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Canine distemper virus in wildlife in south-western Europe

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Abstract

Multi-host pathogens emerging and re-emerging at the wildlife–domestic animal interface affect wildlife management and conservation. This is the case of canine distemper virus (CDV), a paramyxovirus closely related to human measles virus and rinderpest virus of cattle. With an area of 10,603 km^2, Asturias region in Atlantic Spain is a hotspot of carnivore diversity, which includes the largest Eurasian brown bear (*Ursus arctos arctos*) population and one of the largest wolf (*Canis lupus*) populations in south-western Europe. In 2020–2021, we recorded mortality due to distemper in four carnivore species including three mustelids (Eurasian badger *Meles meles*, European marten *Martes martes* and European polecat *Mustela putorius*) and one canid (red fox, *Vulpes vulpes*). Clinical signs and pathology were similar across species and consistent with the emergence of a highly pathogenic viral strain, with CDV antigen mainly located in the central nervous system, lungs, spleen and lymph nodes. A molecular study in eight wild carnivore species, also including the Iberian wolf, Eurasian brown bear, American mink (*Neovison vison*) and stone marten (*Martes foina*), revealed 19.51% (16/82) of positivity. Phylogenetic analysis demonstrated that CDV belonged to the previously described European lineage. A retrospective serosurvey (2008–2020) showed a high seroprevalence of CDV antibodies (43.4%) in 684 analyzed badgers, indicating a long-term though not stable viral circulation in this multi-host community. The possible triggers of the 2020–2021 outbreak and the implications for carnivore management and conservation are discussed.

KEYWORDS
canine distemper virus (CDV), epidemiology, pathology, serology, south-western Europe, wildlife

1 | INTRODUCTION

Emerging and re-emerging viral diseases shared between wildlife and domestic animals are continually spreading to new geographic locations, influenced by human activities and environmental change (Howard & Fletcher, 2012; Marston et al., 2014). This is the case of emerging paramyxoviruses. The family *Paramyxoviridae* comprises four recognized subfamilies (*Avulavirinae*, *Metaparamyxovirinae*, *Orthoparamyxovirinae* and *Rubulavirinae*) (ICTV, 2021). The subfamily *Orthoparamyxovirinae* comprises eight genera which include

Morbillivirus, *Respirovirus* and *Henipavirus* genera that collectively show a broad host range of vertebrates from fish to mammals, with transmission primarily occurring via the respiratory route (Afonso et al., 2016). For centuries, viruses of the genus *Morbillivirus* have caused devastating outbreaks in humans and animals. This genus includes the single-stranded RNA viruses: canine morbillivirus (canine distemper virus [CDV]), cetacean morbillivirus, feline morbillivirus, human measles morbillivirus, phocine morbillivirus, rinderpest morbillivirus of cattle and small ruminant morbillivirus (ICTV, 2021).

CDV shows high pathogenic potential and causes distemper (Loots et al., 2017). The virus is distributed worldwide and can affect both domestic and wild mammals, especially carnivores (Martínez-Gutiérrez & Ruiz-Saenz, 2016). CDV threatens the conservation of scarce or endangered wild carnivores (Gilbert et al., 2015; Williams et al., 1988). In fact, CDV outbreaks involving high mortality have occurred worldwide in recent years in canids, mustelids, felids, ursids and even non-human primates (Balboni et al., 2021; Kennedy et al., 2019; Loots et al., 2017; Origgi et al., 2012; Pope et al., 2016; Sakai et al., 2013; Sun et al., 2010).

In Europe, the virus has been linked to mortality of Eurasian badger (*Meles meles*), stone marten (*Marten foina*) or red fox (*Vulpes vulpes*) in Denmark, Switzerland and Italy (Akdesir et al., 2018; Balboni et al., 2021; Di Sabatino et al., 2016; Garigliany et al., 2018; Hammer et al., 2004; Origgi et al., 2012). In contrast, in south-western Europe reports of clinical cases of distemper among carnivores are scarce (Gortázar, 1997; López-Peña et al., 2001; Sobrino et al., 2008). Nevertheless, several serological or viral nucleic acid surveys in the Iberian Peninsula indicate that wild populations of red fox, Iberian wolf (*Canis lupus*) and Iberian lynx (*Lynx pardinus*) are in contact with the virus (Meli et al., 2010; Oleaga et al., 2015; Rosa et al., 2020; Sobrino et al., 2008), suggesting CDV is endemic.

CDV causes similar clinical signs in wildlife as it does in domestic dogs (Deem et al., 2000). However, the disease course may vary across species and outbreaks as a function of strain virulence as well as host age and immune status (Loots et al., 2017; Zhao et al., 2015). Generally, clinical signs are neurological, respiratory and gastrointestinal and include fever, anorexia, depression, rash, nasal and ocular discharge, coughing, conjunctivitis, vomiting and diarrhoea (Rendon-Marín et al., 2019). Progressive disease is associated with convulsion, lack of coordination, seizure and death. The virus can be found in every secretion of an infected animal during the acute phase, usually at 1 week after infection (Appel, 1987). Animals can be infected by CDV through the oral or nasal route. CDV exhibits a profound lymphotropism and it comes into contact with tonsils and the upper respiratory tract, where it multiplies within macrophages (Zhao et al., 2015). The virus also infects dendritic cells which carry the virus to the draining lymph nodes where activated T and B cells are infected, resulting in virus amplification and initiation of the primary viremia with further spread via the lymphatic system to secondary lymphoid organs and target tissues such as the central nervous system (CNS), gastrointestinal tract, kidney or liver (secondary viremia) (Loots et al., 2017; Zhao et al., 2015). Therefore, microscopic lesions as well as intracytoplasmic and intranuclear inclusion bodies can be observed in any of these affected tissues (Loots et al., 2017).

With an area of 10,603 km^2, Asturias region in Atlantic Spain is a hotspot of carnivore diversity, which includes the largest Eurasian brown bear (*Ursus arctos arctos*) population and one of the largest wolf populations in south-western Europe. In 2020–2021, we confirmed mortality due to distemper in four carnivore species – three mustelids (Eurasian badger, European marten *Martes martes* and European polecat *Mustela putorius*) and one canid (red fox). Here, we characterized the clinical and pathological manifestations of the disease, investigated the presence of CDV in eight wild carnivore species and studied the molecular features of the virus. We also investigated the seroprevalence and spatiotemporal distribution of CDV in free-ranging badgers from the same region from 2008 to 2020. Our findings may help clarify the circulation of the virus in this multi-host community and assess the risk of interspecies transmission in a region of exceptional relevance for wildlife conservation.

2 | MATERIALS AND METHODS

2.1 | Study area

The study was performed in the region of Asturias, Spain (south-western Europe). Asturias is characterized by an Atlantic climate with a temperature range from −4 to 8°C in the coldest months and abundant precipitation throughout the year (1400–2100 mm per year) (Ninyerola et al., 2005). The region is flanked to the north by the Cantabrian Sea and to the south by the Cantabrian Mountain Range. More than 30% of the territory is forest consisting mainly of oaks, beech and birch woods, which give refuge to a varied community of wild animals, including 12 terrestrial carnivores. Among large carnivores, wolves belong to the north-western Iberian wolf population, which is the largest population in western Europe (Chapron et al., 2014), estimated at 217−405 individuals (Mateo-Tomás et al., 2019). The brown bear is listed on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species and catalogued as in danger of extinction (McLellan et al., 2016), with a population of approximately 300 individuals. Medium size carnivores such as foxes or badgers are abundant and widespread (Acevedo et al., 2014). The population densities of other species such as European marten or European polecat are unknown.

2.2 | The outbreak

2.2.1 | Clinical study

Thirteen ill subjects [badger (n = 7), fox (n = 4), European marten (n = 1) and European polecat (n = 1)] were collected by wildlife officers and taken to the Wildlife Rehabilitation Center of the Principality of Asturias (WRC) after the Center received telephone calls from individuals who had observed the animals in the field to be exhibiting abnormal behaviour, such as loss of fear of humans, disorientation, lethargy, ataxia and/or other neurologic signs. They were admitted in

TABLE 1 Available data of animals showing compatible signs of distemper disease

Animal	Date	Species	Area in Asturias	Sex	Age
1	13 June 2018	Red fox	Central	Female	Subadult
2	29 March 2019	Red fox	Eastern	Male	Adult
3	19 May 2020	Red fox	Central	Male	Adult
4	19 June 2020	Badger	Eastern	Male	Subadult
5	12 August 2020	Badger	Western	Female	Subadult
6	15 August 2020	Red fox	Central	Male	Subadult
7	3 September 2020	Badger	Central	Male	Subadult
8	5 September 2020	Badger	Central	Male	Adult
9	9 September 2020	Badger	Central	Female	Subadult
10	11 November 2020	Badger	Central	Female	Adult
11	19 November 2020	Badger	Central	Female	Adult
12	25 March 2021	European marten	Eastern	Male	Adult
13	20 April 2021	European polecat	Eastern	Male	Adult

Note: Animals 1 and 2 died before the 2020–2021 outbreak.

the Center for treatment and sanitary surveillance between May 2020 and April 2021, except for two of the red foxes that were admitted in 2018 and 2019. Available data of the 13 animals are shown in Table 1. Age was determined based on their body size and teeth. The animals either naturally died ($n = 7$) or received humanitarian euthanasia ($n = 6$) because of their severe clinical status or poor prognosis. Animals were preserved at 4°C, and complete post-mortem examination of each carcass was conducted at the laboratory within 24 h.

2.2.2 | Pathology

Necropsy was performed on all 13 animals, and gross lesions were recorded during the post-mortem examination. Routine tissues and sections of brain and spinal cord were collected, fixed in 10% formalin and processed for histological evaluation. Sections (4 μm) from each block were stained with haematoxylin and eosin.

2.2.3 | Immunohistochemistry

Serial paraffin-embedded sections (3 μm) from the 13 subjects, prepared as described in Section 2.2.2, were stained with a monoclonal primary antibody against CDV. The sections were dewaxed, and endogenous peroxidase activity was blocked by incubating sections with 0.5% hydrogen peroxide in distilled water for 30 min. Next, samples were microwaved for 20 min in citrate buffer (pH 6.0) for antigen retrieval, then blocked for 20 min with 5% horse normal serum and 0.1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) in a humidified chamber. The tissue sections were incubated overnight at 4°C in a humidified chamber with a commercial monoclonal antibody (mouse anti-raccoon dog CDV monoclonal antibody, clone DV2-12, reference number MBS215321; MyBiosource®, San Diego, CA, USA) diluted in TBS containing 0.1% BSA. In pilot studies, we tested different dilutions of the primary antibody (1:200; 1:500, 1:1000; 1:1500). The dilution 1:1500 was selected based on known CDV-positive and CDV-negative control samples.

Afterwards, slides were washed with TBS, incubated with a secondary antibody (Vector Laboratories®, Burlingame, CA, USA) diluted 1:200 in TBS containing 0.1% BSA for 30 min, washed with TBS and then incubated with the avidin–biotin–peroxidase complex reagent method (ABC Standard, Vector Laboratories®, Burlingame, CA, USA) in TBS for 30 min. Labelling was visualized using Vector® NovaRED™ peroxidase substrate kit SK-4800 (Vector Laboratories®, Burlingame, CA, USA) for 2–5 min. Slides were counterstained with Mayer's haematoxylin, dehydrated and mounted with DPX (Sigma–Aldrich®, St. Louis, MO, USA). For each run, negative and positive controls were used. The negative control consisted of a slide from each tissue processed in the absence of the primary antibody. Lymph node tissue from a CDV-infected dog was used as a positive control. Immunolabelling was evaluated qualitatively as follows: low (few cells were immunostained in a 200× magnification field), moderate (a moderate number of cells were immunostained in a 200× magnification field) and high (most of cells were immunostained in a 200× magnification field).

2.3 | Nucleic acid detection and sequencing

A molecular study was performed in all sick animals ($n = 13$). Additionally, CNS samples were collected from 69 wild carnivores found dead between November 2020 and June 2021 as part of an official passive surveillance program: European marten ($n = 52$), Iberian wolf ($n = 6$), Eurasian brown bear ($n = 4$), American mink (*Neovison vison*, $n = 3$), red fox ($n = 1$), stone marten ($n = 1$), Eurasian badger ($n = 1$) and European polecat ($n = 1$). Viral RNA was isolated from the brains of the 82 animals using the SpeedTools RNA virus extraction kit

(Biotools, Madrid, Spain). Quantitative polymerase chain reaction (qPCR) was performed using the Canine Distemper Virus kit (Genesig, Primer Design, Camberley, UK) and High ScripTools-QUANTIMIX Easy Probes Master Mix (Biotools, Madrid, Spain) for reverse transcription (RT) and qPCR, in a StepOnePlus Real-Time PCR System (Thermofisher Scientific, Paisley, UK) using positive and negative controls included in the CDV diagnostic kit.

The partial sequencing of the H gene [420 base pairs (bp)] was performed on the PCR positive samples to identify the different genotypes circulating during the outbreak. RT was performed (High Capacity cDNA Reverse Transcription kit, Applied Biosystems, Foster City, CA, USA) with 10 μl RNA, incubated at 25°C for 10 min, followed by 37°C for 120 min, with enzyme inactivation at 85°C for 5 min. For PCR amplification, we used specific forward (5′-CTTGCTTGCTATCACTGGAG-3′) and reverse (5′-TTTTGAAATCAAAGACATGG-3′) primers (Origgi et al., 2012). PCR was performed using Premix Ex Taq™(TaKaRa) with a 25 μl total reaction volume, which contained 1 μl of cDNA, 1 μl each of 10 μM primers, 12.5 μl 2 × Reaction Mix and RNase-free water. The thermal cycle was performed for initial RT, followed by denaturation at 95°C for 30 s and 35 cycles of a denaturation step at 95°C for 5 s, an annealing step at 52°C for 30 s, an extension step at 72°C for 30 s and a final extension step at 72°C for 7 min. Amplicons from both the PCRs were analyzed by electrophoresis in 2% agarose gel stained with GelRed® Nucleic Acid Gel Stain (Biotium Inc., Fremont, CA, USA).

PCR products were first purified by the NucleoSpinExtract II kit (Machery-Nagel, Düren, Germany) and were then sequenced using both primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), purified and run in an automatic capillary sequencer ABI 3500 (Applied Biosystems) according to the manufacturer's instructions. DNA sequences were edited, aligned and assembled in the BioEdit 7.2.5 program. The sense and antisense sequences were aligned, and consensus sequences were created using the default alignment parameters. A phylogenetic tree of 66 CDV strains, including 375-bp sequences from all lineages described so far, was constructed using ClustalW and neighbour-joining algorithm, Kimura-2 parameter model (MEGAX software, Kumar et al., 2018). The consensus tree was generated from 1000 bootstrap resampling. Pairwise distances between sequences were also calculated using the Kimura-2 parameter model (MEGA X software, Kumar et al., 2018).

2.4 | Long-term serological study in badgers (2008–2020)

2.4.1 | Sampling

A total of 684 badgers from Asturias were necropsied for post-mortem examination from 2008 to 2020 within an active and passive surveillance program for tuberculosis (see Blanco Vázquez et al., 2021). The distribution of these animals across the years was as follows: 2008 (n = 21), 2009 (n = 37), 2010 (n = 68), 2011 (n = 45), 2012 (n = 20), 2013 (n = 23), 2014 (n = 43), 2015 (n = 22), 2016 (n = 92), 2017 (n = 57), 2018 (n = 74), 2019 (n = 102) and 2020 (n = 80).

Of these animals, 99 were trapped badgers and the other 585 animals were killed in road traffic accidents and found by gamekeepers in the region. Serum samples (2 ml) were taken from the jugular vein, heart or thoracic cavity, collected in vacutainer tubes and frozen at −20°C before processing.

The location, sex, age (subadult/adult) and weight were recorded for trapped and road-killed badgers. In 60 individuals, data on sex and age were not available.

2.4.2 | Serology

The analysis of badger serum samples for antibodies against CDV was performed using the commercial diagnostic kit Ingezim Moquillo IgG (Eurofins-Ingenasa®, Madrid, Spain) that uses the recombinant N protein from CDV as antigen, following the recommendations of the manufacturer but with some modifications. Briefly, the secondary antibody was changed to a specific anti-badger antibody – horseradish peroxidase-conjugated CF2/HRPo anti-badger IgG (Goodger et al., 1994) – at a dilution of 1:5000. All other steps in the procedure were performed as recommended by the manufacturer. All samples were analyzed in duplicate. The results were expressed as optical density (OD) values, and positivity was defined as an OD exceeding the plate's cut-off value of mean $OD_{positive\ controls} \times 0.2$. Positive and negative controls included in the kit were used in each run. Additionally, we included as positive and negative controls sera from a sick and a healthy badger, respectively.

2.4.3 | Statistical analysis

A generalized linear model was developed to identify factors associated with the presence or absence of antibodies against CDV in badgers. The presence/absence of antibodies against CDV was the response variable, whereas geographical area, sex, age, death cause and sampling year were explanatory factors. A logistic link function was applied, and a binomial error distribution was assumed. Data were analyzed using SPSS 17.0 (IBM, Chicago, IL, USA). Additionally, the 95% confidence interval (95% CI) was calculated for each year and the lower and upper limits were delimited (Kohn & Senyak, 2021). Comparations among the years were performed by the Fisher's exact test using GraphPad Prism v.8.0 (GraphPad Software Inc, San Diego, CA, USA).

3 | RESULTS

3.1 | Distemper in wildlife

3.1.1 | Clinical signs

Among 13 sick animals submitted alive to the WRC, the most frequent clinical signs were ataxia (n = 11, Video S1), tremors/seizures (n = 7, Video S2), blindness or severe vision alteration (n = 5; only in

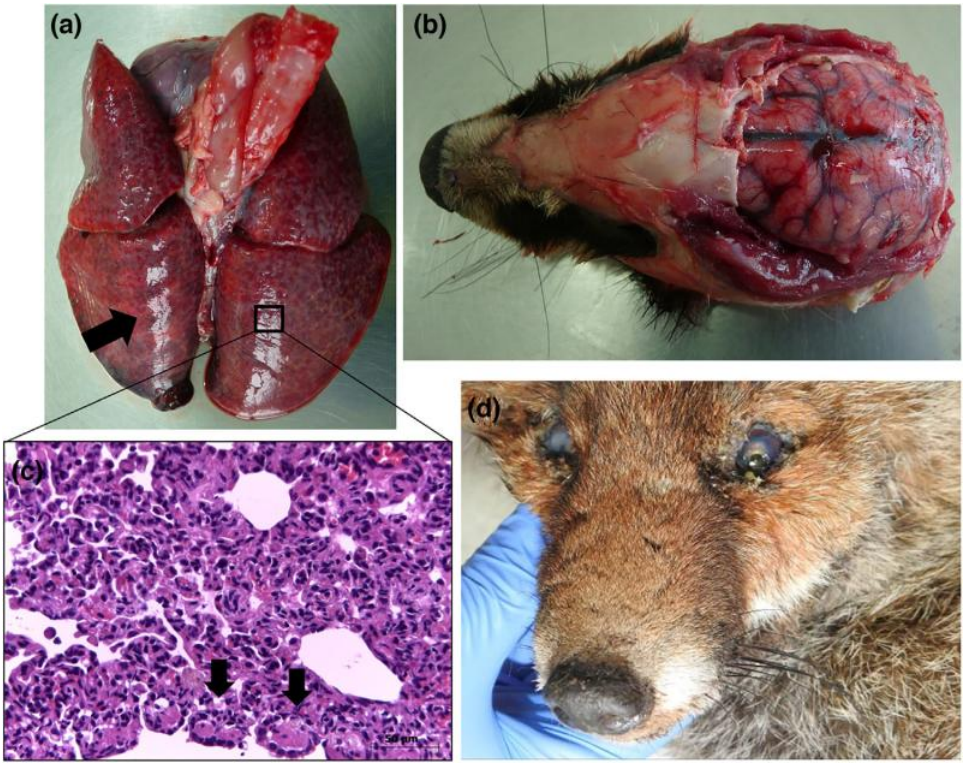

FIGURE 1 Pathological findings in free-ranging badger and fox with distemper disease. (a) Lungs. The lungs of a sick badger appear diffusely enlarged due to interstitial pneumonia, and costal marks (arrow) are observed in the caudal left lobe. Congestion is also observed. (b) Brain. Congestion is observed in a sick badger. (c) Histological view of the interstitial pneumonia consisting of lymphocytes, plasma cells and macrophages, together with numerous syncytial cells (arrows) of epithelial origin. Congestion is also observed. Tissue is stained with haematoxylin and eosin. Bar = 50 microns. (d) Eyes. Blindness or severe vision alteration in both eyes is observed in a sick fox

badger and red fox) and ocular/nasal discharge associated with conjunctivitis/rhinitis ($n = 3$). Diarrhoea was also observed in two badgers, whereas dyspnoea and jaw snapping were reported in one.

3.1.2 | Pathology

Post-mortem examination revealed gross lesions mainly in lungs, CNS and spleen (Figure 1). Lung lesions were observed in all studied animals and consisted of several degrees of congestion and/or haemorrhage and enlargement, often affecting the whole parenchyma and showing consolidated areas in two cases. Ten of 13 studied animals presented severe to moderate congestion in the cerebral cortex, whereas spleen congestion, darkening and apparent structure/consistency loss was detected in nine animals. Less frequent alterations included the presence of hyperkeratosis in nose/footpads; ocular alterations (Figure 1); haemorrhagic lesions in the kidney parenchyma; self-inflicted bites in forelimbs, apparently produced during seizures; and hemopericardium. Five of the studied animals showed poor body condition or cachexia.

Histological lesions were observed in all studied animals and mostly affected the CNS, lungs and lymphoid tissue (spleen and lymph nodes). CNS lesions consisted of non-purulent meningoencephalomyelitis involving the following types of damage in various areas of the brain and spinal cord: meningitis, neuronophagia, neuronal degeneration and necrosis, demyelination, perivascular cuffing and multifocal gliosis. The inflammatory infiltrate was composed mainly of lymphocytes and microglial cells. Lesions were present in grey and white matter in the cerebral cortex, midbrain, cerebellum, medulla oblongata and cervical spinal cord, but they were more severe in the corpus striatum, thalamus and hypothalamus in mustelids and in cerebellum in fox. Loss of Purkinje cells was evident in the cerebellum, with proliferation of Bergmann glial cells and, occasionally, atrophy of the granular and molecular layers. Eosinophilic intranuclear inclusion bodies were observed in a few neurons.

The lungs showed interstitial pneumonia consisting of an inflammatory infiltrate of lymphocytes, plasma cells and macrophages, as well as numerous syncytial cells of epithelial origin (Figure 1). Eosinophilic intranuclear and intracytoplasmic inclusion bodies were observed, as well as karyorrhexis and karyolitic phenomena mainly in the bronchial and bronchiolar epithelium. Multifocal hypertrophy of muscle in bronchi and bronchioles was also observed. Vascular walls showed damaged endothelium with presence of small thrombi and fibrin in the lumen. In one badger, a large focus of necrosis was observed.

Lymphoid tissue depletion was present in lymph nodes and spleen, where multifocal necrosis of lymphocytes was observed, together with intranuclear inclusion bodies in lymphocytes and macrophages. Cellular necrosis phenomena were also detected in skin; trachea, stomach,

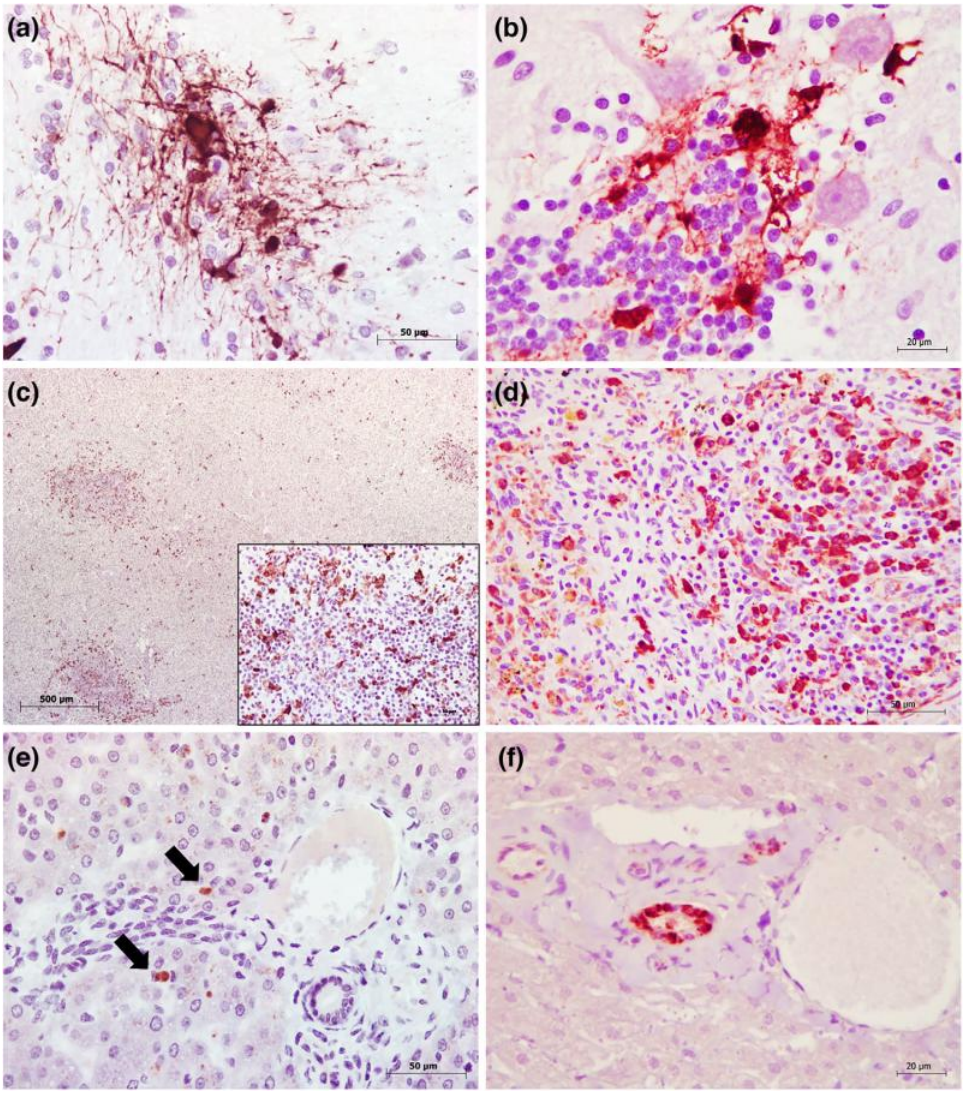

FIGURE 2 Immunohistochemical detection of canine distemper virus (CDV) antigen (stained red) in tissue sections in wildlife. Tissues were counterstained with haematoxylin. (a) Badger: thalamus. High immunolabelling is detected in neurons in cell bodies and axons. (b) Fox: cerebellum. Detail of cells in molecular and granular layers and Purkinje cells showing the virus antigen in cell bodies and axons. (c) Badger: spleen. High immunolabelling is associated mainly with the white pulp. Inset: detail of white pulp showing immunostained lymphocytes and macrophages. (d) Fox: spleen. High immunolabelling is associated with both the white and red pulp. (e) Badger: liver. Immunolabelling is detected within a few hepatocytes and Kupffer cells (arrows). (f) European marten: liver. Low labelling is associated with epithelial cells of the bile duct of the portal space. Bar = 20 microns (b and f), 50 microns (a, d, e and inset) and 500 microns (c)

small intestine, urinary bladder and biliary bladder mucosal epithelium; epithelial cells of the proximal and distal tubules in the kidney; and acinar cells from the exocrine pancreas. In the liver, degeneration and necrosis were observed in scarce hepatocytes and some Kupffer cells.

3.1.3 | Immunohistochemistry

In the 13 animals, virus immunolabelling was detected, albeit in different amounts, in all tissues studied (Figures S1–S4). In badger and marten, immunostaining in the CNS was higher in the corpus striatum, thalamus and hypothalamus than in other regions of the brain (Figure 2). Moderate immunolabelling was observed in neurons, Purkinje cells (cerebellum) and lymphocytes and glial cells forming the perivascular cuffs, as well as in glial foci located in cortical regions, midbrain, cerebellum, medulla oblongata and cervical spinal cord. In contrast, in foxes the immunostaining was higher in the cerebellum (located in Purkinje cells and in cells in molecular and granular layers) than in other regions of the brain (Figure 2). Within neurons and Purkinje cells, immunolabelling was observed in cell bodies and axons. In ventricles and the cervical spinal cord, ependymal cells were constantly immunostained.

High immunolabelling was observed in the epithelial cells of bronchi and bronchioles, as well as in macrophages and lymphocytes in the inflammatory infiltrate. Lymphoid tissues contained high levels of viral antigen. In lymph nodes, high immunostaining was detected in

lymphocytes and macrophages in cortical and medullar regions. In the spleen, high labelling was associated mainly with white pulp in badger and marten, and with both the white and red pulp in fox (Figure 2).

The virus was also detected from moderate to high in the skin epidermis; in epithelial cells of the proximal and distal tubules (also in endothelial cells of glomeruli in fox) in the kidney; in mucosal epithelium of the trachea, stomach, small intestine, urinary bladder and biliary bladder; and in acinar cells from the exocrine pancreas. In liver, low CDV antigen was detected in a few hepatocytes and Kupffer cells in badger and fox, and in the epithelial cells of the bile duct of the portal space in marten (Figure 2).

In the polecat, low immunolabelling was observed only in CNS and small intestine. Negative controls did not show any immunolabelling (Figure S5).

3.2 | RT-PCR (qPCR) and sequencing

A total of 16 (19.51%) individuals were positive for CDV by RT-PCR: 11 of 13 sick individuals and five of 69 wild carnivores found dead between November 2020 and June 2021 during passive surveillance. The positive cases included seven badgers (six of them were sick badgers), four foxes (all sick), four European martens (one of them was the sick marten and the remaining were from passive surveillance) and one stone marten (from passive surveillance). Sequences were obtained from 11 animals, revealing four different sequences with the following GenBank accession numbers: MZ169061, MZ605429, MZ605430 and MZ605431. The sequences showed 98% similarity with the closest canine morbillivirus strain (KY214447.1), isolated from a wolf in Portugal in 2017. All four sequences clustered together in the phylogenetic tree (Figure 3), and they showed very low distance between them. GenBank accession number, location, species, year and lineage, following Meli et al. (2010), of the 66 used sequences in the phylogenetic tree can be found in Table S1. Additionally, pairwise genetic distance between sequences is shown in Table S2.

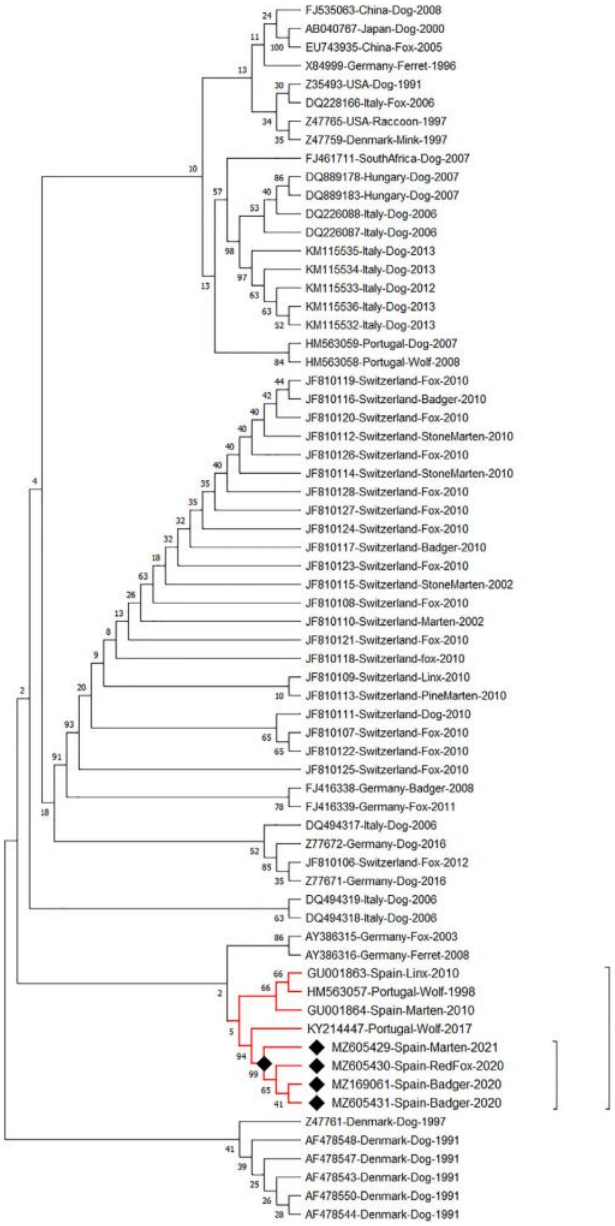

FIGURE 3 Phylogenetic analysis of canine distemper virus (CDV) strains based on partial H gene sequence (375 bp) from 66 sequences. Sequences are described following this naming scheme: GenBank accession number–location–species–year (see Table S1). Red branches indicate a putative Iberian sub-lineage (long black branch). Sequences obtained in this work are highlighted with a black diamond (short black branch)

3.3 | Antibodies against CDV in free-ranging badgers from 2008 to 2020

A total of 297 out of the 684 badgers (43.4%) included in the serological study were positive by CDV ELISA. The mean annual seroprevalence ranged from 25% in 2020 to 63.64% in 2015. Fisher's tests between years indicated numerous significant differences (Table S3), suggesting important prevalence fluctuations through time with maxima in 2008, 2012, 2015, 2017 and 2018 and minima in 2014 and 2020 (Figure 4a). The rate of seropositivity varied with geographic region (Figure 4b): 24 of 77 (31.2%) badgers from the Western region were positive, compared to 78 of 221 (35.3%) from the Central area and 195 of 386 (50.5%) from the Eastern area. The rate of seropositivity was 41.2% (124/301) among males and 41.8% (135/323) among females. The rate of seropositivity was 42.7% (197/461) among adults and 38.0% (62/163) among subadults. The results of individual sera distributions of each group can be found in Figure S6. Results of GLM (Table 2) revealed no sex- or age-related differences in the probability of presenting antibodies against CDV. Nevertheless, both sampling year ($\chi^2 = 34.77, p = .01$) and area ($\chi^2 = 15.97, p < .001$) were confirmed to be statistically significant factors according to GLM, with badgers from the Eastern region showing a higher probability of seropositivity than those from Western and Central areas. On the other hand, trapped badgers also showed a statistically significant higher

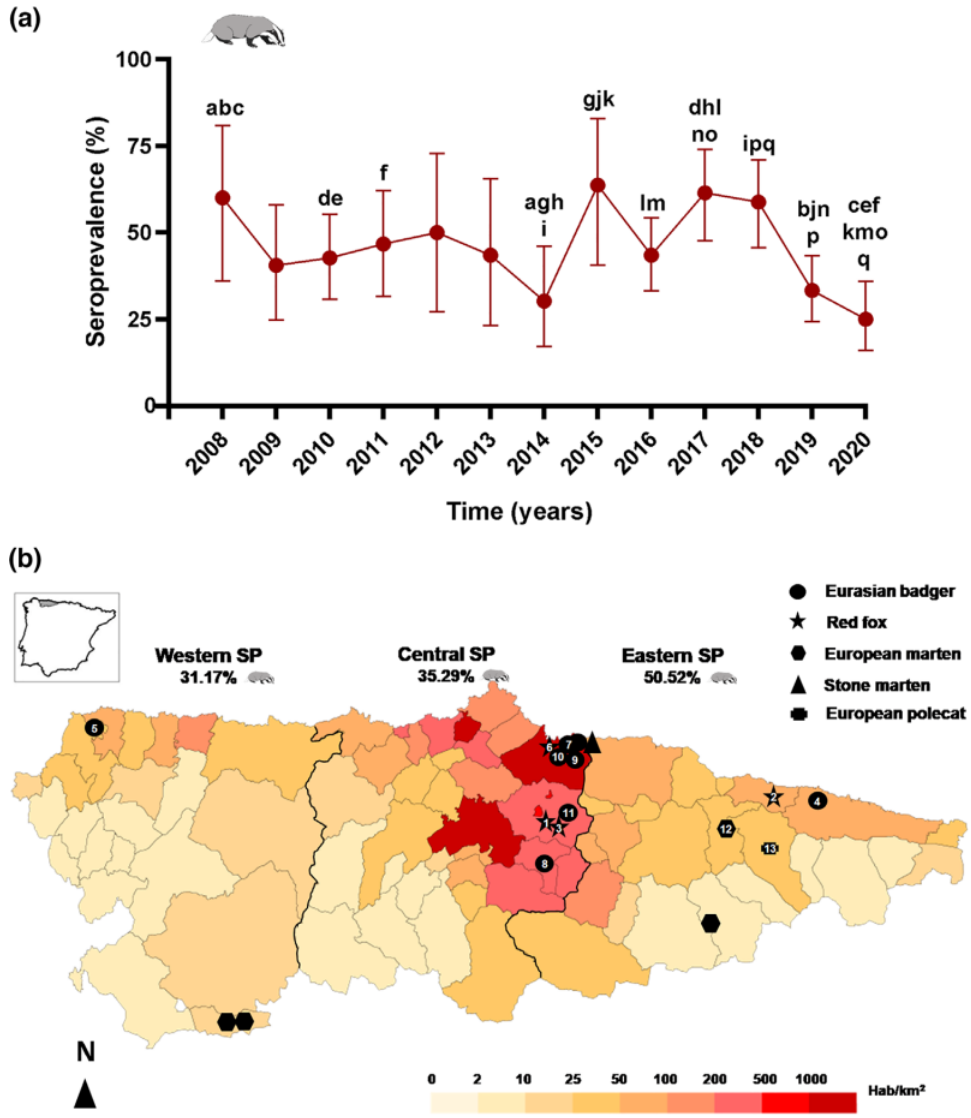

FIGURE 4 Distemper in wildlife in Asturias (south-western Europe). (a) Seroprevalence of canine distemper virus among badgers by year (2008–2020) based on a qualitative ELISA. The lines in each point indicate the lower and upper limits calculated by the 95% confidence interval (95% CI). For each year, the same letters above different points indicates significant differences between the seroprevalence for each year ($p < .05$). Comparations among the years were performed by the Fisher's exact test. (b) Map of human population density in Asturias and seroprevalence (SP) of badgers in the Western, Central and Eastern areas of the region. The three regions are demarcated with black lines. The map is coloured according to human population density from beige to maroon to reflect inhabitants per kilometre square. Black forms indicate the geolocation of the wild species with clinical distemper disease or positive to canine distemper virus by molecular techniques. The numbers inside the forms are related to animals showing distemper disease in Table 1

probability of being seropositive than those killed in road traffic accidents ($\chi^2 = 41.25$, $p < .001$).

4 | DISCUSSION

We witnessed a mortality episode caused by CDV in several wild carnivore species and used a large sample of routinely collected badger serum samples to gain additional insights into the dynamics of an infectious disease, distemper, in a well-preserved and diverse carnivore community in south-western Europe. Pathology and nucleic acid detection confirmed that distemper (CDV infection) caused mortality in four mustelids (European badger, European marten, stone marten, and polecat) and one canid (red fox) between 2018 and 2021. The detection of consistently high antibody prevalence in badgers over the period 2008–2020 suggests that CDV circulation is maintained in wildlife in this region.

Previous studies also confirmed CDV antibodies in fox (22%) and wolf (19%) in the region (Oleaga et al., 2015; Sobrino et al., 2008). Despite high seroprevalence in wolves, few wolves showing clinical signs compatible with distemper have been reported in Asturias. Further, CDV infection has not been confirmed by RT-PCR, although it was

TABLE 2 Generalized linear model for the presence of antibodies against canine distemper virus (CDV) reported in badger serum samples

CDV ELISA	
Explanatory variables	χ^2, *d.f.*, Param. estimates (± SE), *p*
Sex	0.007, 1, 0.01 ± 0.18, .93
Age	0.15, 1, −0.08 ± 0.20, .69
Year	34.77, 12, 2008 = −1.38 ± 0.57, 2009 = −0.50 ± 0.45, 2010 = −0.40 ± 0.38, 2011 = −0.43 ± 0.44, 2012 = −1.22 ± 0.52, 2013 = −0.20 ± 0.54, 2014 = −0.12 ± 0.43, 2015 = −1.61 ± 0.53, 2016 = −0.71 ± 0.34, 2017 = −1.51 ± 0.38, 2018 = −0.57 ± 0.39, 2019 = −0.01 ± 0.36, **.01**
Area	15.97, 2, Western = 1.07 ± 0.31, Central = 0.54 ± 0.19, **<.001**
Death cause	41.25, 1, 1.79 ± 0.28, **<.001**

Note: Parameter estimates for the levels of fixed factors were calculated considering a reference value of 0 for the level male in the variable 'Sex', for the level subadult in the variable 'Age', for the level 2020 in the variable 'Year', for the level Eastern in the variable 'Area' and for the level capture in the variable 'Death cause'. Significant *p*-values are highlighted in bold. *d.f.*: degrees of fredom; SE: standard error.

confirmed by immunohistochemistry in one wolf with sarcoptic mange coinfection (Oleaga et al., 2015).

4.1 | Implications for carnivore conservation

In the context of conservation, distemper may have an impact on population size (Lloyd-Smith et al., 2005; Viana et al., 2015). Collectively, research suggests that CDV circulates in a diverse carnivore community where it might also spill over into endangered species such as the brown bear. Fatal cases of distemper have been described in close species such as a young free-ranging black bear (*Ursus americanus*) in the United States (Cottrell et al., 2013) and six captive giant pandas (*Ailuropoda melanoleuca*) in China (Feng et al., 2016). We are unaware of reports of distemper in Eurasian brown bear populations, and anti-CDV antibodies have been detected in 12% of brown bears in Slovakia and 37% in Italy (Di Francesco et al., 2015; Vitásková et al., 2019). Those data confirm that the virus is circulating among European brown bear populations and that the surveillance for possible CDV cases in this species should not be neglected.

4.2 | Long-term dynamics and infection maintenance

Despite the continuous presence of antibodies against CDV in badgers since at least 2008, no badger or other wild species with neurological or other symptoms compatible with CDV arrived at the WRC until 2018, suggesting that CDV is well adapted to wildlife, with subclinical manifestations in most animals and without severely affecting population dynamics. Temporal fluctuations in seroprevalence in badgers indicate either cycles of increasing population susceptibility coinciding with periodic outbreaks (e.g. in 2020-2021) or sporadic infection spillover events, possibly from unvaccinated domestic dogs, because vaccination against CDV is included among standard dog vaccinations in Spain, although it is not obligatory (Colvema, 2021). Cycles of CDV have previously been reported in raccoons (*Procyon lotor*) in North America (Roscoe, 1993) and in lions in Africa (Munson et al., 2008). In addition, we detected the lowest seroprevalence in badgers in 2020, yet an outbreak of the viral disease occurred in the same year, which might reflect the progressive replacement of immune with naive populations, facilitating the outbreak.

Although temporal differences in the search and submission effort by citizens cannot be ruled out, alternative explanations for the apparent increase in pathogenicity include the emergence of a more pathogenic CDV strain or coinfections with other immunosuppressive pathogens (Munson et al., 2008). A more pathogenic CDV strain may be the most probable explanation given the nature of the gross and microscopic lesions in the animals studied, as well as the lack of obvious comorbidities. This would be analogous to how the emergence of a new CDV strain among mustelid populations in central Europe led to an apparent increase in pathogenicity (Origgi et al., 2012). The identified variants are phylogenetically close to others isolated in Europe and can be assigned to the European lineage (Meli et al., 2010; Origgi et al., 2012). Based both on phylogenetic tree and pairwise genetic distances, CDV seems to have a clustering based on geography and collection date (see Figure 3). The fact that all four new sequences cluster together, and very close to other Iberian (Spain and Portugal) sequences, suggests a common origin for the observed outbreak. This result also suggests an endemic pattern of the virus in Iberian Peninsula, with mutations occurring by genetic drift in an RNA virus. More sequencing studies are needed to understand if those results could indicate the existence of an Iberian sub-lineage (Figure 3).

Badgers from which the same CDV strain was identified were geographically separated by approximately 50 km. Given that the average home range for badgers in Asturias is less than 2 km (Acevedo et al., 2014), the presence of other reservoirs such as foxes or martens (or domestic dogs) capable of transmitting the virus to badgers might also be a plausible source of infection. Additionally, 10 (55%) of the 18

positive animals in our study were found in areas with the densest human populations (Figure 4b), where the probability is higher that dogs serve as reservoirs and that humans may come across infected animals and report them. Specifically in densely populated rural areas, vaccination remains as a key preventive measure for the control of this (and others, such as canine adenovirus type 1) domestic–wildlife shared virus circulation, and thus for the indirect protection of sympatric wild carnivores susceptible to those pathogens (Oleaga et al., 2015, 2022). On the other hand, the availability of anthropogenic food sources in rural areas can also attract opportunistic species such as foxes, thus favouring interspecific transmission of contagious diseases.

The acute, highly immunizing nature of CDV infection may suggest that large populations of susceptible hosts are required for persistence, circulation and transmission (Almberg et al., 2010; Viana et al., 2015). Despite the absence of data regarding dog vaccination compliance in Asturian rural areas, it is unlikely that dog and wolf populations can explain by themselves the circulation and long-term maintenance of CDV. In this sense, the presence and higher densities of other susceptible wild carnivores, especially red fox and badger, likely plays a key role in the epidemiology of this viral disease in the field. The inclusion of more host species generally increases the persistence probability of pathogens with density-dependent transmission (Dobson, 2004; McCormack & Allen, 2007) by reducing the critical community size within any one host species and the minimum spatial scale necessary for long-term disease persistence (Almberg et al., 2010). In smaller populations, regular epidemics are typically followed by 'fade-outs' during which infection disappears until reintroduced from outside (Lloyd-Smith et al., 2005).

4.3 | Insights gained from histopathology

Although sick badgers, foxes, the marten and the polecat showed systemic infection, they also showed prominent ataxia, seizure and convulsions, suggesting neurotropism that we confirmed histologically and by immunohistochemistry. In this regard, immunohistochemistry was useful to confirm CDV infection in different species. Our CDV-infected subjects showed similar neurological signs and lesions as badgers, red foxes (Origgi et al., 2012), fennec foxes (*Vulpes zerda*) (Woo et al., 2010) and lions (*Panthera leo*) (Roelke-Parker et al., 1996) in previous studies. Additionally, as previously reported, CNS, lungs and lymphoid tissues were the main target organs in all studied species (Deem et al., 2000). CNS infection by CDV occurs via hematogenous and neurotropic routes (Krakowka, 1989; Vandevelde et al., 1985). The presence of viral antigen within ependymal cells, neurons (including axons) and glial cells in our study confirms the likely spreading of the virus through the cerebrospinal fluid and nerves. In fox, the presence of viral antigen was higher in cerebellum than in other areas of the brain, in contrast with the distribution observed in badger and marten. Thus, cerebellum would be the target area for sampling brain in fox and corpus striatum, thalamus and hypothalamus in mustelids. Differences in target immunostained cells between species might be due to differences in the affinity of the host's viral receptors. However, sample size limitations in this study did not allow us to confirm that hypothesis. The scarce immunolabelling observed in the polecat might suggest a different stage of the disease in which the virus has been cleared from tissues. The histological lesions and viral detection in lungs, gut and kidney suggest the potential excretion of CDV through nasal discharge, faeces and urine, respectively. This could lead to environmental contamination and subsequent transmission through indirect contact with contaminated material, as suggested for other viruses and carnivore species in the region (Oleaga et al., 2022).

4.4 | Sampling effects in badgers

In this long-term serological study, trapped badgers were significantly more likely to test seropositive for CDV than animals killed in road accidents. We cannot exclude that at least part of this difference reflected a greater sensitivity of CDV detection in serum samples from trapped badgers given that the quality of those samples was optimal. At the same time, this may reflect how badgers were sampled within a surveillance program for tuberculosis in the region (see Section 2). Asturias includes a well-studied medium-density badger population (3.81 adults/km^2) (Acevedo et al., 2014). Trapped badgers were captured from settings located close to tuberculosis-infected cattle herds, where densities of adults (5.0 adults/km^2) are higher than in other areas (Acevedo et al., 2019). Thus, circulation of the virus may also be favoured in areas more densely populated with badgers. The higher CDV seroprevalence in Eastern areas than in Central or Western ones may also reflect the geographic distribution of badger populations (Acevedo et al., 2019). Additionally, the proximity to farms of trapped badgers may increase contact between badgers and unvaccinated dogs, increasing the risk of CDV transmission in both directions (Beineke et al., 2015; Kapil & Yeary, 2011).

4.5 | Final remarks

It remains unknown whether the region contains other relevant domestic or wildlife reservoir hosts for the virus, including bats (Drexler et al., 2012). CDV was originally thought to be restricted to canine host species, but this paramyxovirus has a broad range of potential hosts (Nambulli et al., 2016), and its recent emergence into non-human primate species is a relevant cause for concern (Sakai et al., 2013; Sun et al., 2010). Integrated monitoring and investigation of the mechanisms of long-term CDV maintenance will be essential to optimize disease management under the One Health concept. Collaboration of citizens for the detection of sick animals in the field and veterinary assistance in WRCs are also key tools for the early detection of disease outbreaks (Kelly et al., 2021).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. All studies of badgers were performed in accordance with relevant guidelines and regulations. All experimental protocols in trapped badgers were approved by the licensing committees from the Government of the Principality of Asturias, Spain (license numbers 010/07-01-2011, PROAE 20/2015, and PROAE 47/2018).

DATA AVAILABILITY STATEMENT

All study data are included in the article and the Supporting Information.

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