs in a hare infected with RHDV2/GI.2. F) Good body condition, as evidenced by the presence of fat around the kidneys, in an individual infected with RHDV2/GI.2.

2022). Active surveillance was supported by volunteer hunters; however, their hunting activities were neither evenly distributed across the territory nor consistent in intensity each year, leading to variation in sample size. Additionally, not all the individuals were tested using molecular analyses, which represent the most sensitive and specific laboratory technique (Abrantes and Lopes, 2021). Consequently, some infected hares, those negative for antibodies or in the early stages of infection without lesions, may have been misclassified as uninfected. For hunted individuals, collecting tissue samples was not feasible, so misdiagnosis could also be possible. Nonetheless, any false negatives, likely limited in number, would not significantly alter our study's conclusions. Despite these limitations, 44 out of 50 serological samples from *Lagovirus*-infected individuals confirmed by molecular analyses also tested positive using virological ELISA. This likely reflects the high viral load during the viremic phase, indicating that this serological technique provides reliable results. Hence, these methods could serve as a valuable alternative or complementary diagnostic tool for epidemiological field studies.

5. Conclusions

The pathological findings of this study reveal significant differences in the clinical course of *Lagovirus* infections in hares. EBHSV/GII.1 appears to be well-adapted to the European brown hare, allowing for more prolonged survival and potentially contributing to its persistence within hare populations. In contrast, the rapid and severe progression of RHDV2/GI.2 infections and low seropositivity rates support the hypothesis that hares act as spillover hosts for RHDV2/GI.2 from rabbits. Despite a decade of potential co-evolution between hares and RHDV2/GI.2, there is no evidence of adaptation in hares. They continue to exhibit acute disease, limited immune response, with probable inefficient transmission between hares, making it unlikely that the European brown hare currently acts as a reservoir for RHDV2/GI.2. However, in areas where hares and rabbits are sympatric, hares could still play a role in re-transmitting the virus to rabbits.

Nonetheless, ongoing surveillance is crucial, as mutations or recombination events could overcome existing host-specific barriers, increasing the risk of an epizootic. These distinctions in pathology and epidemiology further highlight the importance of targeted surveillance

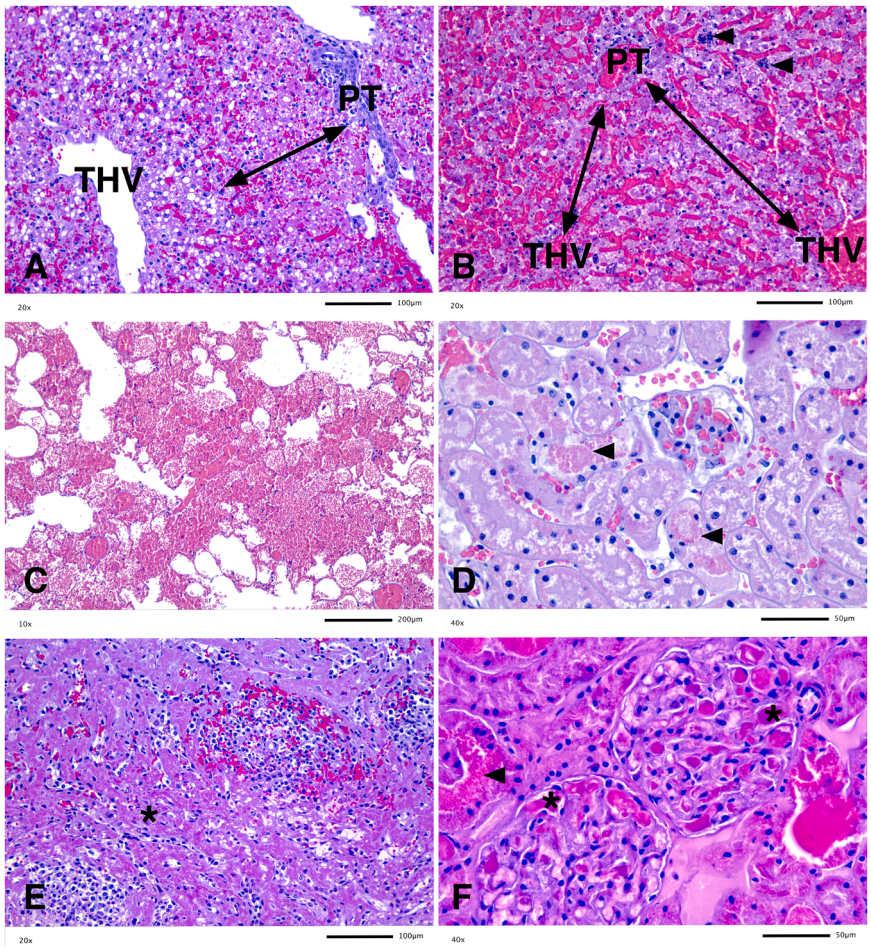

Fig. 4. European brown hare (*Lepus europaeus*), various animals. A) Liver. Hare infected with European brown hare syndrome virus (EBHSV/GII.1). Periportal to midzonal areas of coagulative necrosis (), with mononuclear inflammation and marked vacuolar degeneration of the remnant hepatocytes (THV, terminal hepatic vein; PT, portal tract). B) Liver. Hare infected with rabbit haemorrhagic disease virus 2 (RHDV2/GI.2). Massive coagulative necrosis (), sinusoidal haemorrhages and occasional mineralisation of the hepatocyte's mitochondria (arrowheads). C) Lung. Hare infected with RHDV2/GI.2. Multifocal alveolar haemorrhages. D) Kidney. Hare infected with EBHSV/GII.1. Diffuse areas of tubular degeneration with occasional multifocal haemoglobin casts within the lumen (arrowheads). E) Spleen. Hare infected with EBHSV/GII.1. Fibrinoid-like material deposited on the red pulp (*). F) Kidney. Hare infected with RHDV2/GI.2. Occasional microthrombi in the lumen of glomerular capillaries (*) and haemoglobin casts within the lumen of the tubules (arrowhead).

and management strategies to mitigate the impact of *Lagovirus* infections in hare populations. The north-eastern Spain constitutes the southernmost limit of the European brown hare, where information regarding the population status is limited. The co-circulation of both lagoviruses, occasional local surges in European rabbit populations within hare habitats, and intensive agricultural practices may threaten the viability of European brown hare populations at the southern edge of their distribution in Europe.

Funding

This work has benefited from the financial aid of research grants funded by the Spanish Ministry of Science and Innovation [IberLagoHealth project, PID2023–151954NB-I00, LagoHealth project, REF: PID2019–111080RB-C21, and LAGMED project PRIMAS2–11-PCI2019–103698]. The passive surveillance programme is supported by the Subdirecció General d'Activitats Cinegètiques i Pesca Continental from the Department of Agriculture, Livestock, Fisheries, and Food (DARPA) of the Government of Catalonia. Fundação para a Ciência e Tecnologia (FCT, Portugal) supported the Junior Researcher grant of Ana M. Lopes [CEECIND/01388/2017] and the LAGMED project [REF: PRIMA/0003/2018].

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Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used OpenAI - ChatGPT to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the content of the published article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank the Rural Rangers of the Cos d'Agents Rurals from the Catalonia government and hunters for their invaluable collaboration in the wildlife passive surveillance programme in Catalonia and for reporting and bringing cases to our facilities at the veterinary faculty (SEFaS). We also thank, in particular, the collaboration of Maria Josep Vargas, Montserrat Rubió and Albert Sanz, technical managers of the Catalan authorities linked to this surveillance programme. Serology was performed at the WOAH Reference Laboratory for rabbit haemorrhagic disease (IZSLER, Italy), using laboratory technical work from Mrs. Alessandra Previdi and Silvia Brodini.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2025.110478.

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