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RAPID COMMUNICATION

Spillover event of recombinant *Lagovirus europaeus*/GI.2 into the Iberian hare (*Lepus granatensis*) in Spain

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Abstract

Viruses that affect lagomorphs have decades of reported history of spillover events. One of these viruses is the causative agent of the so-called rabbit or 'lagomorph' haemorrhagic disease (e.g. *Lagovirus europaeus*/GI.1 and *L. europaeus*/GI.2). In particular, *L. europaeus*/GI.2 has shown a great capacity to recombine with existing lagoviruses. In fact, it has replaced the former GI.1 genotype in the wild, and recently, an increase on spillover events has been detected among several lagomorph species including European and North American species of hares. In this study, we report for the first time the infection of a wild Iberian hare with GI.2 (RHDV2/b), potential shedding and associated histopathological alterations. We identify the recombinant GI.4P-GI.2 as causative of the infection and discuss plausible causes regarding the origin of the spillover event and its potential consequences for the Iberian hare wild populations, which is an endemic species of the Iberian Peninsula as well as an important game and prey species for many predators, including endangered species.

KEYWORDS
epidemiology, host jump, Iberian Peninsula, rabbit haemorrhagic disease, RHD

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1 | INTRODUCTION

Myxomatosis (MYX), rabbit haemorrhagic disease (RHD) and European brown hare syndrome (EBHS) are among the most important viral diseases that affect lagomorphs. MYX is caused by myxoma virus (MYXV) belonging to the genus *Poxvirus* (family *Poxviridae*), whereas RHD and EBHS are caused by rabbit haemorrhagic disease virus (RHDV) and European brown hare syndrome virus (EBHSV), respectively, belonging to the genus *Lagovirus* (family *Caliciviridae*). RHD was originally caused by *Lagovirus europaeus*/GI.1 (hereafter GI.1; following the nomenclature proposed by Le Pendu et al., 2017 but not yet accepted by ICTV). A new genotype, *L. europaeus*/GI.2 (hereafter GI.2; according to Le Pendu et al., 2017), emerged in 2010 in France (Le Gall-Reculé et al., 2011) and seems to have replaced the former GI.1 genotype in the wild (Lopes, Correia, et al., 2015), which is now rarely detected. GI.2 also has a great capacity to recombine with existing lagoviruses (e.g. Abrantes et al., 2020; R. N. Hall et al., 2018; Lopes, Correia, et al., 2015).

Epidemiological data collected for more than 40 years from several countries around the world demonstrate that MYXV, RHDV (GI.1) and EBHSV have a limited and high specific host spectrum, such as forest rabbit (*Sylvilagus brasiliensis*) for MYX (Aragão, 1927), European rabbit (*Oryctolagus cuniculus*) for RHD (Abrantes et al., 2012) and European brown hare (*Lepus europaeus*), and to a lesser extent mountain hare (*L. timidus*) and Italian hare (*L. corsicanus*), for EBHS (e.g. Lavazza et al., 1996). However, viruses that affect lagomorphs have decades of reported history of spillover events. Indeed, emergence of MYXV as a pathogenic virus for the European rabbit was due to a spillover from forest rabbits to domestic European rabbits in Uruguay in 1896 (Aragão, 1927). A more recent natural recombinant MYXV caused the first outbreak of MYX in Iberian hare (*L. granatensis*) in the Iberian Peninsula (García-Bocanegra et al., 2019). Spillover events of RHDV from rabbits to several hare species have also been documented, particularly for GI.2 (e.g. Camarda et al., 2014; Le Gall-Reculé et al., 2017; Lopes et al., 2014; Neimanis, Ahola, et al., 2018; Puggioni et al., 2013; Velarde et al., 2017). Additionally, spillover events have been detected among other lagomorph species, including eastern cottontails (*Sylvilagus floridanus*) (Asin et al., 2021; Rouco et al., 2020; Stokstad, 2020). Surprisingly, GI.2 has not been detected in Iberian hare wild populations.

The Iberian hare is an endemic Iberian species and is an important prey for many endangered predators such as the Spanish imperial eagle (*Aquila adalberti*), the Iberian lynx (*Lynx pardinus*) and the Iberian wolf (*Canis lupus signatus*) (Purroy, 2011). Moreover, it is one of the most important small game species in Spain (e.g. >1 million hares harvested during 2011 season, MAPA, 2020). However, Iberian hare populations have undergone a severe decline likely due to a combination of different factors such as predation and hunting, habitat loss and agricultural intensification (Carro & Soriguer, 2017). Furthermore, diseases may have also played an important role in this decline, especially in the last few years. For example, in Spain, the number of hares hunted during the 2018 season resulted in 47% less animals than the 2011 hunting season (i.e. 531,191 hares, MAPA, 2020), coinciding with the first outbreak of MYX in hares.

In this study, we report for the first time the infection of a wild Iberian hare with *L. europaeus*/GI.2/RHDV2/b and associated histopathological alterations. We identify the recombinant GI.4P-GI.2 (P stands for the polymerase) as causative of the infection and discuss plausible causes regarding the origin of the spillover event and its potential consequences for the Iberian hare wild populations.

2 | MATERIAL AND METHODS

On 20 November 2020, a female adult Iberian hare (Ref: LG20001) was found dead lying on the ground in a hunting state near Sant Jaume dels Domenys (41°17′59″N 1°33′34″E) in the province of Tarragona (Northeast of Spain). The animal was transported refrigerated by Rural Rangers to the Veterinary Faculty of the Universitat Autònoma de Barcelona (UAB) and submitted to necropsy and pathological investigation under the Passive Surveillance Program for game species in the region. The area is characterized by a semiarid agricultural landscape dominated by vineyards where populations of European wild rabbits, European brown hares and Iberian hares coexist. Simultaneously, mortality in rabbits was also detected in the area. A week later, one dead adult female rabbit (Ref: OC20-044) was also submitted for necropsy to the UAB within the same surveillance program.

A systematic necropsy was performed in both animals, with all significant findings noted, and the following samples collected in sterile microtubes: blood, brain, submandibular lymph nodes, thymus, trachea, lungs, liver, spleen, heart, kidney, skeletal muscle, ovaries, femoral bone marrow, duodenum and faeces. All tissue samples were frozen at −20°C for subsequent molecular analyses. Blood from the heart and major vessels was collected and centrifuged at 3000 x *g* for 10 min to separate the serum. Tissue samples were fixed in 4% neutral buffered formalin and processed and embedded in paraffin for routine microscopic examination. Sections of 3–4 μm thick were stained with Mayer's haematoxylin and eosin.

All tissue sections from the Iberian hare were evaluated through immunohistochemistry (IHC) to detect the presence and localization of lagovirus antigen using a monoclonal antibody against the protein VP60 of RHDV (from INGENASA, Ref. I1A2; for full IHC methods, antibodies and reagents description, see Supporting Information).

Samples of liver, spleen, duodenum, and faeces of the Iberian hare, liver and spleen from one of the foetuses, and of liver and spleen of the European rabbit were sent to the Research Centre in Biodiversity and Genetic Resources (CIBIO, Vairão, Portugal) for molecular characterization. Samples from the Iberian hare and from the European rabbit were analysed independently. A portion of each tissue (~30 mg) was homogenized in a rotor-stator homogenizer (Mixer Mill MM400; Retsch) at 30 Hz for 5 min. RNA was extracted from hare tissues with the AllPrep DNA/RNA Mini Kit (Qiagen) and from rabbit tissues with the RNeasy Mini Kit (Qiagen). Reverse transcription was performed using oligo (dT) as primers and SuperScript III Reverse Transcriptase (Invitrogen). These procedures followed the manufacturers' instructions. A diagnostic polymerase chain reaction (PCR) was performed, which targeted GI.1 and GI.2 lagoviruses and

amplified a partial fragment of the RdRp and the VP60. In parallel, LG20001 was screened for the presence of EBHSV with specific EBHSV primers (conditions available upon request). Then, a primer-walking approach was used to obtain the complete lagovirus coding sequences as described elsewhere (Lopes, Dalton, et al., 2015) with some modifications. PCR products were purified and sequenced on an automatic sequencer ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems) with the amplification primers and internal primers to obtain overlapping fragments (primers available from the authors upon request). Blast searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed to identify genotype/variant.

To confirm viral shedding in the Iberian hare, RNA was extracted from the faeces. One hundred milligram of pellets was dispersed in 1.5 ml of phosphate-buffered saline (PBS) and soaked for 30 min at 4°C. The sample was then centrifuged at 10,000 x *g* for 30 min, and 200 μl of the supernatant was recovered for RNA extraction using the QIAamp MinElute Virus Spin Kit (Qiagen). cDNA synthesis was performed as described above. A fragment of the p16 was amplified by PCR using the primers RHDV0001F (5′ GTGAAAGTTATGGCGGCTATGTCG 3′) and RHDV0201R (5′ GCCACATTTGTCACATGTCTCCAG 3′). PCR conditions are available from the authors upon request. The amplicon was sequenced as described above using the reverse primer.

The Iberian hare and European rabbit RHDV sequences obtained in this study were aligned in BioEdit software version 7.0.370 (T. A. Hall, 1999) with all the publicly available complete coding sequences of *L. europaeus* from GenBank (see Table S1 for a list of the sequences used). These sequences included genotypes GI.1-4, and the final dataset consisted of 320 sequences, 7369 nucleotides in length. Sequences were aligned with the ClustalW tool available in BioEdit software followed by visual inspection. The dataset was screened for recombination with the RDP software version 4.4 (Martin et al., 2015) by seven methods (RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq) with the following parameters: Sequences were set to linear, Bonferroni correction, highest acceptable *p*-value of .05. Only recombination events detected by three or more methods were considered. Phylogenetic analysis was carried out separately for the non-structural (nucleotides 1–5295; p16, p23, 2C-like helicase, p29, VPg, Protease and RdRp) and the structural encoding genomic regions (nucleotides 5296–7369; VP60 and VP10). Maximum-likelihood phylogenetic trees were inferred in MEGAX (Kumar et al., 2018) using the best model of nucleotide substitution determined in the same software for the different genome partitions, according to the lowest AICc value (Akaike information criterion, corrected). Support for each cluster was obtained from 1000 bootstrap replicates. The partial deletion (95%) option was used for handling missing data and gaps.

An indirect enzyme-linked immunosorbent assay (ELISA) was performed to detect the presence of anti-GI.1 or anti-GI.2 antibodies in the serum of the Iberian hare as described in Bárcena et al. (2015) with some modifications. Briefly, GI.1 and GI.2 purified virus-like particles were adsorbed to Nunc-Immuno MicroWell 96-Well plates (VWR) at 1 ng/μl in carbonate-bicarbonate buffer and incubated at 4°C overnight. Wells were blocked with 5% non-fat milk/PBS; a 1:200 serum dilution (pre-incubated at 37°C for 15 min) was added to the wells in duplicate, followed by the addition of an HRP-conjugated goat anti-rabbit IgG (H+L) at 1:4000. All incubations were performed at 37°C for 1 h. The plate was stained with TMB ELISA substrate (Abcam) for 7 min, and the reaction was stopped with 1 M phosphoric acid; absorbance was read at 450 nm. In between incubations, wells were washed with 0.05% Tween 20 in PBS. Positive, negative and blank controls were included in the assay.

3 | RESULTS AND DISCUSSION

The hare was identified as an adult female Iberian hare of 2.6 kg based on phenotypic morphometric characters; genetic analysis further confirmed the species identification (see supporting information). The animal had minimal fat deposits in the mesentery and around the kidneys and was pregnant with three foetuses of a mean crown-rump length of 9.5 cm and 62 g of body weight (apparently viable). Main findings on the necropsy revealed a slightly enlarged pale friable liver with an enhanced reticular pattern and a few focal haemorrhages, mild splenomegaly, petechial haemorrhages in the thymus and markedly congested/haemorrhagic cervical lymph nodes and tracheal mucosa. These lesions were similar to those described in other hare species infected with GI.2, such as in the single case of an Italian hare (Camarda et al., 2014), in the several cases identified in Sardinian Cape hares (*L. capensis mediterraneus*, Puggioni et al., 2013), in European brown hares (Velarde et al., 2017), and in mountain hares (Neimanis, Ahola, et al., 2018) and also indistinguishable to hares suffering from EBHS (Le Gall-Reculé, 2017).

The microscopic pathology analysis of the hare confirmed severe hepatocellular loss and necrotic/apoptotic dissociated hepatocytes in haemorrhagic periportal to midzonal areas (Figure 1) with a mild to moderate inflammatory infiltrate with mainly lymphocytic cells and few heterophils in portal areas and occasionally around terminal hepatic veins. Although these lesions are consistent with a lagovirus infection in hares, the periportal to midzonal hepatic necrosis distribution is described more frequently in European brown hares and mountain hares infected with EBHSV (GII.1; Gavier-Widén, 1994). So far, a massive hepatic necrosis is the most consistent finding in the different hares infected with GI.2 (Neimanis, Ahola, et al., 2018; Puggioni et al., 2013; Velarde et al., 2017). Other microscopic findings were mild to moderate numbers of active macrophages in the red pulp and congestion, and mild lymphocytolysis with tingible body macrophages in several follicles of the congested cervical lymph nodes. Multifocal acute extensive haemorrhages were seen in the thymus. In the lungs, only occasional acute focal haemorrhages were observed. The results of the IHC in the tissues from the hare revealed that the distribution of the antigen was similar to that described in both natural and experimental infection of RHD (Neimanis, Pettersson, et al., 2018) and in mountain hares naturally infected with GI.2 (Neimanis, Ahola, et al., 2018). Indeed, the antigen was detected mainly in the liver and spleen. In the liver, it was clearly associated with periportal and midzonal dying hepatocytes as fine to coarse intracytoplasmic stippling (Figure 1). Other positive cells were kupffer cells, occasional inflammatory cells in portal

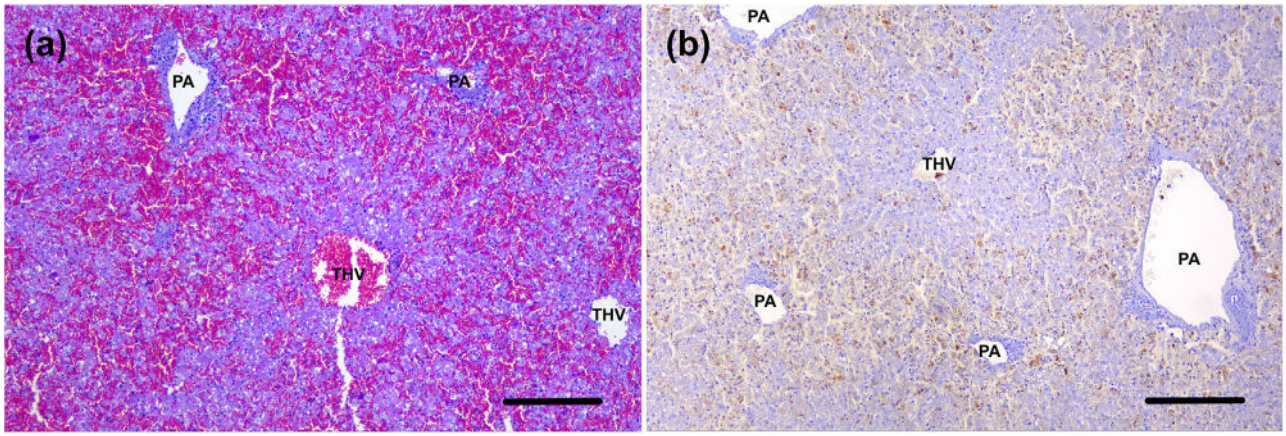

FIGURE 1 Microscopic lesions and viral antigen detection in the liver of a GI.2 infected Iberian hare (*Lepus granatensis*). (a) Acute to subacute haemorrhagic periportal to midzonal hepatocellular necrosis and apoptosis (PA, portal area; THV, terminal hepatic venule) and mild inflammatory portal infiltrates (H&E stain, bar = 200 μm). (b) Immunohistochemical visualization of viral capsid antigen (brown) follows the same periportal to midzonal distribution (bar = 200 μm)

infiltrates, possibly histiocytic cells, macrophages in the red pulp of the spleen, a few macrophages in the cervical lymph nodes and in the bone marrow, scattered cells in the alveolar septa in the lungs and intravascular leukocytes.

In all three foetuses, there were acute multifocal subepicardial and myocardial haemorrhages and marked periacinar congestion in the liver with mild vacuolar hepatocellular changes. Mild multifocal haemorrhages were seen in the lung of one foetus. Yet, viral antigen was not detected in the placenta or in tissues from the foetuses, either by IHC or PCR. Vertical–transplacental transmission has been detected for EBHS but not for RHD. Indeed, mild hepatitis, necrotizing placentitis and viral nucleic acids detection through PCR have been described in an outbreak of EBHS in captive hares (Drews et al., 2011), but this transmission has not been demonstrated in RHDV infections (Duff & Gavier-Widén, 2012). It is possible that the placenta may act as an efficient physical and immunological barrier for RHDV, though this requires further investigation. The mechanisms underlying this difference were not studied, as well as if there is an association with a longer course of the disease. Because the virus was neither detected in the foetus through IHC nor by PCR, we hypothesize that the haemorrhages in the foetuses could be the result of hypoxic damage to the endothelial cells in the latest stages of the disease or the result of intravascular coagulation disorder affecting both the doe and the foetuses. The slight vacuolation seen in the liver cells was considered a very unspecific mild change that can be seen as the result of hypoxia, metabolic toxicity or lipid mobilization.

The analysis of a single animal does not allow a reliable comparison of the pathology associated with GI.2 infection in Iberian versus European brown hares. Nonetheless, it is interesting to note the clear periportal to midzonal necrosis and the presence of a moderate inflammatory infiltrate seen in this animal. In the European brown hares, a massive necrosis and null to minimum inflammation is the most common finding (Velarde et al., 2017). This could suggest a longer course of the disease in the Iberian hare than in the European brown hare. Nonetheless, anti-GI.1 or anti-GI.2 IgG antibodies were not found by serology (data not shown). Overall, the macroscopic and microscopic lesions observed in the hare were highly consistent with an EBHS-like disease and similar to those reported for other hare species infected with GI.2 (e.g. Camarda et al., 2014; Le Gall-Reculé, 2017; Neimanis, Ahola, et al., 2018; Puggioni et al., 2013, Velarde et al., 2017).

For the rabbit, the main findings were a pale liver, marked splenomegaly (8 cm long), haematuria, diffuse congestion of the lungs and brown irregular areas of increased firmness in the caudal lung lobes consistent with verminous pneumonia. Microscopically, marked autolysis hampered the evaluation, but a clear necrotizing hepatitis with hepatocellular dissociation, multifocal lytic foci, and massive disseminated intravascular coagulation, consistent with RHD (Abrantes et al., 2012), was seen.

No other possible causes of the disease were seen in the necropsy and the histopathological study, and the death of this Iberian hare was clearly associated with the viral infection. Moreover, the genome characterization confirmed the presence of GI.2 in both individuals, the hare and the rabbit, and discarded EBHSV infection of LG20001. Complete coding sequences were obtained using a primer-walking approach from the liver of the Iberian hare and the spleen of the rabbit, as amplification from these tissues was most effective. Sequences were deposited in GenBank under the accession numbers MZ203092 (LG20001) and MZ203093 (OC20-044). The sequence of the lagovirus infecting the Iberian hare was 7360 nucleotides (nt) long and presented several in-frame deletions: 6 nt in the p16 and 3 nt in the 2C-like helicase, similarly to the MRCV strain (GenBank accession number GQ166866), GI.4 strains and GI.4P-GI.2 recombinant strains; 3 nt in the VP60, shared with strains from genotypes GI.1, GI.2 and GI.3. The coding sequence of the lagovirus infecting the European rabbit was similar to that described for the Iberian hare, although we were not able to amplify the p16, part of p23 and part of 2C-like helicase

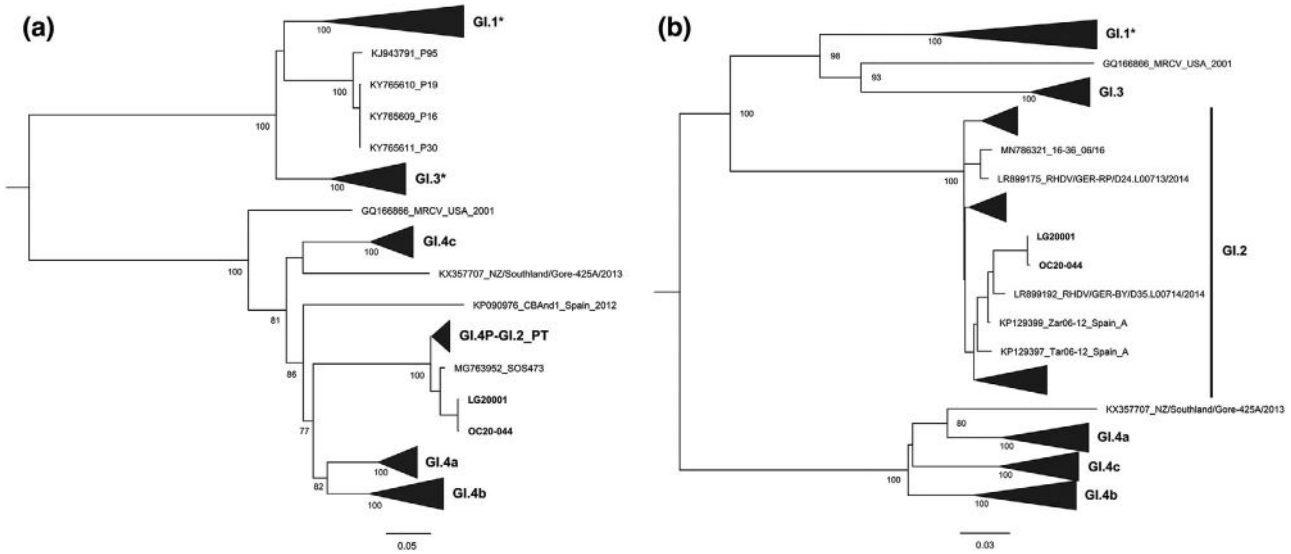

FIGURE 2 Maximum likelihood (ML) phylogenetic trees for (a) the non-structural genes ($n = 320$ sequences; nucleotides 1–5295; nucleotide substitution model GTR+G+Γ4) and (b) the structural genes ($n = 320$ sequences; nucleotides 5296–7369; nucleotide substitution model GTR+G+Γ4). Horizontal branch lengths are drawn to scale of nucleotide substitutions per site, and the trees are mid-point rooted. The percentage of trees in which the associated taxa clustered together was determined from 1000 bootstrap replicates and is shown next to the branches (only bootstrap values ≥ 70 are shown). Sequences obtained in this study appear in bold. Clusters marked with * include recombinant sequences (GI.1bP-GI.2 and GI.3P-GI.2 in (a) and GI.xP-GI.1b in (b)). GenBank accession numbers of the sequences used are listed in the supporting information (Table S1)

(nucleotides 1–786 and 1399–1831). Despite their close relatedness, these sequences were not 100% identical, which discarded the possibility of sample contamination, and was suggestive of a spillover, most likely from rabbit to the Iberian hare. Indeed, eight nucleotide differences exist between the two sequences: C/T at positions 1887, 2376 and 6672, A/G at positions 2358 and 7261, T/C at positions 3804 and 3826 and G/A at position 6564. One of these nucleotide differences corresponds to a non-synonymous substitution (S/P) at amino acid position 1276 in RdRp. Screening of the sequences for recombination revealed that the lagovirus sequences retrieved from both species were recombinants with a recombination breakpoint close to the RdRp/VP60 junction (data not shown). Accordingly, maximum likelihood (ML) trees were inferred separately for the non-structural and structural encoding regions (nucleotides 1–5295 and 5296–7369, respectively). For the non-structural part (Figure 2a), the LG20001 and OC20-044 strains clustered together with strong support (bootstrap value of 99) within a highly supported group (bootstrap value of 100) of previously identified GI.4P-GI.2 strains (Lopes, Dalton, et al., 2015; Silvério et al., 2018). These strains are all included in a major cluster grouping GI.4 strains (bootstrap value of 81). For the structural part (Figure 2b), the LG20001 and OC20-044 strains also clustered together (bootstrap value of 100) within the highly supported GI.2 cluster (bootstrap value of 100). The phylogenetic results are in agreement with the recombination analysis results and confirm that LG20001 and OC20-044 strains are GI.4P-GI.2 recombinant strains. This study and others show that lagoviruses spillover infections are not restricted to one type of recombinant, as hares have been infected by GI.4P-GI.2 and GI.1bP-GI.2 recombinants (R. N. Hall et al., 2018; Le Gall-Reculé et al., 2017; Mahar et al., 2018).

Virus shedding in the Iberian hare was confirmed by sequencing of a partial fragment of p16 (100% identical to the Iberian hare virus sequence) in the faeces. Yet, until March 2021, no other dead Iberian hares have been found in the study area. An earlier study showed infection of *L. granatensis* with a GI.1 strain (Lopes et al., 2014), and in both cases (i.e. Lopes et al., 2014 and the current study), there were no obvious associated outbreaks in the Iberian hare population, but only isolated spillover events, suggesting that the virus is still not well adapted to the new host to cause onward transmission. Notably, the previous event was also caused by a recombinant strain, between a lagovirus from a group that diverged ~13% from known strains and had never been reported, and GI.1b (i.e. GI.xP-GI.1b; Lopes et al., 2017). Yet, the role of recombination in these spillover events remains to be determined.

In the last few years, the Iberian hare populations had drastically decreased by the impact of MYX (García Bocanegra et al., 2020). Therefore, the burden of a new emerging disease such as RHD, with the impact that it has shown in other wild leporids populations (Stokstad, 2020) could place the species in a worrying situation regarding its conservation. In this sense, it is worth to study the role that species-specific factors may play in the epidemiology of GI.2 on the Iberian hare, such as viral load, clinical course of the disease, differences in susceptibility of the species to the infection or the specific recombinant virus found. These non-exclusive reasons may explain why, despite the wider geographical distribution of the Iberian hare in comparison

to the European brown hare in the Iberian Peninsula and the active search for the virus in this species, no more cases have been detected so far and warrants future investigations.

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ETHICAL STATEMENT

This study did not involve purposeful killing of animals. All analysed Iberian hares were animals found dead in the study hunting areas. No animals were specifically hunted for this study and ethical approval by an Institutional Animal Care and Use Committee was not deemed necessary. Samples were collected by authorised gamekeepers and hunters with the correct permits and licenses and with the permission of landowners. Samples were collected following the protocol established for Rabbit/Hare Haemorrhagic Disease in Catalonia, and liver samples were submitted first to the regional laboratory (Laboratori de Sanitat Animal de Catalunya, LaSAC) and to the National Reference Laboratory (Laboratorio Central de Veterinaria de Algete in Madrid). Both bodies confirmed that samples from the hare and from the rabbit were positive to GI.2.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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