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VIGILANCIA EPIDEMIOLÓGICA EN LAGOMORFOS EN ANDALUCÍA

DOCTORAL THESIS

EPIDEMIOLOGICAL SURVEILLANCE IN LAGOMORPHS IN ANDALUSIA

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TÍTULO DE LA TESIS: Vigilancia Epidemiológica en lagomorfos en Andalucía

DOCTORANDA: Dña. Leonor Natividad Camacho Sillero

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

La Tesis Doctoral titulada "Vigilancia Epidemiológica en lagomorfos en Andalucía", que ha realizado la doctoranda Dña. Leonor Natividad Camacho Sillero, dio comienzo en el curso 2013/2014. Durante este periodo, la doctoranda ha realizado una estancia de tres meses en el Dipartimento di Scienze Veterinarie de la Università degli studi di Torino (Italia).

El objetivo general de esta Tesis Doctoral es ampliar el conocimiento sobre las principales enfermedades víricas (mixomatosis y enfermedad hemorrágica del conejo) que afectan a las poblaciones de lagomorfos silvestres de la Península Ibérica. Para la consecución de este objetivo general, se han planteado los siguientes objetivos específicos: 1) Monitorización de la nueva variante del virus de la enfermedad hemorrágica del conejo (GI.2) en conejo silvestre (*Oryctolagus cuniculus*) en Andalucía. 2) Monitorización espacio-temporal de la mixomatosis en conejo silvestre en ecosistemas mediterráneos de España. 3) Descripción del primer brote de mixomatosis detectado en liebre ibérica (*Lepus granatensis*). 4) Monitorización de los brotes epidémicos causados por el virus mixoma emergente en liebre ibérica (h-MYXV) en España.

Tres de los estudios realizados durante el desarrollo de la presente Tesis Doctoral ya han sido publicados en revistas indexadas en el JCR:

- **Camacho-Sillero, L.**, Caballero-Gómez, J., Gómez-Guillamón, F., Martínez-Padilla, A., Agüero, M., San Miguel, E., Zorrilla, I., Rayas, E., Talavera, V., García-Bocanegra, I. (2019). Monitoring of the novel rabbit haemorrhagic disease virus type 2 (GI.2) epidemic in European wild rabbits (*Oryctolagus cuniculus*) in southern Spain, 2013-2017. Veterinary Microbiology 237:108361. doi: 10.1016/j.vetmic.2019.07.013. Índice de impacto: 3,030. Posición en la categoría: 7/142 (D1/T1/Q1). Categoría: Veterinary Science.

- García-Bocanegra, I., **Camacho-Sillero, L.**, Risalde, M.A., Dalton, K.P., Caballero-Gómez, J., Agüero, M., Zorrilla, I., Gómez-Guillamón, F. (2019). First outbreak of myxomatosis in Iberian hares (*Lepus granatensis*). Transboundary and Emerging Diseases 66(6): 2204-2208. doi:10.1111/tbed.13289. Índice de impacto: 4.188. Posición en la categoría: 4/142 (D1/T1/Q1). Categoría: Veterinary Sciences.

- García-Bocanegra, I., **Camacho-Sillero, L.**, Caballero-Gómez, J., Agüero, M., Gómez-Guillamón, F., Ruiz-Casas, J.M., Díaz-Cao, J.M., García, E., Ruano, M.J., de la Haza, R. (2020). Monitoring of emerging myxoma virus epidemics in Iberian hares (*Lepus granatensis*) in Spain, 2018-2020. Transboundary and Emerging Diseases 68(3):1275-1282. doi:10.1111/tbed.13781. Índice de impacto: 5,005. Posición en la categoría: 4/146 (D1/T1/Q1). Categoría: Veterinary Sciences.

Así mismo, actualmente, uno de los trabajos incluidos en esta Tesis Doctoral se encuentra en revisión:

- **Camacho-Sillero, L**., Cardoso, B., Beato-Benítez, A., Gómez-Guillamón, F., Díaz-Cao, JM., Jiménez Martín, D., Caballero-Gómez, J., Castro-Scholten, S., Cano-Terriza, D., García-Bocanegra, I. (2022). Spatiotemporal monitoring of myxomatosis in European wild rabbit (*Oryctolagus cuniculus*) in Spanish Mediterranean ecosystems. Transboundary and Emerging Diseases. En revisión.

Una vez redactada, la presente Tesis Doctoral ha sido revisada, reuniendo a mi juicio todos los requisitos necesarios para su lectura y defensa.

Y, para que conste, en cumplimiento de las disposiciones vigentes, se expide el presente informe y se autoriza a la presentación de la Tesis Doctoral.

Córdoba, 2 de abril de 2021

Firma del director

Fdo.: Ignacio García Bocanegra

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A mi familia, la de sangre

y la que se elige.

ÍNDICE GENERAL

RESUMEN/SUMMARY

RESUMEN

La vigilancia epidemiológica se define como la recolección sistemática, análisis e interpretación de datos sanitarios necesarios para la planificación, implementación y evaluación de medidas de lucha frente a las enfermedades, así como la difusión de la información generada. En veterinaria, la vigilancia epidemiológica aborda el conocimiento continuo y sistemático del estatus sanitario de las poblaciones animales, así como de los factores de riesgo a los que están expuestas las poblaciones. Según la metodología de obtención de información, la vigilancia puede clasificarse en activa o pasiva. Esencialmente, la vigilancia epidemiológica activa trata de medir o cuantificar la presencia de un determinado patógeno en una población, mientras que la vigilancia epidemiológica pasiva se sustenta en la comunicación de cualquier sospecha de enfermedad para determinar el agente etiológico implicado. Con el objetivo de establecer las medidas de prevención y lucha adecuadas en cada momento, un programa de vigilancia epidemiológica (ya sea activa o pasiva) debe ser dinámico y estar sujeto a modificación constante.

En la actualidad, los programas de vigilancia epidemiológica que se realizan en Europa son numerosos y se encuentran bien establecidos tanto para los seres humanos como para animales domésticos. En humanos, las primeras medidas que pueden considerarse como acciones de vigilancia, tuvieron lugar durante las grandes epidemias que afectaron a Europa en los siglos XIV y XV, aunque los programas como tal se desarrollaron mucho más tarde, en la segunda mitad del siglo XX. En paralelo, debido al estrecho contacto entre las personas y los animales domésticos, estos programas fueron surgiendo también en ganadería. Sin embargo, el interés por la vigilancia de las enfermedades en fauna silvestre es mucho más reciente. En España, los primeros programas de vigilancia comienzan a desarrollarse a finales de siglo, en los años 80. A partir de ese momento se incrementa el interés por conocer el papel epidemiológico de la fauna silvestre en la transmisión de enfermedades relevantes para su conservación, así como para la sanidad animal y la salud pública, y con ello, la necesidad de desarrollar programas de vigilancia orientados también a estas especies.

Las especies de lagomorfos silvestres más relevantes en la Península Ibérica, en términos de abundancia e interés cinegético, son el conejo silvestre (*Oryctolagus cuniculus*) y la liebre ibérica (*Lepus granatensis*). Entre las principales causas implicadas en la modulación de sus poblaciones se encuentran las enfermedades infectocontagiosas. En este sentido, la Enfermedad Hemorrágica del Conejo (EHC) y la mixomatosis destacan como los principales factores responsables del declive poblacional de estas especies en la última década. La aparición de la

nueva variante del virus de la EHC (GI.2) o las modificaciones genéticas del virus de la mixomatosis (ha-MYXV), han ocasionado cambios importantes en la epidemiología y patogenia de estas enfermedades víricas. El estudio de estos nuevos escenarios requiere de la implementación de programas de vigilancia sanitaria que permitan detectar de forma rápida (mediante vigilancia pasiva) la circulación de estos virus, monitorizar (empleando vigilancia activa) su distribución y evolución espacio-temporal, y evaluar su impacto en las poblaciones de lagomorfos silvestres en los ecosistemas mediterráneos ibéricos. El **objetivo general** de la presente Tesis Doctoral es ampliar el conocimiento sobre las principales enfermedades víricas (EHC y mixomatosis) que han afectado al conejo silvestre y a la liebre ibérica en la Península en la última década. Para ello, se han desarrollado varios estudios distribuidos en cuatro capítulos.

En el **Capítulo 1** se llevó a cabo un programa de vigilancia pasivo para monitorizar los brotes de GI.2 en las poblaciones de conejo silvestre en Andalucía durante el periodo 2013-2017. En este estudio se analizaron un total de 96 cotos de caza menor o zonas protegidas en las que se detectó elevada mortalidad. El primer brote se observó en junio de 2013. El número de brotes aumentó considerablemente en 2013 y 2014, observándose una tendencia decreciente durante los años siguientes. La distribución espacial de GI.2 no fue homogénea, ya que la mayoría de los brotes se detectaron en la zona occidental de la región. Se confirmó circulación de GI.2 en los cinco años evaluados, detectándose la mayoría de los brotes en los meses de invierno y primavera. Durante el periodo de estudio, se obtuvieron muestras de hígado de 190 conejos encontrados muertos en 87 de las 96 áreas analizadas. Los análisis moleculares confirmaron la presencia de ARN de GI.2 en muestras de 185 de los 190 (97,4%) conejos analizados. El estudio filogenético realizado en 11 muestras de hígado obtenidas en diferentes provincias entre 2013 y 2017 reveló una elevada homología genética con las cepas de GI.2 detectadas previamente en España, Francia y Portugal. Los resultados obtenidos sugieren una amplia distribución espacial y una circulación endémica de GI.2 en las poblaciones de conejo silvestre en Andalucía durante el periodo 2013-2017. Nuestro estudio constituye un paso importante para comprender la emergencia y propagación de GI.2 en el este de España y proporcionarán información relevante para el desarrollo de programas de vigilancia en Europa.

El objetivo del **Capítulo 2** fue establecer un programa de vigilancia epidemiológica activa para determinar la seroprevalencia del virus de la mixomatosis (MYXV), la prevalencia de infección de este virus, así como los patrones espaciotemporales y factores de riesgo asociados a su circulación en las poblaciones de conejo silvestre en ecosistemas mediterráneos del sur de España.

Para ello, se muestrearon un total de 2376 animales durante cuatro periodos temporales: 2009-2012 (P1), 2012-2015 (P2), 2015-2018 (P3) y 2018-2021 (P4). Se encontraron anticuerpos frente al MYXV en el 59,6% (1424/2376; IC95%: 58,0-61,9%) de conejos silvestres utilizando un ELISA indirecto comercial. Se detectó al menos un animal seropositivo en 131 (96,3%) de los 136 cotos de caza menor muestreados. Se confirmó infección por MYXV en 94 de 1063 (8,8%; IC95%: 7,3-10,7) conejos mediante PCR. No se encontró circulación del MYXV recombinante de la liebre (ha-MYXV) en los conejos silvestres analizados durante P4, periodo de comienzo de circulación de este virus emergente en las poblaciones de liebre ibérica. Se identificaron cinco clústeres espacio-temporales estadísticamente significativos de alta seroprevalencia utilizando un modelo de Bernoulli; uno en el P2 y cuatro en el P3. El análisis, empleando modelo lineal generalizado mixto para la seropositividad al MYXV, identificó la temporada (otoño), la edad (adulto y subadulto), los brotes de mixomatosis durante el mes anterior al muestreo, la temperatura media anual y la seropositividad al Virus de EHC como potenciales factores de riesgo. De forma similar, los brotes de mixomatosis durante el mes anterior al muestreo, la seropositividad a MYXV y la presencia de lesiones compatibles con mixomatosis fueron factores asociados con la infección por MYXV. Los resultados indican una elevada exposición, una distribución generalizada pero no homogénea y una circulación endémica de MYXV en las poblaciones de conejos silvestres en Andalucía durante la última década. La prevalencia de anticuerpos frente al MYXV presentó fluctuaciones durante el año y a lo largo de los periodos de estudio, mostrando variaciones en la inmunidad de las poblaciones de conejos silvestres en los ecosistemas mediterráneos, lo que podría incrementar el riesgo de re-emergencia del MYXV en poblaciones inmunológicamente desprotegidas. El presente estudio destaca la importancia de la vigilancia activa a largo plazo para una mejor comprensión de la epidemiología del MYXV en lagomorfos silvestres.

En el **Capítulo 3** se describe el primer brote de mixomatosis en liebre ibérica. Entre mediados de julio y finales de septiembre de 2018 se detectaron alrededor de 530 animales muertos en Andalucía. La tasa de mortalidad media aparente fue del 56,7% y la tasa de letalidad media estimada fue del 69,2%. Los resultados histopatológicos y moleculares confirmaron la infección por ha-MYXV en todas las liebres analizadas. Desde nuestro conocimiento, este es el primer brote de mixomatosis que causa una elevada mortalidad en liebres y la primera descripción de un brote de mixomatosis en la liebre ibérica. La ausencia de casos en conejos silvestres simpátricos sugiere diferencias en la susceptibilidad entre ambas especies de lagomorfos a la cepa del virus implicada en el brote. Tras el primer caso, el número de zonas afectadas aumentó

considerablemente afectando a la mayor parte de la Península Ibérica donde está presente la liebre ibérica.

El objetivo del **Capítulo 4** fue describir la evolución espacio-temporal y los principales hallazgos epidemiológicos de los brotes causados por ha-MYXV en liebre ibérica en España. En el periodo 2018-2020, se confirmó mediante PCR, la infección por MYXV en un total de 487 liebres ibéricas procedentes de 372 áreas. Se detectaron brotes de ha-MYXV en la mayoría de las regiones españolas donde habita la liebre ibérica. La distribución espacial no fue homogénea, concentrándose la mayoría de los brotes en el sur y centro de España. La detección de brotes consecutivos durante 2019 y 2020, sugieren una circulación endémica en España de este virus emergente. El estudio retrospectivo realizado justo después del primer período epidémico (2018-2019) reveló que el virus podría haber estado circulando desde junio de 2018. El número de brotes comenzó a aumentar en julio, alcanzando su pico epidémico durante la primera quincena de agosto y octubre, y disminuyó bruscamente hasta enero de 2019. La tasa de mortalidad media aparente fue del 55,4% (mediana: 70,0%). Los resultados obtenidos indicaron una elevada susceptibilidad de la liebre ibérica a la infección por ha-MYXV, pero una aparente resistencia en las especies de liebres presentes en España y una limitada circulación del ha-MYXV en las poblaciones de conejo silvestre. El ha-MYXV ha tenido un importante impacto en el estado sanitario de las poblaciones de liebre ibérica en España, con las consecuentes implicaciones para la sanidad animal y la conservación de esta especie. El presente estudio contribuye a una mejor comprensión de la epidemiología del ha-MYXV y proporcionará información relevante para el desarrollo de medidas de control frente a este virus emergente.

En esta Tesis Doctoral se aportan nuevos conocimientos sobre las dos enfermedades infecciosas (mixomatosis y EHC) que mayor impacto han tenido en las poblaciones de conejo silvestre y liebre ibérica en la Península Ibérica durante la última década. Los resultados obtenidos proporcionan una visión general sobre la evolución espacio-temporal de estas enfermedades infecciosas y resaltan la importancia de los programas de vigilancia pasiva y activa para una mejor comprensión de la epidemiología del GI.2, MYXV y ha-MYXV.

SUMMARY

Epidemiological surveillance is defined as the systematic collection, analysis, and interpretation of health data necessary for the planning, implementation, and evaluation of disease control measures, together with the dissemination of the information generated. In the veterinary field, epidemiological surveillance is directed at providing systematic ongoing information about the health status of animal populations and the risk factors to which these populations are exposed. Depending on the methodology used to gather information, surveillance can be classified as active or passive. Essentially, active surveillance aims to measure or quantify the presence of a given pathogen in a population, whereas passive surveillance involves the rapid reporting of any suspected disease and investigating the causative agent. In order to establish the appropriate prevention and control measures at any given time, epidemiological surveillance programmes (both active and passive) should be dynamic and subject to constant adjustment.

Nowadays, there are numerous well-established epidemiological surveillance programmes in Europe for both human beings and domestic animals. In the case of humans, the first surveillance measures were instituted during the major epidemics that affected Europe in the 14th and 15th centuries, although surveillance programmes as such only developed much later, in the second half of the 20th century. In parallel, epidemiological surveillance programmes started to be developed to survey the health of domestic animals, due to their close contact with humans. Interest in wildlife disease surveillance, however, is a much more recent phenomenon. In Spain, the first surveillance programmes began to be developed in the 1980s. Interest subsequently focussed on the epidemiological role of wildlife species in the transmission of diseases of relevance to their conservation, as well as to animal and public health. As a result, surveillance programmes evolved to include these species as well.

The main wild lagomorph species in the Iberian Peninsula in terms of abundance and hunting interest are European wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*), although populations have been declining over the last decade. Rabbit haemorrhagic disease (RHD) and myxomatosis are among the main factors involved in the regulation of their populations and are also the main ones explaining the current decline of their populations. The appearance of the new variant of RHD virus (GI.2) and the genetic modifications of myxoma virus (ha-MYXV) have led to important changes in the epidemiology and pathogenesis of these viral diseases. To study these new scenarios, it is necessary to implement health surveillance programmes that can rapidly detect (by passive surveillance) the circulation of these viruses,

monitor (by active surveillance) their distribution and spatiotemporal evolution, as well as assess their impact on wild lagomorph populations in Mediterranean ecosystems. The overall objective of this PhD thesis is to increase knowledge about the main viral diseases (RHD and myxomatosis) that have affected wild rabbits and Iberian hares in the Iberian Peninsula over the last decade. With this purpose in mind, several studies were developed and are presented here under four main headings.

Chapter 1: A passive surveillance programme was conducted to monitor outbreaks of GI.2 in wild rabbit populations in Andalusia between 2013 and 2017. This study included a total of 96 game estates or protected areas where high mortality rates were detected. The first outbreak of GI.2 was notified in June 2013. While the number of outbreaks increased sharply in 2013 and 2014, a decreasing trend was observed in the following years. The spatial distribution of GI.2 was not homogeneous, with most outbreaks being detected in the westernmost part of the region. Circulation of GI.2 was confirmed in all five years evaluated, with most outbreaks being detected in the winter and spring months. During the study period, liver samples were obtained from 190 rabbits found dead in 87 of the 96 areas tested. Molecular analysis confirmed the presence of GI.2 RNA in samples from 185 of the 190 (97.4%) rabbits tested. The phylogenetic analysis conducted on 11 liver samples obtained from different provinces between 2013 and 2017 revealed high genetic homology with GI.2 strains previously detected in Spain, France, and Portugal. The results obtained suggested a wide spatial distribution and endemic circulation of GI.2 in wild rabbit populations in Andalusia between 2013 and 2017. Our study represents an important step towards understanding the emergence and spread of GI.2 in Andalusia and provides information relevant to the development of surveillance programmes in Europe.

Chapter 2: The aim of this study was to establish an active epidemiological surveillance programme to determine myxoma virus (MYXV) seroprevalence and the prevalence of infection, as well as the spatiotemporal patterns and factors associated with its circulation in wild rabbit populations in the Mediterranean ecosystems of southern Spain. A total of 2,376 animals were sampled over four time periods: 2009-2012 (P1), 2012-2015 (P2), 2015-2018 (P3) and 2018-2021 (P4). Antibodies against MYXV were detected by a commercial indirect ELISA in 59.9% (1,424/2,376; 95%CI: 58.0-61.9) of wild rabbits. At least one seropositive animal was detected on 131 (96.3%) of 136 game estates sampled. MYXV infection was confirmed by PCR in 94 of 1,063 (8.8%; 95%CI: 7.3-10.7) wild rabbits. Infection with recombinant MYXV (ha-MYXV) was not found in any wild rabbits tested in P4, the period when ha-MYXV emerged in the Iberian hare. Five

statistically significant spatiotemporal clusters of high seroprevalence were identified using a Bernoulli model: one in P2 and four in P3. A generalized linear mixed model (GLMM) analysis identified sampling season (autumn), age (adults and juveniles), outbreaks of myxomatosis in the month prior to sampling, mean annual temperature, and seropositivity to rabbit haemorrhagic disease virus as factors potentially linked with MYXV seropositivity. GLMM analysis identified outbreaks of myxomatosis in the month prior to sampling, MYXV seropositivity and the presence of lesions compatible with myxomatosis as factors associated with MYXV infection. The results indicate high exposure, widespread but non-homogeneous distribution, and endemic circulation of MYXV in wild rabbit populations in southern Spain over the last decade. Prevalence of antibodies against MYXV showed fluctuations both within the year and over the study periods, revealing variations in the immunity of wild rabbit populations in Mediterranean ecosystems that could increase the risk of MYXV re-emergence in immunologically naïve populations. The present study highlights the importance of long-term surveillance to obtain a better understanding of the epidemiology of MYXV in wild lagomorphs.

Chapter 3: This study describes the first outbreak of myxomatosis in Iberian hares. Between mid-July and the end of September 2018, about 530 animals were found dead in Andalusia. The mean apparent mortality rate was 56.7% and the estimated mean case-fatality rate was 69.2%. Histopathological and molecular results confirmed ha-MYXV infection in all hares tested. To the best of our knowledge, this was the first outbreak of myxomatosis to cause high mortality in hares and the first description of a myxomatosis outbreak in Iberian hares. The fact that no cases were reported in sympatric wild rabbits suggests differences in susceptibility to ha-MYXV between the two lagomorph species. After the first cases, the number of affected areas increased considerably, and most of the Iberian Peninsula where the Iberian hare is present is currently involved.

Chapter 4: The objective of this study was to describe the spatiotemporal evolution and main epidemiological findings of outbreaks caused by ha-MYXV in Iberian hares in Spain. Between 2018 and 2020, MYXV infection was confirmed by PCR in a total of 487 hares from 372 areas. Outbreaks of ha-MYXV were detected in most Spanish regions where the Iberian hare is present. Spatial distribution was not homogeneous, with most outbreaks being concentrated in south and central areas of the country. The detection of consecutive outbreaks in 2019 and 2020 suggests an endemic circulation of this emerging virus in Spain. This retrospective study conducted soon after the first epidemic period (2018-2019) revealed that the virus may have been circulating since June 2018. The number of outbreaks started to increase in July of that year, reaching its epidemic peak

between the first half of August and October, and then declined sharply until January 2019. The mean apparent mortality rate was 55.4% (median: 70.0%). The results obtained indicate high susceptibility of Iberian hares to ha-MYXV infection, but apparent resistance in other hare species present in Spain, and limited circulation of ha-MYXV in wild rabbit populations. ha-MYXV has had a major impact on the health status of Iberian hare populations in Spain, with corresponding implications for the health status and conservation of this species. The present study provides new insights into the epidemiology of ha-MYXV and information relevant to the development of control measures against this emerging virus.

The results of this doctoral thesis present new knowledge on the two infectious diseases (myxomatosis and RHD) that have had the greatest impact on wild rabbit and Iberian hare populations in the Iberian Peninsula over the last decade. The results obtained provide an overview of the spatiotemporal evolution of these infectious diseases and highlight the importance of passive and active surveillance programmes in order to obtain a better understanding of the epidemiology of GI.2, MYXV and ha-MYXV.

INTRODUCCIÓN

INTRODUCCIÓN

1. Generalidades de los lagomorfos

El Orden Lagomorpha incluye 91 especies divididas en dos familias: la familia *Ochotonidae*, que incluye las picas o conejos de roca, y la familia *Leporidae*, en el que se agrupan los jackrabbits, conejos y liebres. Esta última familia comprende más de una treintena de especies de liebres (género *Lepus*) y 29 especies de conejo (Chapman y Flux, 2008; Fontanesi y cols., 2016). Estas especies tienen gran importancia económica por constituir una fuente de alimentación para el ser humano, por ser especies relevantes en producción animal y en la actividad cinegética, por emplearse como animales de compañía y en experimentación animal, así como por las pérdidas debidas a los daños producidos en la agricultura. Igualmente, destaca su relevancia ecológica como moduladores del paisaje y por ser especies de presa en la cadena alimentaria de un elevado número de depredadores.

El conejo silvestre o conejo de monte (*Oryctolagus cuniculus*) y la liebre ibérica (*Lepus granaetnsis*) son dos especies endémicas de la Península Ibérica. Debido a su abundancia y amplia distribución, estos lagomorfos están considerados los más representativos de los ecosistemas mediterráneos ibéricos (Delibes-Mateos y cols., 2008). Las elevadas densidades de estas especies, condicionadas por sus características biológicas y su diversidad genética, han favorecido su capacidad de adaptación a una gran variedad de condiciones ecológicas. De hecho, el conejo silvestre es el mamífero con mayor distribución en la Península Ibérica. Su elevada adaptabilidad se debe, por un lado, a la cecotrofia que le permite aprovechar los nutrientes de alimentos de baja calidad (Hirakawa, 2001), confiriéndole cierta ventaja frente a otros herbívoros a la hora de colonizar hábitats marginales o soportar, de forma transitoria, condiciones ambientales adversas (Ramírez, 2016) y, por otro lado, a su elevada prolificidad por el tamaño de las camadas y las características sexuales de la especie asociadas a una rápida madurez sexual y celo post-parto, entre otras (Wood, 1980; Soriguer, 1981). En este sentido, la prolificidad del conejo silvestre le ha permitido colonizar una gran diversidad de ecosistemas, alcanzando territorios alejados de su área de distribución original. En algunas zonas de Australia, donde el conejo silvestre fue introducido en 1859, esta especie compite con éxito con otras especies autóctonas, donde la ausencia de depredadores naturales que regulen las poblaciones de conejos, la ha convertido en plaga, ocasionando un gran impacto económico y ambiental, con pérdidas anuales de 285 millones de euros derivadas del control de sus poblaciones en este país.

De forma similar, algunas especies de liebres también son abundantes en Europa, donde son especies cinegéticas importantes en muchos países, incluido España. En la Península Ibérica habitan tres especies de liebres: la liebre parda europea (*Lepus europaeus*) que se localiza en el norte y noreste de España, la liebre del piornal (*L. castroviejoi*) que se restringe a algunas zonas de la Cordillera Cantábrica y la liebre ibérica (*L. granatensis*) que es una especie endémica de la Península Ibérica y la más relevante en términos de abundancia y distribución, estando presente en la mayor parte de la Península Ibérica (MTERD, 2019).

1.1. Importancia de los lagomorfos silvestres en España

La importancia de los lagomorfos silvestres en los ecosistemas mediterráneos ibéricos puede contemplarse desde diferentes puntos de vista. Para la actividad cinegética, el conejo silvestre y la liebre ibérica están consideradas, junto con la perdiz roja (*Alectoris rufa*), las especies más relevantes de la caza menor en España. El interés socio-económico que generan estos lagomorfos radica en los ingresos que proporciona en muchas zonas rurales. En Andalucía, se estima que la caza del conejo y la liebre, las dos únicas especies de lagomorfos presentes en esta región, aporta unos 150 millones de euros al año al producto interior bruto de la región, generando más de 47.000 puestos de trabajo y favoreciendo de manera directa e indirecta a otros sectores como el turismo. Por lo tanto, esta importancia cinegética y socio-económica, determina un marcado interés por mantener estables las densidades de poblaciones de estos lagomorfos.

Por otro lado, el conejo y la liebre ibérica se consideran especies ecológicas clave en la Península Ibérica por sus las elevadas densidades poblacionales y por su amplia distribución espacial. Estas especies constituyen la base de la cadena trófica de más de 30 especies de depredadores en los ecosistemas mediterráneos ibéricos, incluyendo especies amenazadas como el lince ibérico (*Lynx pardinus*), el felino más amenazado del mundo, o el águila imperial ibérica (*Aquila adalberti*) (Ferrer y Negro, 2004; Delibes-Mateos y cols., 2014).

Por último, cabría destacar el valor de los lagomorfos silvestres como moduladores de paisaje por su efecto fitófago y, en el caso del conejo silvestre, como excavadores del terreno. Debido a la dispersión de semillas a través de las heces durante el pastoreo, estas especies alteran la cobertura vegetal contribuyendo al enriquecimiento de la diversidad de la flora (Junta de Andalucía, 2019). Además, las galerías y madrigueras de los conejos suponen un importante refugio para numerosos vertebrados e invertebrados.

1.2. Tendencias poblacionales de los lagomorfos silvestres en España

Las poblaciones de conejo silvestre y de liebre ibérica en la Península Ibérica han disminuido significativamente a lo largo de los últimos 50 años (Ballesteros y cols., 1996; Villafuerte y cols., 1998), mostrando actualmente, una distribución heterogénea en esta región. Aunque en algunas áreas de España, el conejo silvestre mantiene elevadas densidades, considerándose como una especie plaga por los daños que causa en la agricultura, en otras regiones, las poblaciones de conejos han desaparecido prácticamente, incluyendo en zonas donde alguna vez fueron abundantes.

Los datos recogidos a partir de las bolsas de caza (número de individuos capturados durante una temporada cinegética), muestran una tendencia decreciente en el número de conejos y liebres abatidos en los últimos años en España, con variaciones entre comunidades autónomas (Figura 1).

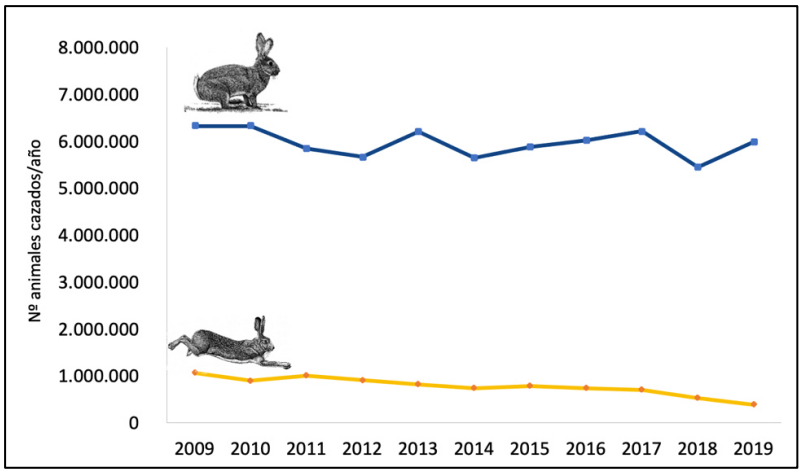

Figura 1.- Evolución del número de conejos silvestres y liebres ibérica cazados en España (Fuente: AEC, 2019).

En Andalucía, las densidades poblacionales de conejo silvestre han mostrado fluctuaciones en las últimas décadas, con una tendencia decreciente en los últimos años (Figura 2). En algunas regiones, las densidades de esta especie siguen siendo muy abundantes, estando consideradas como zonas de emergencia cinegética por los daños que ocasionan en la agricultura. Sin embargo, en muchas áreas del territorio andaluz, el conejo silvestre ha experimentado un marcado descenso, con poblaciones muy reducidas o prácticamente inexistentes (Junta de Andalucía, 2019) (Figura 3).

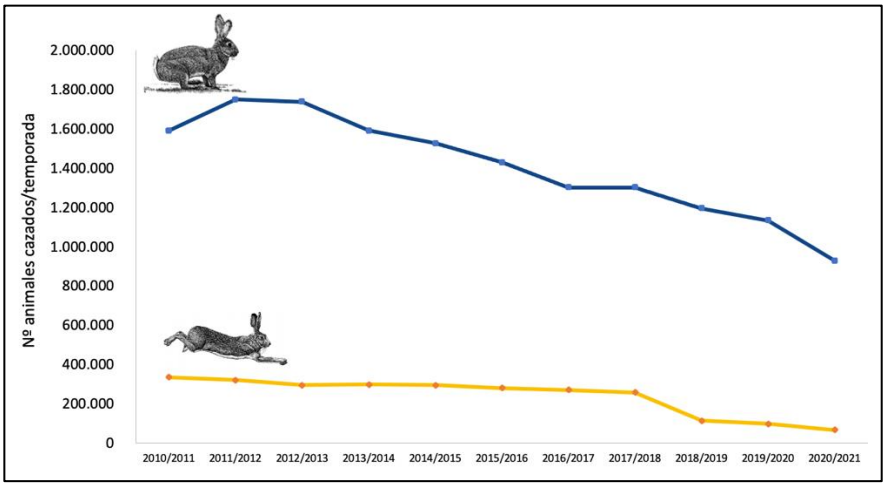

Figura 2.- Evolución del número de conejos silvestres y liebres ibérica cazados en Andalucía (Fuente: CAGPDS, 2021).

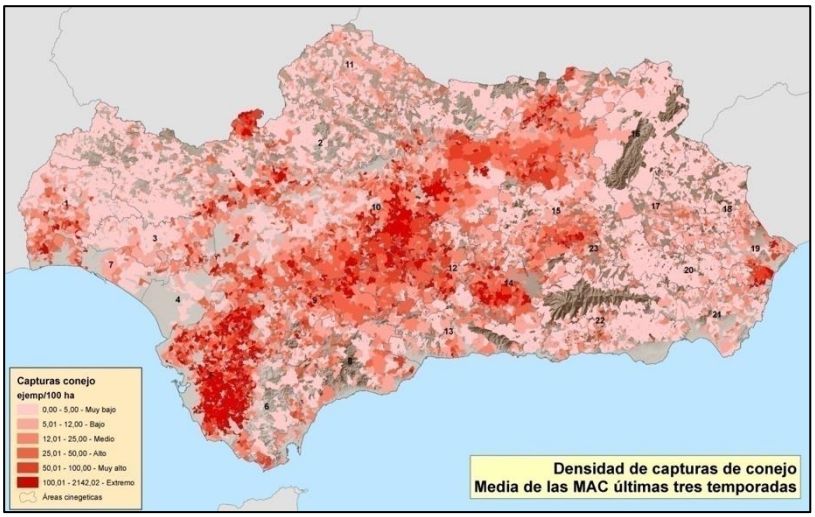

Figura 3.- Mapa de abundancia del conejo silvestre en Andalucía (Fuente: CAGPDS, 2021).

Con respecto a la liebre ibérica, la tendencia poblacional, al igual que la del conejo, ha ido en declive. Los escasos estudios realizados hasta la fecha en zonas concretas como el Parque Nacional de Doñana, Navarra o el sur de Portugal, manifiestan una disminución en las densidades de liebres ibéricas en estas zonas (Alves y Rocha, 2003; Carro y Soriguer, 2017). Aunque no existen datos de evolución de censos en Andalucía, los resultados obtenidos a partir de la bolsa de caza muestran una tendencia decreciente en las poblaciones en la última década (CAGPDS, 2021). Este descenso es especialmente evidente en la temporada 2018-2019, debido al impacto del brote epidémico de mixomatosis detectado por primera vez en esta especie (García-Bocanegra y cols., 2019).

1.3. Factores moduladores de las poblaciones de lagomorfos silvestres en España

Los principales factores limitantes de las poblaciones naturales de lagomorfos silvestres están directamente relacionados con la degradación del hábitat, el uso de productos fitosanitarios, el cambio climático, la presión cinegética, la depredación y las enfermedades (Ballesteros y cols., 1996; Carro y Soriguer, 2007).

La reproducción de los lagomorfos depende en gran medida de la calidad de la alimentación, la cual está asociada con el hábitat y las condiciones climáticas. La pérdida o alteración de hábitats adecuados para los conejos y las liebres, particularmente asociados a la intensificación de la agricultura, a los cambios de usos del suelo o a la deforestación, son factores relevantes en la disminución de sus poblaciones en muchas regiones de España (Villafuerte y cols., 1997). Asimismo, se han descrito factores climáticos como periodos de sequía o inundaciones, como limitantes ambientales de las poblaciones de estas especies.

Tanto el conejo silvestre como la liebre ibérica están sometidos con frecuencia a una presión cinegética intensiva, condicionando en gran medida sus parámetros demográficos. Aunque la adecuada gestión permite que las poblaciones soporten estas presiones asociadas a la actividad cinegética, la caza gestionada de forma inapropiada puede poner en peligro la estabilidad demográfica de estas especies. En este sentido, se ha demostrado que la caza ilegal junto con una gestión cinegética intensiva puede ocasionar importantes descensos poblacionales en estas especies (Ballesteros y cols., 1996).

Otro factor que modula las poblaciones de lagomorfos silvestres es la presión de los depredadores. La depredación, aunque no suele ser una causa directa de la disminución de las poblaciones, sí se considera un factor limitante para la recuperación, particularmente en aquellos casos en los que las densidades de estas especies han una disminución por factores naturales o antropogénicos (Trout y Tittensor, 1989). En un estudio previo realizado por Sánchez-García y cols. (2012) se identificó a los depredadores, en particular al zorro rojo (*Vulpes vulpes*), como la principal causa de mortalidad de las liebres ibéricas en el noreste de España. De forma similar, en Alemania, también se sugirió que la depredación asociada a esta misma especie tiene una influencia relevante en la tasa de supervivencia de la liebre europea (Goretzki y cols., 1999).

Sin embargo, de los factores implicados en la reducción de las poblaciones de lagomorfos silvestres en España inicialmente citados, cabe destacar las sucesivas epizootias que han afectado a estas especies en las últimas décadas. Entre las diferentes enfermedades que afectan al conejo silvestre, la mixomatosis y el virus de la EHC, son sin duda, las principales causas de mortalidad

asociadas a la disminución poblacional de esta especie (Villafuerte y cols., 1995). Se estima que ambas enfermedades han provocado un descenso de las poblaciones de conejo silvestre en las últimas décadas de hasta un 27% en Portugal (Ferreira y cols., 2010) y de hasta un 73% en algunas regiones de España (Virgós y cols., 2005). Las liebres también pueden verse afectadas por diversas patologías de etiología infectocontagiosa. Los principales agentes implicados en la mortalidad en liebre europea son el síndrome de la liebre parda europea (SLPE o del inglés EBHS), la cisticercosis, la pasteurelosis o la tularemia, entre otros (Wibbelt y Frölich, 2005). Sin embargo, la información sobre el estado sanitario de las liebres ibéricas sigue siendo muy limitada. En el estudio realizado por Sánchez-García y cols. (2012) en esta especie, los procesos infecciosos fueron la segunda causa de mortalidad después de la depredación. Recientemente, se han confirmado casos de EHC asociados a la nueva variante (GI.2), tanto en liebre europea como en liebre ibérica en el noreste de España (Velarde y cols., 2016; Velarde y cols., 2021). Asimismo, en el verano de 2018, se detectó una elevada mortalidad asociada al MYXV recombinante de la liebre (ha-MYXV) en poblaciones de liebres ibéricas en varios cotos cinegéticos localizados en Andalucía (García-Bocanegra y cols., 2019). El número de brotes de mixomatosis en esta especie aumentó rápidamente afectando a la mayoría de las regiones de la Península Ibérica donde habita (RASVE, 2022).

2. Mixomatosis

2.1. Definición

La mixomatosis es una enfermedad infecciosa y altamente contagiosa, producida por el MYXV (género *Leporipoxvirus*, familia *Poxviridae*) (Fenner, 2000), que afecta principalmente al conejo europeo y, excepcionalmente, a otras especies del género *Lepus*. El MYXV se transmite principalmente mediante la picadura de vectores artrópodos hematófagos (pulgas y mosquitos) o por contacto directo. Clínicamente se caracteriza por la aparición de tumefacciones mesenquimatosas denominadas mixomas, localizadas principalmente en las regiones cefálica y ano-genital.

2.2. Etiología

El MYXV presenta forma de paralelepípedo y posee una membrana lipídica trilaminar, dos cuerpos laterales, un núcleo bicóncavo y un genoma de ADN de doble cadena y 250 nm de longitud. Además, está rodeado por una membrana externa formada por antígenos solubles de naturaleza fosfolipídica (Joubert y cols., 1972) (Figura 4). El virus presenta diferentes proteínas estructurales codificadas por genes que determinan la virulencia de las diferentes cepas. Entre ellas, destacan tres inhibidores de la proteinasa sérica (Serpins): la "Serp1" asociada a efectos antiinflamatorios, la "Serp2" relacionada con la virulencia, la respuesta antiinflamatoria y los efectos antiapoptósicos y la "Serp3" que representa un factor de virulencia del virus de forma sinérgico con las dos anteriores.

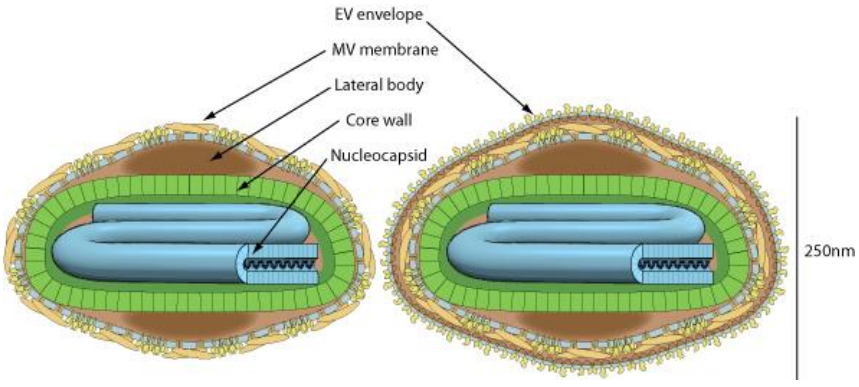

Figura 4.- Representación esquemática de la estructura del MYXV (**Fuente**: *Swiss Institute of Bioinformatics, 2014).*

2.3. Historia

La mixomatosis se describió por primera vez en 1896 en Uruguay por Guiseppe Sanarelli, en conejos europeos (Bertagnoli y Marchandeau, 2015). El MYXV circula de forma natural en los conejos de cola de algodón (*Sylvilagus* spp.) de Sudamérica, en los que la infección cursa generalmente de forma asintomática o con la aparición de fibromas cutáneos localizados. En 1950, el MYXV se empleó como herramienta de control biológico en Australia en un intento por disminuir las poblaciones de conejos silvestres, consideradas una plaga en este país. Al tratarse de una población no expuesta con anterioridad, el virus causó inicialmente una importante reducción de la población de conejos, alcanzando un descenso de las poblaciones cercano al 90% (Fenner y Ratcliffe, 1965). Sin embargo, a medida que fueron pasando los años, la atenuación de las cepas de MYXV y el desarrollo de inmunidad de las poblaciones de conejos, ocasionó un incremento en las densidades de conejos en las siguientes décadas.

En Europa, el virus se introdujo ilegalmente en Francia en 1952. El médico francés Armand Delille inoculó con MYXV y liberó intencionadamente dos conejos silvestres en su finca agrícola, con el fin de disminuir la densidad de la especie e intentar controlar los daños que producían estos animales en sus viñedos. El virus se diseminó rápidamente por Francia y por otros países europeos, estimándose un avance anual de 450 km (Kerr y Best, 1998). En 1953, llegó a Reino Unido originando disminuciones en las poblaciones silvestres similares a las acaecidas inicialmente en Australia. Los primeros casos de mixomatosis en España se detectaron durante los meses de septiembre y octubre de ese mismo año en la provincia de Gerona (Sánchez y cols., 1954), si bien, la aparición de la enfermedad epizoótica no tuvo lugar hasta septiembre de 1954. La mixomatosis causó cambios significativos en las poblaciones de conejos silvestres y una reducción sustancial en sus densidades, llegando al punto de extinción en algunas regiones de la Península Ibérica (Villafuerte y cols., 1994; Calvete, 2006). Sin embargo, la mortalidad fue disminuyendo progresivamente en las siguientes décadas debido a un aumento de la resistencia genética, al desarrollo de inmunidad adquirida y al contacto con una cepa del virus atenuada (Fenner y Fantini, 1999). Actualmente, la mixomatosis es endémica en la mayoría de los países europeos, incluido España, con brotes epizoóticos que pueden originar elevadas mortalidades en poblaciones susceptibles, principalmente durante los meses de verano y otoño (Villafuerte y cols., 2017; Rosell y cols., 2019).

2.4. Epidemiología

La mixomatosis afecta a los lagomorfos, es decir, conejos y liebres. El conejo americano (*Sylvilagus brasiliensis*) está considerado como el reservorio natural del virus (OIE, 2018). En esta especie, la infección cursa generalmente de forma asintomática, mientras que, en el conejo europeo, tanto doméstico como silvestre, produce la mixomatosis. Durante los brotes en conejo silvestre en Europa, también se han detectado esporádicamente casos clínicos de mixomatosis en liebre europea en diferentes países como Francia e Irlanda, y más recientemente en Gran Bretaña (Collins, 1955; Barlow y cols., 2014). En España, hasta la fecha, sólo se ha confirmado mixomatosis en la liebre ibérica (RASVE, 2022), pero no en liebre europea y tampoco en liebre de piornal, las otras dos especies de liebres presentes en nuestro país.

Aunque la mixomatosis afecta a conejos de cualquier edad, los gazapos de entre 40-60 días son los más susceptibles a la infección (Ross y Tittensor, 1986). Con respecto al sexo, se ha descrito una mayor presencia de signos clínicos en las hembras gestantes a término y recién paridas. No afecta al hombre (no es una zoonosis) y tampoco se ha detectado en otras especies animales.

El MYXV es un virus termolábil, se inactiva a 37°C durante una hora o a 55°C durante un minuto. Sin embargo, es muy resistente a la mayoría de los desinfectantes habituales, el frío y la congelación. Además, en condiciones adecuadas de humedad y temperatura, el MYXV puede permanecer viable durante más de 200 días en cadáveres de animales infectados. El virus se inactiva con cloroformo, éter y formol al 2% (Joubert y cols., 1972). Según la virulencia, las cepas de MYXV se clasifican en lentógenas (de baja virulencia), mesógenas (de virulencia intermedia) y velógenas (de elevada virulencia) (Cameron y cols., 1999).

La mixomatosis es una enfermedad estacional. Los casos aparecen principalmente durante los meses de primavera, verano y otoño, coincidiendo con la mayor presencia de vectores competentes (pulgas y mosquitos), así como con la mayor densidad de hospedadores susceptibles (mayor densidad de gazapos jóvenes) (Calvete y cols., 2002). Los brotes más graves están asociados a veranos húmedos con temperaturas suaves, ya que se favorece el desarrollo de los vectores y el virus puede permanecer activo durante un mayor periodo de tiempo.

La principal vía de contagio del MYXV es la percutánea por picadura de artrópodos vectores. En el caso de las liebres parece que las pulgas juegan un papel menor en la transmisión en comparación con los conejos, donde la especie *Xenopsylla cunicularis* actúa como principal vector biológico al ser la especie de pulga más frecuente en los conejos silvestres de España

(Ósacar-Jiménez y cols., 2001). Dentro de las vías de contacto indirecto, también se ha descrito transmisión de MYXV mediante fómites, comederos y bebederos, cadáveres y ropa (Merchant y cols., 2003). En las granjas en las que se practica inseminación artificial, se ha detectado infección por semen procedente de machos infectados (Sánchez-García y cols., 2019). También se ha descrito el contagio directo por contacto de un animal enfermo con uno sano, a través de secreciones nasales o conjuntivales eliminadas por los animales afectados, o con virus vehiculados en el aire por partículas de polvo o humedad, dando lugar a las formas atípicas de la mixomatosis.

2.5. Clínica y lesiones

La mixomatosis presenta dos formas clínicas: la forma clásica o nodular y la forma amixomatosa o respiratoria. A su vez, según el curso de la enfermedad, dentro de la forma clásica, se describen tres formas de evoluciones clínicas (Rosell, 2000):

1) Forma aguda: forma clínica asociada normalmente a cepas velógenas en poblaciones que no han tenido contacto previo con el virus, pudiendo llegar las tasas de morbilidad y mortalidad al 90%.

2) Forma subaguda: es la forma más común, confiriendo resistencia en algunas poblaciones de conejos y asociada a la circulación de cepas mesógenas.

3) Forma crónica: asociada a conejos adultos que han estado en contacto con cepas mesógenas o lentógenas del virus. Esta forma de presentación es también frecuente en las fases finales de los brotes epizoóticos. Muchos de los animales acaban recuperándose, quedando como portadores del virus.

Por otro lado, la forma atípica de la mixomatosis es una forma clínica que se caracteriza por presentar una sintomatología predominantemente respiratoria y ausencia de mixomas (Farsang y cols., 2003).

En conejos, una de las lesiones más frecuentes que podemos encontrar asociadas a la forma clásica son los nódulos cutáneos o tumefacciones mesenquimatosas denominadas mixomas, que aparecen generalmente en las zonas cefálica, auricular y ano-genital (Figura 5A). Otras lesiones frecuentemente observadas incluyen la blefaritis, blefaroconjuntivitis, rinitis y edema cefálico y ano-genital (Figura 5B). Al mismo tiempo, se presenta inmunosupresión que permite la aparición de infecciones bacterianas secundarias (Rosell, 2000).

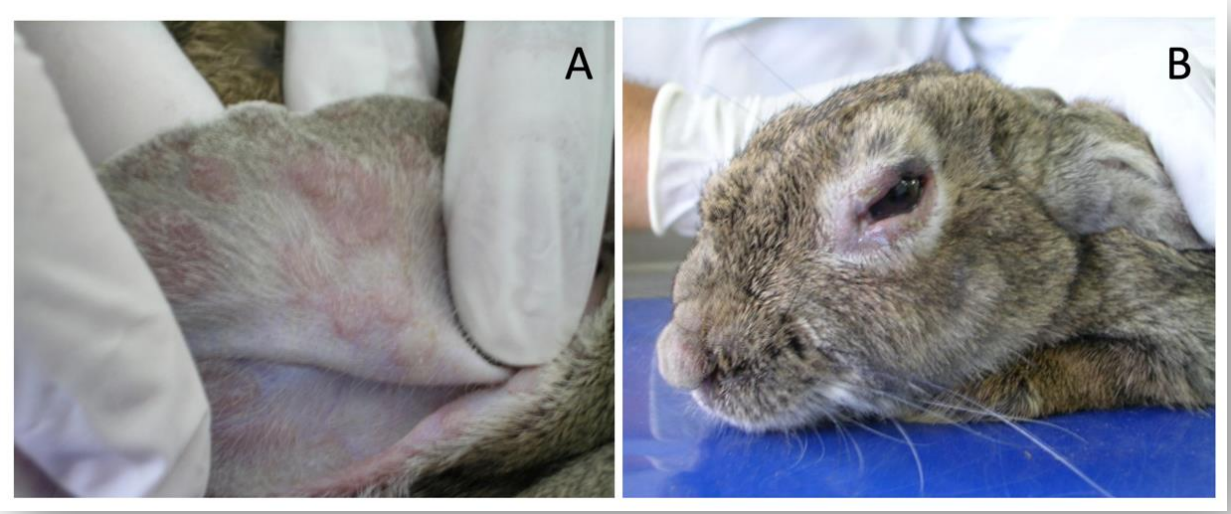

Figura 5.- Presencia de mixomas (Figura 5A), edema y blefaroconjuntivitis (Figura 5B) en conejo silvestre infectado por MYXV (Fuente: Ignacio García Bocanegra).

En la liebre ibérica, las lesiones encontradas con mayor frecuencia son: inflamación de los ojos y párpados (blefaroconjuntivitis) (Figura 6A), presencia de sangre en nariz (epistaxis) (Figura 6B), inflamación de la región de la boca y nariz (edema oronasal) e inflamación de la zona del ano y genitales (edema anogenital) (RASVE, 2022). Los principales signos clínicos asociados a mixomatosis en liebre ibérica incluyen: postración, pataleos, convulsiones y delgadez (caquexia) (Sánchez-García y cols., 2019).

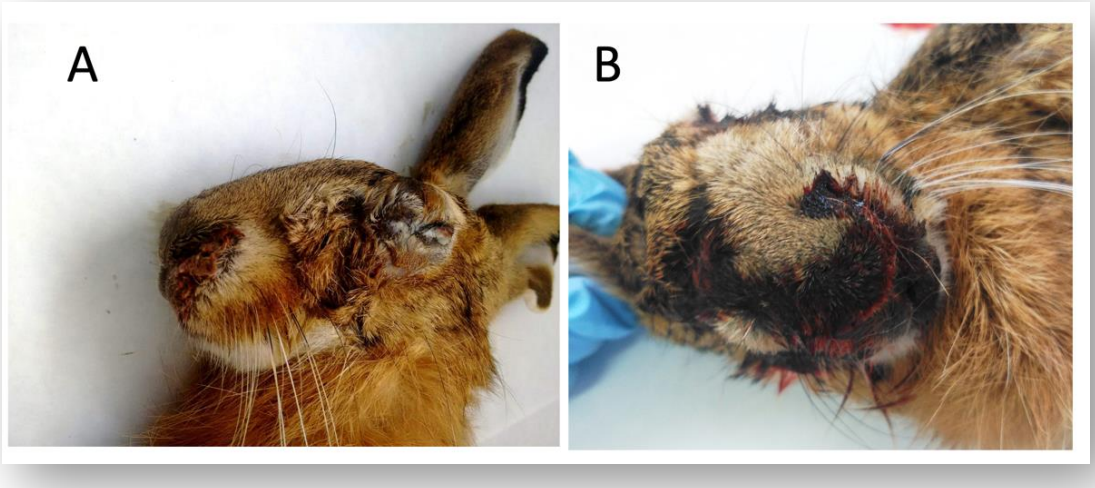

Figura 6.- Presencia de blefaroconjuntivitis (Figura 6A) y epistaxis (Figura 6B) en liebre ibérica infectada por MYXV (Fuente: Ignacio García Bocanegra).

2.6. Diagnóstico

Para el diagnóstico presuntivo de la mixomatosis se deben tener en cuenta factores relacionados con la época de aparición de casos, edad de los animales, antecedentes en la zona, densidad de vectores, así como los signos clínicos y lesiones observadas en los animales afectados. La presencia de lesiones y clínica compatible es muy orientativa, sobre todo en las formas clásicas de mixomatosis. Sin embargo, deberemos realizar un diagnóstico diferencial incluyendo otras enfermedades que tengan un curso similar, entre ellas, incluiremos la pasteurelosis del conejo, la EHC, blefaritis por *Staphylococcus aureus*, fibromatosis y toxemia por gestación. En cualquier caso, ante la sospecha de un caso de mixomatosis se recomienda confirmar la enfermedad realizando un diagnóstico de laboratorio, que puede ser directo o indirecto. La técnica más utilizada es la detección de ADN mediante PCR convencional o a tiempo real. La presencia de cuerpos de inclusión intracitoplasmáticos en queratinocitos mediante cortes histológicos y examen histopatológico puede reforzar el diagnóstico presuntivo (OIE, 2018). Por otro lado, el diagnóstico indirecto se basa en la detección de anticuerpos mediante técnicas inmunoenzimáticas (ELISA), inmunofluorescencia indirecta, seroneutralización o inmunodifusión en gel de agar (OIE, 2018).

2.7. Lucha

La mixomatosis es una enfermedad de declaración obligatoria (RASVE, 2022). No existe tratamiento específico, por lo que la lucha se basa principalmente en la profilaxis. Su erradicación es complicada debido a la presencia de reservorios silvestres y a la transmisión mediante diferentes especies de vectores artrópodos.

Entre las principales medidas de profilaxis sanitaria para evitar la entrada del virus en la explotación, así como su diseminación dentro de la granja o a otras explotaciones, destacan: eliminar los animales enfermos o sospechosos, así como los gazapos de las reproductoras sacrificadas, limpiar y desinfectar las jaulas donde hubo animales enfermos, realizar cuarentena de reposición, instalar redes protectoras contra insectos, realizar adecuados programas de desinsectación, desinfección y desratización, mantener una buena ventilación (evitar la acumulación de polvo) y controlar la entrada de personas, aves o roedores en la explotación (Rosell, 2000).

En animales silvestres, las medidas son mucho más complejas como en la mayoría de las enfermedades. Sin embargo, es importante evitar translocaciones y repoblaciones de liebres y conejos silvestres no controlados sanitariamente, limpiar y desinfectar todo el material y utensilios utilizados que hayan estado en contacto con los animales y en caso de encontrar animales

muertos por el virus, enterrarlos a suficiente profundidad y cubrirlos con cal viva, o bien introducirlos en una bolsa hermética y depositarla en un contenedor para SANDACH. Asimismo, la desinsectación de las entradas de las madrigueras empleando piretroides puede ser una medida útil en los cotos de caza.

La inmunoprofilaxis es la principal herramienta para el control de la mixomatosis en conejos domésticos. Sin embargo, existe controversia en cuanto a su uso en animales silvestres. Algunos autores han sugerido un efecto protector limitado debido a la inmunosupresión temporal asociada al estrés de los animales por la captura y manipulación (Marlier y cols., 2000; Cabezas y cols., 2006; Ferreira y cols., 2009). Sin embargo, otros autores han reportado una seroconversión postvacunación en conejos silvestres (Guitton y cols., 2008; Arenas y cols., 2012).

Actualmente existen en el mercado dos tipos de vacunas frente al MYXV, ambas son vacunas vivas atenuadas: las vacunas heterólogas, elaboradas con el virus del fibroma de Shope (agente causal de la fibromatosis) (Spibey y cols., 2012) y las vacunas homólogas, desarrolladas con cepas lentógenas (ej. Cepa SG33) de MYXV atenuadas mediante pases en cultivos celulares. También existe en el mercado una vacuna combinada que incluye MYXV y virus de la EHC (cepa AG88 inactivada).

En la liebre ibérica, los resultados preliminares obtenidos a partir de vacunas homólogas comerciales parecen apuntar a cierto grado de protección. A su vez, se está desarrollando una vacuna específica contra la nueva cepa (ha-MYXV) que afecta a la liebre ibérica (Crespo, 2021). Dado que no es factible en condiciones de campo vacunar a un porcentaje de la población suficientemente elevado como para controlar eficazmente la enfermedad, en el caso de obtenerse una vacuna efectiva frente al ha-MYXV sería una herramienta destinada a la inmunización de liebres capturadas para repoblaciones y traslocaciones. Por ello, la vacuna debería de ser combinada igualmente con medidas de gestión de los cotos (Sánchez-García y cols., 2019).

3. Enfermedad hemorrágica del conejo (EHC)

3.1. Definición

La EHC (del inglés RHD) es una enfermedad infecciosa altamente contagiosa producida por un calicivirus (familia *Caliciviridae*) del género *Lagovirus* que afecta a lagomorfos domésticos y silvestres y se caracterizada por presentar una rápida difusión con elevadas tasas de morbilidad y mortalidad (OIE, 2018b).

3.2. Historia

La EHC se describió por primera vez en la República Popular de China en 1984, en un lote de conejo de Angora importados desde Alemania (Liu y cols., 1984). El virus de la EHC (EHCV) llegó a Europa dos años después (sur de Italia) (Cancellotti y Renzi, 1991) y a España en el año 1988 (Argüello y cols., 1988). En los primeros años tras su detección en este país, el virus causó elevadas mortalidades en explotaciones de conejos domésticos, así como en poblaciones de conejos silvestres (Villafuerte y cols., 1995). Tras la primera detección en Italia en 1986, el EHCV se extendió al resto de Europa, llegando a ser endémico en diferentes países, incluido España. En la misma década se detectaron brotes de EHC en el norte de África. Asimismo, en 1988 se confirmaron los primeros casos de EHC en América, en concreto en México, a partir de la importación de carne infectada de conejos procedentes de China (House y cols., 1990). En Australia y Nueva Zelanda, donde el conejo se considera una especie plaga, el EHCV fue introducido deliberadamente por los agricultores en un intento de controlar las poblaciones de este lagomorfo (Cooke, 2002).

En Francia, en el año 2010, emergió un nuevo genotipo de EHCV (GI.2, también denominado, del inglés, anteriormente como RHDV2 o RHDVb) (Le Gall-Reculé y cols., 2011; 2013). En los años siguientes a su aparición, este genotipo emergente se expandió a otros países europeos, llegando a España en el 2011. El GI.2 presentó una expansión mucho más rápida que las cepas de EHCV clásicas, alcanzado otros continentes incluyendo Australia, África, América y Oceanía durante la última década (Rouco y cols., 2019).

3.3. Etiología

Inicialmente, el EHCV se clasificó dentro del denominado “complejo de la enfermedad hemorrágica de los lepóridos” junto al síndrome de la liebre parda europea (SLPE), un virus, detectado por primera vez en Suecia a principios de la década de 1980 antes del primer brote de EHCV, que afecta a diferentes especies de liebres (Le Gall-Reculé y cols., 2001), aunque también se

ha detectado en conejos del género *Sylvilagus,* los cuales actúan como fondo de saco epidemiológico, hospedadores accidentales o *spillover hosts* (OIE, 2020). A pesar de la estrecha relación genética (un 70% de similitud) y de causar signos clínicos y lesiones similares al EHCV, ambos virus se consideran especies diferentes (Wirblich et al., 1994; Lavazza et al., 1996). Así pues, la familia *Caliciviridae* incluye el género *Lagovirus,* que integra los dos calicivirus de los lagomorfos; el EHCV y el SLPEV (Le Gall y cols., 2001).

El EHCV es un virus ARN monocatenario y polaridad positiva de 7437 nuecleótidos. Es un virus desprovisto de envoltura, de morfología esférica con depresiones en la superficie en forma de cálices y un tamaño aproximado de entre 35-40 nm de diámetro. El material genético se encuentra en una nucleocápside icosaédrica compuesta por 32 capsómeros distribuidos en 3 icosaedros simétricos (Thouvenin y cols., 1997). Su genoma se compone de dos marcos de lectura abiertos (ORF, del inglés *Open Reading Frame*) ligeramente superpuestos: la ORF1, que comprende los nucleótidos 10 a 7044, origina la proteína estructural mayor VP60, de 257 KDa y con elevada capacidad inmunógena, así como otras proteínas no estructurales (muchas de ellas con función biológica desconocida); y la ORF2, que incluye los nucleótidos 7025 a 7378, origina la proteína estructural menor VP10, proteína reguladora de la diseminación viral, que incrementa los niveles de replicación y promueve la apoptosis (Pacho, 2018). Las frecuentes mutaciones de los calicivirus se relacionan con las elevadas tasas de error de sus polimerasas durante la replicación (Alda y cols., 2010) y con la existencia de una región hipervariable (denominada E) donde se localizan la mayor parte de las mutaciones (Capucci y cols., 1998; Oem y cols., 2009).

Diversos autores han postulado que el origen de los calicivirus patógenos podría deberse a la recombinación genética entre varios calicivirus no patógenos (Forrester y cols., 2006; McIntosh y cols., 2007; Abrantes y cols., 2008; Le Gall-Reculé y cols., 2013) o por un salto de especie a partir de un reservorio desconocido (Merchán y cols., 2011; Abrantes y cols., 2012). Además de las recombinaciones entre grupos genéticos (Forrester y cols., 2007), también se han observado entre diferentes especies víricas (Almeida y cols., 2015; Lopes y cols., 2017), lo que muestra la adaptabilidad y rápida capacidad de una evolución de estos virus (Pacho, 2018). Atendiendo a la clasificación propuesta recientemente por Le Pendu y cols. (2017), los EHCV se dividen en cuatro genotipos: genotipo GI.1, que comprende lagovirus patógenos previamente divididos en grupos filogenéticos GI.1a-GI.1d, los virus no patógenos relacionados con EHCV detectado inicialmente en Europa y posteriormente en Asia, Oceanía y America (OIE, 2018), que se clasifican en los genotipos GI.3 y GI.4, y el nuevo GI.2.

3.4. Epidemiología

El hospedador natural del EHCV es el conejo europeo, tanto doméstico como silvestre. Dependiendo de la cepa o genotipo de EHCV implicado, así como del estado inmunitario de la población afectada, se describen valores de morbilidad entre el 30 y el 100% y mortalidad entre el 40 y el 100%, oscilando entre el 5y el 70% en el caso del GI.2 en esta especie (OIE, 2018b; Villafuerte y cols., 1995). Las especies del género *Lepus* también pueden intervenir en la epidemiología de este virus, actuando como fondo de saco epidemiológico, hospedadores accidentales o *spillover hosts*. En este sentido, el GI.1 ha sido aislado en liebre ibérica en Portugal (Lopes y cols., 2014) mientras que el GI.2 ha sido detectado en diferentes especies del género *Lepus*, incluidas *Lepus capensis* (Puggioni y cols., 2013)*, L. corsicanus* (Camarda y cols., 2014)*, L. europaeus* (Hall y cols., 2016; Velarde y cols., 2016; Le Gall-Reculé y cols., 2017), *L. timidus* (Neimanis y cols., 2018) y *L. granatensis* (Lopes y cols., 2014; Velarde y cols., 2021). Aunque las liebres no parecen desempeñar un papel relevante en la epidemiología del la EHC, no se descarta un posible salto de especie en el futuro debido a la elevada capacidad de mutación/recombinación de los calicivirus (Velarde y cols., 2021). Por otro lado, aunque en estudios experimentales con EHCV no se detectado clínica ni replicación vírica en diferentes especies de mamíferos, incluidos roedores, rumiantes, équidos y primates (Galassi, 1991; Xu, 1991; Ohlinger y cols. 1993), sí se ha confirmado la presencia de anticuerpos frente a EHCV, así como eliminación de virus a través de las heces en roedores (*Mus spretus* y *Apodemus sylvaticus*) y perro y gatos domésticos, lo que sugiere un posible papel de estas especies en la epidemiología y mantenimiento de EHCV (Zhegn y cols., 2003; Merchán y cols., 2011; Rocha y cols., 2017).

La EHC es una enfermedad denso-dependiente. El virus se transmite de forma horizontal (Chasey y cols., 1994), por contacto directo con individuos infectados o con sus aerosoles, por vía oral, oro-nasal, conjuntival y parenteral (Rosell y cols., 1990; Abrantes y cols., 2012). La eliminación del virus se realiza mediante las secreciones/excreciones de individuos infectados (Pacho, 2018), por lo que tanto el animal vivo como ejemplares muertos por EHC pueden constituir fuentes de infección (Ohlinger y cols., 1993; Cooke y cols., 2000). En zonas de alta densidad de conejo donde la EHC es endémica, las madrigueras también juegan un papel importante en la epidemiología de la enfermedad debido al elevado número de conejos que mueren en su interior (Cooke, 2002). También se ha sugerido la transmisión indirecta mediante insectos hematófagos que actúan como vectores mecánicos, así como la transmisión por fómites, alimentos, agua o elementos de la cama y jaula contaminados (Abrantes y cols., 2012; OIE, 2018).

La principal vía de entrada del virus es la oral-nasal. También se ha reproducido experimentalmente la infección por EHCV por vía intravenosa, intramuscular, cutánea, subcutánea, intraperitoneal, intratorácica, heridas y laceraciones cutáneas (Capucci y cols., 1990; 1991; Galassi, 1991; Ohlinger y cols., 1993).

Entre los factores determinantes del EHCV relacionados con el hospedador, el virus y el medioambiente, podemos señalar:

- Edad: tiene una clara influencia en la presentación de la enfermedad. Aunque pueden resultar infectados conejos de cualquier edad, la infección por GI.1 es subclínica en los de menos de 6–8 semanas. Este genotipo afecta a animales mayores de 40-45 días de vida sin vacunar, siendo los más susceptibles aquellos con edades comprendidas entre 2,5 meses y 4,5 meses, seguidos por hembras reproductoras, animales mayores de 60 días y finalmente los animales menores de dos meses (García-Bocanegra, 2010). La aparente resistencia innata al EHCV en animales jóvenes, parece estar asociada a la inmunidad pasiva adquirida vía transplacentaria desde las madres seropositivas. Esta inmunidad puede persistir hasta las 13 semanas de vida, disminuyendo a lo largo del tiempo (Cooke y cols., 2000). Por el contrario, el GI.2 puede afectar a animales menores de 30 días (Dalton y cols., 2012), llegando incluso a infectar a gazapos de 11 días de vida (Dalton y cols., 2014).
- Estado inmunitario: factores como el estrés, parasitaciones (coccidios o criptosporidios), infecciones bacterianas (pasterelosis) o la inmunosupresión causada por una infección previa de mixomatosis (Jeklova y cols., 2008) pueden predisponer al desarrollo clínico de la EHC (Marlier y cols., 2000b; Marchandeau y cols., 2004). La inmunidad adquirida vía materna favorece el mantenimiento de títulos inicialmente altos de anticuerpos que disminuyen con el tiempo. Sin embargo, en poblaciones silvestres localizadas en zonas endémicas de EHCV, estos niveles se mantienen elevados a lo largo de la vida del individuo debido a la exposición y reexposición al virus (Cooke y cols., 2000).
- Sexo: la mayoría de los investigadores coinciden en que el sexo no es un factor de riesgo. Sin embargo, las hembras presentan prevalencias marginalmente mayores que los machos (Villafuerte y cols., 1994; O'Keefe y cols., 1999), lo que podría estar asociado, en el caso de conejos silvestres, por una mayor utilización de las madrigueras por parte de las hembras.

- Resistencia del virus: el EHCV resiste bien las bajas temperaturas, manteniéndose viable durante 225 días a 4°C. Sin embargo, es sensible a elevadas temperaturas, inactivándose a 37°C durante 4 horas ó 50°C durante 1 hora. Con respecto a los agentes químicos, el EHCV es resistente al éter y el cloroformo, el pH ácido, mientras que el tratamiento con formaldehído al 0'4% a temperatura ambiente elimina su infectividad, pero mantiene su inmunogenicidad (Xu y cols., 1988). También se inactiva con hidróxido sódico al 1%.
- Virulencia: se ha demostrado que la circulación de cepas no patógenas de EHCV es otro factor que puede favorece la inmunización de las poblaciones de conejos silvestres (White y cols., 2001).
- Estacionalidad: la EHC presenta un carácter estacional concentrándose los brotes durante los meses de invierno y primavera, disminuyendo en la época estival (Marchandeau y cols., 1998; Calvete y cols., 2002). En España, Calvete y cols (2000, 2002) observaron el pico de mayor mortalidad durante la segunda mitad de la época reproductiva, coincidiendo con la mayor proporción de hembras gestantes y con un mayor uso de las madrigueras por parte de los individuos jóvenes y subadultos.
- Vectores: algunos autores relacionan el incremento de casos de EHC con el aumento de las densidades de vectores (probablemente *Spilopsyllus cuniculi*) coincidiendo, igualmente, con la época de cría del conejo (Lugton, 1999). En este sentido, Lenghaus y cols. (1994) determinaron que las pulgas *Spylopyllus cuniculi* y *Xenopsylla cunicularis,* y los mosquitos *Culex annulrostris*, son capaces de transmitir el EHCV en condiciones de laboratorio. A pesar de ello, la mayoría de los autores coinciden que los insectos no juegan un papel relevante en la transmisión del EHCV (Calvete y cols., 2002).

3.5. Patogenia

El EHCV es un virus pantotropo que infecta las células del sistema monocito-macrófago, replicándose inicialmente en el núcleo de las células infectadas y atravesando la membrana nuclear para madurar en el citoplasma en fases posteriores de la infección. Tras la entrada del EHCV en un organismo, el virus llega al hígado vía hemática, donde tiene lugar una primera replicación durante las primeras 12 horas post infección (hpi) (Prieto y col., 2000). La replicación del virus tiene lugar en el citoplasma de los hepatocitos durante las 36-48 hpi (Pacho, 2018). Tras un periodo de incubación de entre 24 y 72 hpi, aparece la fase de viremia, presentando un marcado tropismo por el endotelio vascular y reticular de este y otros órganos parenquimatosos (Gelmetti y cols., 1998). Como consecuencia, aparecen lesiones a nivel endotelial y se produce lisis

celular que causan un cuadro de coagulación intravascular diseminada (CID). La CID se desencadena por el desequilibrio entre producción y eliminación de los factores de coagulación, con un aumento de trombina, síntesis de fibrina, una mayor agregación plaquetaria disminuyendo su recuento en sangre, aumentando la trombosis (Ferreira y cols., 2006). Estos trombos contribuyen al daño endotelial con la consecuente producción de hemorragias (Teifke y cols., 2002). Tras la replicación en el citoplasma de los hepatocitos, llega por vía sanguínea a otros órganos parenquimatosos como bazo, riñón o pulmón (Prieto y cols., 2000; Kerr y Donnelly, 2013) provocando una disfunción sistémica generalizada y desencadenando un desequilibrio electrolítico (Chen y cols., 2008). Posteriormente el virus se disemina por el organismo, alcanzando tejidos como linfonodos, intestino, timo, tejido cardíaco y sistema nervioso central (Ramiro-Ibáñez y cols., 1999; van de Bildt y cols., 2006). Finalmente, las alteraciones de todos estos órganos propician un fallo multiorgánico y, con frecuencia, la muerte del animal (Pacho, 2018).

3.6. Clínica y lesiones

Dependiendo de la evolución de la EHC, se describen a varias formas clínicas de presentación. La forma sobreaguda y aguda son las formas clínicas más frecuentes (>90% de los casos) asociadas al GI.1, mientras que las formas subagudas y crónicas tienen tasas de presentación del 5-10% en las infecciones por este genotipo (Teifke y cols., 2002). Sin embargo, el GI.2 presenta un curso más largo (3-5 días), frecuentemente de tipo subagudo o crónico (OIE, 2018b).

En la forma sobreaguda los animales infectados prácticamente no manifiestan signos clínicos muriendo de manera repentina, en ocasiones precedida de convulsiones y opistótonos a las 12-36 hpi (Tesouro-Vallejo y cols., 1990). En la forma aguda, los animales comienzan con un aumento de temperatura entre 40°C y 41,5°C, disminuyendo posteriormente hasta llegar a 32,5°C, momentos antes de la muerte (Ohlinger y cols., 1993; Du, 1990). En esta forma clínica, los animales experimentan signos nerviosos inespecíficos como ataxia, incoordinación, parálisis del tercio posterior, convulsiones, opistótonos y disnea. La muerte suele producirse a las 48-72 hpi (Pacho, 2018; Abrantes y cols., 2012). La forma subaguda presenta una sintomatología similar a la aguda, aunque los menos acusada y el porcentaje de conejos que sobrevive al contacto con el virus y se recuperan, es mayor (Pacho, 2018; García-Bocanegra, 2005). Los animales afectados presentan leucopenia, hipertermia, depresión y anorexia durante 2-3 días (Gelmetti y cols., 1998). En la forma crónica, los animales apenas manifiestan signos clínicos presentando un curso de 1-2

semanas. Tras este período, la mayor parte de los animales seroconvierten y sobreviven al curso de la enfermedad (Abrantes y cols., 2012).

En los cadáveres se observa sangre y/o espuma sanguinolenta sin coagular en orificios nasales (Figura 7A), consecuencia de la congestión y el edema a nivel pulmonar (Ohlinger y cols., 1993). En ocasiones puede observarse ictericia alrededor del pabellón auricular, mucosas externas y tejido subcutáneo (Rosell y cols., 1990). Internamente, es característica la presencia de lesiones hemorrágicas de tipo petequial, equimosis, hemorrágicas y congestión en los diferentes órganos (Lucidi, 1991).

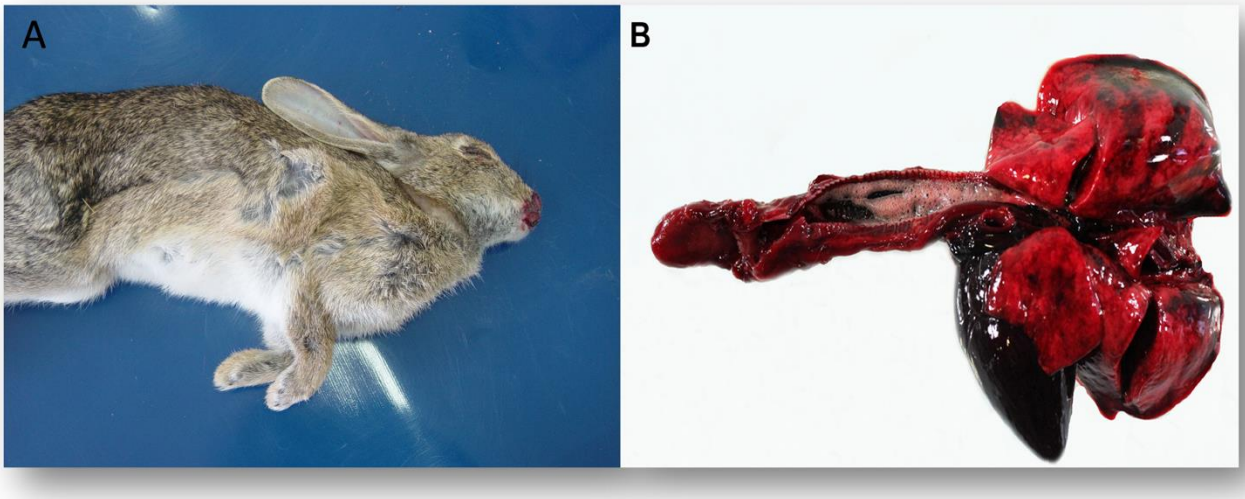

Figura 7.- Presencia de epistaxis (Figura 7A) y congestión, edema y hemorragia pulmonar (Figura 7B) en conejo silvestre infectado por EHCV (Fuente: Ignacio García Bocanegra).

El hígado es el órgano más afectado por el EHCV. Macroscópicamente, este órgano presenta hepatomegalia, con un aumento de coloración por la congestión y un marcado patrón lobulillar en infecciones sobreagudas (Tesouro-Vallejo y cols., 1990). En la forma aguda, se observa un órgano pálido y friable (Kimura y cols., 2001). Las células hepáticas muestran diferentes fases de daño celular, siendo frecuente la aparición de picnosis, cariorrexis, cariolisis y vacuolas (Teifke y cols., 2002; Abrantes y cols., 2012; Duarte y cols., 2015). Los pulmones se encuentran edematosos y congestivos (Figura 7B) (Prieto y cols., 2000; OIE, 2018b) con áreas de hepatización roja en las formas clínicas agudas y contenido espumoso y sanguinolento al corte además de petequias y hemorragias de extensión variable en la superficie del órgano dependiendo de si la clínica es sobreaguda, aguda y/o subaguda (Calvete y cols., 2002). Estas hemorragias pueden ser intraalveolares o perivasculares, observándose igualmente casos de trombosis (Kerr y Donnelly,

2013). En tráquea, las lesiones más comunes incluyen sufusiones hemorrágicas en forma de sábana con áreas congestivas e hiperémicas en mucosa y exudado sanguinolento (Lee y Park, 1987; Kerr y Donnelly, 2013). El bazo se presenta aumentado de tamaño (esplenomegalia), con punteado rojizo oscuro, hiperemia, cariorrexis ocasional, depósitos de hemosiderina y leucopenia (Abrantes y cols., 2012). En riñones también puede observarse el aumento de tamaño, hiperemia y hemorragias a nivel glomerular y medular, trombosis, dilatación de túbulos, infiltración linfocítica y degeneración del epitelio tubular (Abrantes y cols., 2012), mientras que, a nivel cardíaco, es frecuente la presencia de sangre coagulada a nivel auricular (Kerr y Donnelly, 2013).

Las alteraciones producidas por el GI.2 son similares a las de las cepas clásicas, siendo también la CID, la lesión más frecuente asociadas a este genotipo. Sin embargo, en la infección por GI.2 en conejo, es característica la presencia del virus en el intestino (Dalton y cols., 2012). El hígado se observa pálido o congestivo con focos de necrosis focal o difusa (Duarte y cols., 2015). El bazo está aumentado de tamaño, congestivo y friable. Los riñones presentan un aspecto congestivo con aumento de la coloración. Los pulmones también presentan congestión, con presencia de petequias o hemorragias que pueden ocupar lóbulos completos, así como edema alveolar y hemorragias (Pacho, 2018).

3.7. Diagnóstico

El diagnóstico presuntivo ante una sospeche de EHC se establece en base a la información epidemiológica, clínica y lesional. Para el diagnóstico diferencial se deben de tener en cuenta otras patologías que se manifiestan con alteraciones vasculares y que cursan de forma sobreaguda o aguda, como la septicemia hemorrágica causada por *Pasteurella multocida* que afecta a conejos y liebres, SLPE en el caso de las liebres, así como aquellas de origen no infeccioso (intoxicaciones, toxemia de gestación o golpes de calor, entre otros) (Pacho, 2018). Esta primera aproximación puede ser útil y valiosa, pero para determinar el agente etiológico debe realizarse un diagnóstico laboratorial específico. Entre los órganos empleados para el diagnóstico directo de la EHC, se incluyen: hígado y bazo como principales órganos diana, así como pulmón y riñón (Abrantes y Lopes, 2021).

Existen diversas técnicas para la detección de EHCV. Entre las técnicas directas empleadas para la detección de EHCV, podemos señalar las siguientes:

- Microscopía electrónica de tinción negativa: fue de las primeras técnicas utilizadas, aunque presenta una limitada sensibilidad determinada por la concentración de partículas víricas en el tejido analizado (Capucci y cols., 1991; Ferreira y cols., 2005).

- Hemoaglutinación (HA): se basa en la capacidad de hemoaglutinar en presencia de eritrocitos de algunas especies (Capucci y cols., 1991; Parra y Prieto, 1990), aunque en la actualidad su uso es menos frecuente debido a la existencia de técnicas más sencillas, rápidas y específicas. Además, se han identificado variantes de EHCV sin capacidad hemoaglutinante (OIE, 2018).
- ELISA tipo sandwich: es una de las técnicas más empleadas para la detección del EHCV y se han descrito variaciones de la misma (OIE, 2018).
- RT-PCR: se han desarrollado diferentes RT-PCT a tiempo real y a tiempo final, que permite la identificación y diferenciación de los distintos genotipos de EHCV (Nowotny y cols., 1997; Capucci y cols. 1998, Strive y cols., 2009; Le Gall-Reculé y cols., 2013). Esta técnica se ha utilizado además para realizar estudios filogenéticos y generar hipótesis sobre el origen de los lagovirus (Abrantes y cols., 2008; Kerr y cols., 2009; Lopes y cols., 2015; Abrantes y cols., 2020). Por su elevada sensibilidad y especificidad es la técnica más utilizada actualmente para el diagnóstico de la EHC.

Además de las técnicas descritas anteriormente, se han desarrollado otros métodos de diagnóstico directos para la detección del EHCV, entre ellos: inmunotinción, inmunotransferencia, inoculación en conejos, inmunihistoquímica, inbridación *in situ*, inmunofluorescencia directa, Western Blot, test de coagulación asociado a la proteína A purificada de *Staphylococcus* (Sp A COAT), o inmunocromatografía (*Lateral Flow Immunoassay*), entre otros (OIE, 2018; Stoercklé-Berger y cols., 1992; Peshev y cols., 1996 ;Neimanis y cols., 2018; Abrantes y Lopes, 2021)

Dado que la respuesta humoral tiene gran importancia para la protección de los animales frente a la EHC, el título de anticuerpos específicos asociado a una infección natural o tras la vacunación es un factor predictivo de la capacidad de los conejos de resistir la infección por EHCV. Actualmente, existen diferentes técnicas indirectas para detectar exposición o vacunación frente a este virus, destacando la inhibición de la hemoaglutinación (IHA) y las técnicas ELISAs (indirecto y de competición) como las más utilizadas. La IAH presenta una buena sensibilidad, especificidad, seguridad y bajo coste. Sin embargo, debido a la mayor sensibilidad, especificidad, rapidez y facilidad de realización, las técnicas ELISA son los métodos de diagnóstico indirectos más empleados en la mayoría de estudios en los que se trabaja con un elevado número de muestras. Además, los ELISAs permiten identificar anticuerpos frente al EHCV, así como anticuerpos frente a otros lagovirus no-patógenos (Capucci y cols., 1996). Asimismo, también se has desarrollado ELISAs para diferenciar anticuerpos frente a GI.1 y frente a GI.2. Por otro lado, la cuantificación

mediante ELISA de las inmunoglobulinas de isotipo específico del EHCV (IgM, IgA e IgG), ayuda a distinguir entre primo-infecciones, reinfecciones o anticuerpos vacunales (OIE, 2018).

3.8. Lucha

Al igual que la mixomatosis, la EHC es una enfermedad de declaración obligatoria (RASVE, 2022b). No existe tratamiento etiológico, por lo que la lucha se basa principalmente en aplicar las medidas de profilaxis adecuadas. La mayor parte de las medidas de profilaxis sanitaria son iguales a las descritas para la mixomatosis y hacen referencia a conejos de producción o de compañía (Osácar, 1999; Rosell, 2000). Sin embargo, las opciones de intervención en conejo silvestre continúan siendo muy limitadas en la actualidad y se centran en la aplicación de períodos de cuarentena en los animales capturados que se destinan a repoblaciones o translocaciones, limpieza y desinfección del material empleado para la captura y transporte de los animales y la eliminación de cadáveres (Osácar, 1999). La inmunoprofilaxis es una de las principales medidas de lucha en conejo doméstico, si bien en las poblaciones de conejos silvestre, las campañas de vacunación son económica y logísticamente difíciles de implementar y sus efectos se consideran insignificantes a gran escala (Abrantes y cols., 2012), siendo una medida habitualmente limitada a aquellos animales destinados repoblaciones o translocaciones (Arenas y cols., 2006). Actualmente, existen en el mercado vacunas bivalentes que permiten inmunizar frente al EHCV y al MYXV (Pacho, 2018). Estas vacunas incluyen una cepa de MYXV atenuado (cepa 009 o SG33) y expresan la proteína de la cápside del EHCV (VP60) (Rosell y cols., 2000; Boga y cols., 1997). También se han comercializado vacunas inactivadas comerciales frente a G.1 y frente a GI.2. La pauta vacunal frente a estas vacunas suele incluir una primera dosis a partir de las 5 semanas de edad, revacunación a las 6 semanas de primovacunación y dosis de recuerdo cada 6-12 meses dependiendo del producto comercial. Una vez vacunados, los anticuerpos son detectables a partir del día 5-7 postvacunación (Kim y cols., 1989; Hai-bo y cols., 1991).

4. Vigilancia epidemiológica

4.1. Aspectos generales de la vigilancia epidemiológica

La vigilancia epidemiológica es el conjunto de actividades dirigidas a la recopilación, análisis sistemático y continuo de información que posibilitan la monitorización permanente del estatus sanitario de una población y definen los factores de riesgo a los que está expuesta (OIE, 2017). Se trata de un componente fundamental de los programas sanitarios, que permite detectar una enfermedad, estudiando su desarrollo en el tiempo y espacio, con el objetivo de tomar las medidas de lucha correspondientes, en su caso. Atendiendo a la metodología de recogida de información, podemos diferenciar dos tipos de vigilancia epidemiológica:

Vigilancia pasiva: cuyo principal objetivo se basa en recopilar y analizar la información de una manera rutinaria o de forma eventual obtenida, en ocasiones, para otro propósito (Rodríguez, 2014). Este tipo de vigilancia es esencial para determinar de forma precoz las posibles causas de brotes de enfermedad y su potencial impacto en las poblaciones humanas y animales (Mörner, y cols., 2002). Además, la vigilancia pasiva permite detectar de forma precoz la presencia de patógenos emergentes.

Vigilancia activa: incluye las acciones dirigidas a buscar y cuantificar la presencia de un patógeno de manera deliberada (Hoinville y cols., 2013). En este tipo de vigilancia epidemiológica, el personal involucrado busca activamente información sobre la enfermedad que es objeto de investigación. La vigilancia activa permite medir indicadores epidemiológicos de un determinado patógeno (prevalencia, incidencia, factores de riesgo, entre otros), en que las muestras deben tomarse sobre la base de un plan estadístico o probabilístico. Para realizar una vigilancia activa óptima se requiere de un diseño del muestreo mediante métodos estadísticos adecuados para poder establecer el tipo y la cantidad de muestras a tomar con el objeto de que estos muestreos permitan realizar inferencia estadística y sean representativos de la población objeto de estudio. Aunque ambos tipos de vigilancia son necesarios para poder reflejar con veracidad el estatus sanitario de una población, las estimaciones y los análisis epidemiológicos estadísticos estándares pueden aplicarse más eficazmente a los datos de este sistema de vigilancia que en el caso de la vigilancia pasiva.

4.2. Vigilancia epidemiológica en la fauna silvestre

La Organización Mundial en Sanidad Animal (OIE), en el capítulo 1.4 del Código Sanitario de los Animales Terrestres, define la vigilancia epidemiológica de la fauna silvestre como la recogida

sistemática y continua de la información relacionada con la sanidad en las especies silvestres, así como el análisis, gestión y comunicación de la información generada dirigida a la toma de decisiones y el desarrollo de propuestas de medidas para el control de las enfermedades transmisibles que afectan a estas especies (OIE, 2022). Asimismo, la OIE señala que la vigilancia sanitaria en especies silvestres comprende varios componentes básicos, incluida la detección e identificación de enfermedades/patógenos, el análisis y comunicación (que requiere la implicación de epidemiólogos, biólogos y ecólogos); y la gestión de la información (recolección de metadatos de casos/muestras, observación de brotes de mortalidad o enfermedad en las poblaciones silvestres y el envío de datos a los sistemas de notificación correspondientes) (OIE, 2015).

La vigilancia epidemiológica en fauna silvestre permite identificar a las especies silvestres en su hábitat natural y establecer como se distribuyen los patógenos y cuáles son las causas de enfermedad o muerte en estas especies. Los programas de vigilancia epidemiológica en fauna silvestre se caracterizan por incluir un amplio conjunto de especies animales, así como analizar diferentes patógenos, es decir, no se limita a una o unas pocas especies ni a uno o unos pocos patógenos como suele ser habitual en los programas de vigilancia epidemiológicos en animales domésticos o en el ser humano. Los principales objetivos de la vigilancia epidemiológica en fauna silvestre son:

- Determinar qué patógenos y enfermedades están presentes en las especies silvestres de un país, cuáles son sus especies hospedadoras y cuál es su distribución geográfica, incluyendo aquellos patógenos y enfermedades relevantes para los animales domésticos, la salud pública o las propias poblaciones de animales silvestres.
- Detectar, de forma precoz, la presencia de nuevos patógenos y enfermedades o episodios epidemiológicos inusuales que puedan indicar la existencia de una enfermedad emergente.
- Detectar variaciones espacio-temporales en la dinámica de las enfermedades.

4.3. Principales limitaciones de la vigilancia epidemiológica en fauna silvestre.

Existente diferentes factores que dificultan la aplicación de programas de vigilancia epidemiológica en las especies silvestres. Con respecto a los factores biológicos asociados a la fauna silvestre, cabe señalar que, con frecuencia, el acceso a estas especies se ve limitado por diversas características etológicas y ambientales (Giovannini, 2006; Warns-Petit y cols., 2009). Estas circunstancias limitan habitualmente la detección temprana de los posibles brotes de una enfermedad. Por otro lado, el comportamiento huidizo de la mayoría de los animales silvestres,

dificulta considerablemente su captura, la toma de muestras biológicas, así como su posterior recaptura para poder establecer valores de incidencia de enfermedades en las poblaciones silvestres (Fischer y Gerhold, 2003). Además, en el caso concreto de las especies migratorias, los programas de vigilancia pueden verse limitados debido a la posible rapidez en la dispersión de determinados patógenos más allá de las fronteras geográficas, así como por la dificultad de un diseño adecuado de los muestreos.

Otras dificultades inherentes a la vigilancia en fauna silvestre son la toma de muestras en sí, así como su conservación y envío al laboratorio. Estos procesos presentan inconvenientes asociados a la alteración de la muestra desde la recogida hasta el análisis, factor que puede condicionar la validez de los resultados de laboratorio (Boadella y Gortázar, 2011; Arenas-Montes y cols., 2013, Jiménez-Ruiz y cols., 2016). Además, las situaciones de estrés a las que se ven expuestos los animales silvestres durante la captura y manipulación, pueden determinar alteraciones importantes es su estado fisiológico, afectando a los resultados de las pruebas de diagnóstico e incluso ocasionando la muerte de los ejemplares muestreados (Thorne y cols., 2000). También cabe señalar que, aunque las herramientas de diagnóstico directo suelen presentar una sencisbilidad y especificidad similar tanto para las especies domésticas como para la mayor parte de las silvestres (Fischer y Gerhold, 2002), el empleo de técnicas indirectas en las especies domésticas debe estar precedida de una validación de dichas técnicas previa a su utilización en especies silvestres (Boadella y Gortázar, 2011; Stallknecht, 2007). En este sentido, la gran variedad taxonómica de especies silvestres existentes, condicionan con frecuencia la validación de estas técnicas de diagnóstico.

Finalmente, otro factor limitante a tener en cuenta en los programas de vigilancia epidemiológicos en especies silvestres es el asociado al diseño del muestreo, ya sea dirigido a la obtención de prevalencias de enfermedades o a la determinación de factores de riesgo asociados a su transmisión. Habitualmente los diseños para el cálculo del tamaño de muestras en la fauna silvestre suelen realizarse a través de muestreos de conveniencia, lo que implica un sesgo inherente dadas las variaciones en el tamaño de muestra o las variaciones espaciales. Además, las limitaciones en la amplitud del muestreo debidas a razones logísticas o económicas, dificulta la detección de patrones epidemiológicos espaciales entre las poblaciones silvestres (Barroso y cols., 2020).

4.4. Evolución de la vigilancia sanitaria en la fauna silvestre en Europa.

Los primeros sistemas de vigilancia epidemiológica en fauna silvestre datan de mediados del siglo pasado (Mörner y cols., 2002). Sin embargo, no fue hasta principios de los 90 cuando se incrementó el interés por el conocimiento del estado sanitario de la fauna silvestre en Europa (Cardoso y cols., 2021). En las últimas décadas, se han llevado a cabo importantes avances en el sistema de notificación y recopilación de información relacionados con la sanidad en las especies silvestres, gracias en gran medida, a la implementación del Sistema Mundial de Información Zoosanitaria (WAHIS, del inglés World Animal Health Information System) (OIE, 2021b).

Actualmente, la monitorización de enfermedades relevantes en las especies silvestres está considerada como un factor determinante de la estructura y función de los sistemas de vigilancia. Sin embargo, los sistemas de vigilancia en estas especies no son homogéneos a nivel europeo (Lawson y cols., 2021), estando a menudo, restringidos a ciertos patógenos, regiones y/o poblaciones concretas (Kuiken y cols., 2011). No obstante, se han realizado esfuerzos para desarrollar programas de vigilancia en diferentes países europeos y, en la actualidad, es un componente de los sistemas generales de vigilancia de sanidad animal en muchos de estos países (Cardoso y cols., 2021). En este sentido, la OIE ha ido desarrollando un conjunto de orientaciones sobre cómo debe llevarse a cabo la vigilancia de las enfermedades de especies silvestres (OIE, 2010; 2015; 2021).

Ante la evidencia cada vez mayor del papel epidemiológico de la fauna silvestre en el mantenimiento de patógenos transmisibles, la vigilancia sanitaria de estas especies se ha convertido en un aspecto clave, tal y como lo destaca el enfoque de Una Sola Salud (One Health) respaldado por la OMS, la OIE y la FAO (FAO, 2010). Aquellos los países que cuentan con sistemas de vigilancia de enfermedades en especies silvestres se encuentran más capacitados para detectar la presencia de enfermedades infecciosas y adoptar medidas de lucha con mayor antelación y eficacia (Mörner y cols., 2002; OIE, 2021a). En las últimas décadas, diferentes países europeos como Reino Unido, Italia, España, Francia, Suiza, Portugal, Noruega y Finlandia han ido implantando y desarrollando programas de vigilancia epidemiológicos activa y pasiva en especies silvestres. Sin embargo, todavía existe en Europa la necesidad de impulsar la vigilancia de la fauna silvestre a nivel internacional y desarrollar actividades dirigidas que puedan ser puestas en marcha y coordinadas entre los países miembros con facilidad y eficacia (Machalaba y cols., 2021).

4.5. Programas de vigilancia sanitaria para la fauna silvestre en España.

En España, el interés por la vigilancia de las enfermedades que afectan a la fauna silvestre surgió en la década de los 80. Sin embargo, no fue hasta el año 2013 cuando se establece el Programa de Vigilancia Sanitaria en Fauna Silvestre (PVSFS). Este programa de ámbito nacional, contempla la vigilancia tanto activa como pasiva de 21 enfermedades que afectan a diferentes grupos taxonómicos incluidos jabalí, cérvidos, bóvidos, lagomorfos, carnívoros y aves silvestres (MAPA, 2022b).

El PVSFS tiene como objetivos conocer la situación sanitaria de la fauna silvestre en España, prevenir la difusión de enfermedades entre animales domésticos y silvestres y proteger la salud pública. Este programa se enlaza con los programas de organismos internacionales como la OIE o el Centro Europeo para la Prevención y Control de Enfermedades (ECDC), con los programas nacionales del Ministerio de Agricultura, Pesca y Alimentación (MAPA) y con los Planes Regionales de las distintas Comunidades Autónomas, así como con los programas propios que puedan desarrollar otras entidades tales como centros de investigación, universidades, centros de recuperación y ONGs, entre otros. Las autoridades competentes de las Comunidades Autónomas implicadas son las encargadas de la ejecución del PVSFS y los resultados obtenidos se incorporan periódicamente en el informe denominado “Informe sobre resultados del Programa Nacional de Vigilancia en Fauna Silvestre” (MAPA, 2022b).

Todas las actividades de vigilancia activa del PVSFS contemplan muestreos aleatorios en base a una prevalencia específica para cada taxón y enfermedad. Así, el PVSFS incluye los siguientes grupos de especies y enfermedades:

- Jabalíes: infecciones por *B. suis*, tuberculosis animal y triquinelosis.
- Cérvidos: Encefalopatías espongiformes trasmisibles, *Brucella abortus* y *B. mellitensis*, y tuberculosis animal.
- Bovinos: Infecciones por Pestivirus (específicamente para las poblaciones de rebeco), *Brucella abortus* y *B. mellitensis* y sarna sarcóptica.
- Lagomorfos y roedores: tularemia en la liebre y en micrótidos.
- Carnívoros: moquillo, rabia, así como tuberculosis animal en el tejón, y equinococosis/hidatidosis y sarna sarcóptica.
- Aves silvestres: enfermedad de West Nile e influenza aviar altamente patógena.

Asimismo, el tamaño de muestra se distribuye en función de la abundancia de las diferentes especies incluidas en el PVSFS en las seis bioregiones (BRs) en las que se divide el territorio nacional recogidas en este programa (Figura 8). Estas BRs se distribuyen en base a las características bioclimáticas y de distribución y abundancia de la fauna silvestre (MAPA, 2022).

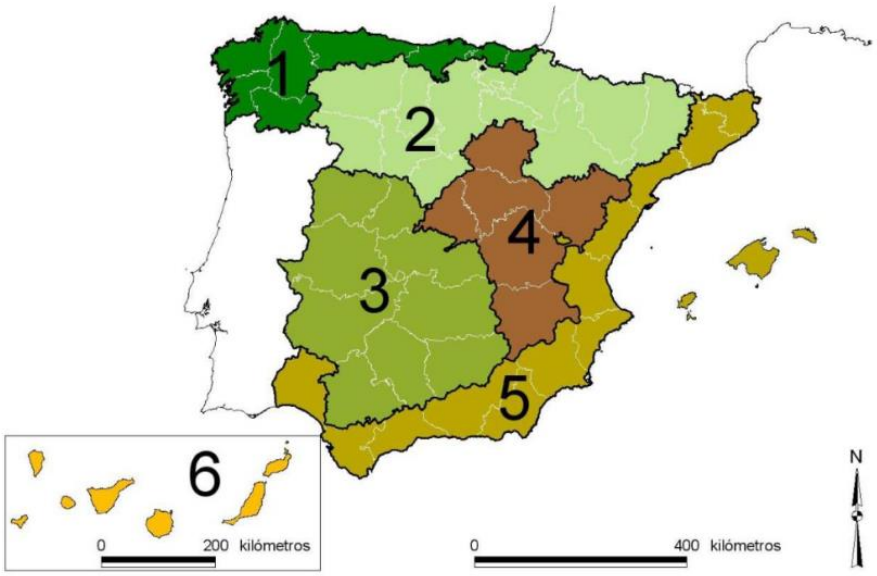

Figura 8.- Principales unidades de muestreo de fauna silvestre en el marco del Plan de Vigilancia Sanitaria (Fuente: MAPA, 2022a).

4.6. Programa de vigilancia epidemiológica de la fauna silvestre en Andalucía.

Andalucía se encuentra entre las Comunidades Autónomas pioneras en la aplicación de los programas de vigilancia epidemiológica de la fauna silvestre. En el año 2009, cuatro años antes de la implantación del PVSFS a nivel nacional, se puso en marcha el Programa de Vigilancia de la Fauna Silvestre de Andalucía (PVE), en base a los establecido en el artículo 7.2 del Decreto 182/2005 del 26 de julio, por el que se aprueba el Reglamento de ordenación de la caza, y en el artículo 16.1 de la Ley 8/2003, del 28 de octubre, de la Flora y la Fauna Silvestres, que especifica la necesidad de instauración de un Programa de Vigilancia Epidemiológica en Andalucía para detectar la aparición de enfermedades y evaluar su evolución con el fin de establecer, con las Consejerías competentes, las medidas de intervención pertinentes (BOE 2003; BOJA 2005).

En base al Decreto 232/2007, Andalucía presenta una zonificación específica que divide el territorio andaluz en unidades territoriales básicas sobre las cuales se establece el PVE, denominadas áreas cinegéticas (AC). Un AC se puede define como un territorio geográfico de

extensión variable, ambiental y cinegéticamente homogéneo, que alberga unas especies cinegéticas cuyas densidades se inscriben dentro de unos márgenes definidos, con una vegetación y unos usos del suelo similares y, a su vez, distintos de otras áreas colindantes. El Plan Andaluz de Caza diferencia un total de 23 ACs con continuidad territorial, características biológicas, físicas y ambientales similares, y presentan especies cinegéticas representativas (Junta de Andalucía, 2007) (Figura 9).

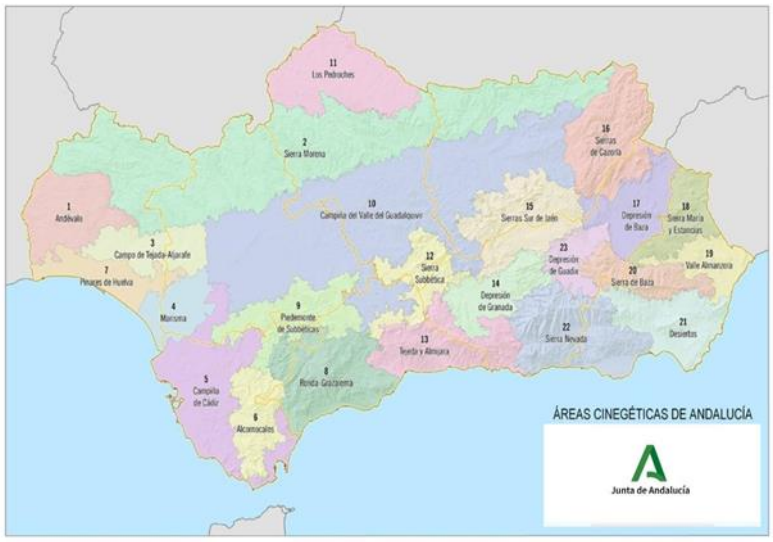

Figura 9.- Áreas cinegéticas de Andalucía. Fuente: Decreto 232/2007; CAGPDS, 2022.

El PVE cuenta con 15 protocolos específicos de especies o grupos de especies, incluyendo especies cinegéticas (cérvidos (ciervo, gamo y corzo), muflón, jabalí, cabra montés, lagomorfos (conejo silvestre y liebre ibérica) y perdiz roja) y protegidas (cetáceos, aves marinas, aves acuáticas, aves estepáricas, aves rapaces, tortugas marinas, tortugas terrestres, murciélagos y pequeños carnívoros). Los diferentes protocolos incluyen la vigilancia de diferentes patógenos bacterianos, víricos y parasitarios seleccionadas por su importancia e impacto sobre las especies silvestres objeto de estudio, así como por el papel epidemiológico de estas especies como reservorios de enfermedades de relevancia para la sanidad animal (enfermedades compartidas) y la salud pública (enfermedades zoonósicas) (CAGPDS, 2022).

El PVE tiene como objetivo general la detección precoz de enfermedades y seguimiento del estado sanitario de la fauna silvestre en el medio natural. Los objetivos específicos incluidos en el PVE son:

- Determinar el estatus sanitario de la fauna silvestre en Andalucía, estableciendo las prevalencias de las enfermedades más relevantes.

- Poner en marcha un dispositivo de Emergencias Sanitarias para la detección precoz de posibles mortandades de especies silvestres, debidas a procesos infecciosos causados por patógenos emergentes o exóticos.
- Determinar la distribución espacio-temporal de las enfermedades más relevantes en la fauna silvestre en Andalucía, estableciendo los factores de riesgo asociados a estas enfermedades.
- Establecer las medidas para la elaboración de programas de lucha de las enfermedades incluidas en el programa, y continuar con el seguimiento de estas y otras enfermedades que vayan considerándose de importancia para las especies silvestres, así como para la sanidad animal y la salud pública.

Como ya se ha comentado, uno de los objetivos del PVE es el de establecer un dispositivo de Emergencias Sanitarias para la detección precoz de enfermedades emergentes o exóticas en fauna silvestre. La detección de éstas, y de otros procesos de diferente naturaleza, se basa en la vigilancia sanitaria pasiva, y lleva asociadas actuaciones que se deben de ejecutar de forma inmediata. Este protocolo denominado "Protocolo de la Red Andaluza de Emergencias Sanitarias de la Fauna Silvestres (RASFAS)" se puso en marcha en el año 2010, encontrándose activo durante todo el año y actuando tanto en caso de mortandades leves (aquellas en las que se detectan tres ejemplares o menos en una misma zona en menos de 24 horas), como en mortandades elevadas (aquellas en las que aparecen más de tres ejemplares enfermos/muertos en un mismo espacio en menos de 24 horas) (CAGPDS, 2022).

Este PVE y su dispositivo de Emergencias Sanitarias (RASFAS) han sido la base sobre la que se ha desarrollado la presente Tesis Doctoral. Gracias a ellos se han podido llevar a cabo diferentes estudios en las dos especies de lagomorfos silvestres presentes en Andalucía, incluidos la monitorización de la GI.2 tras su aparición en conejo silvestre en Andalucía en el año 2013, la vigilancia epidemiológica del MYXV en las poblaciones de conejo silvestre en esta región, la detección de los primeros casos de mixomatosis en liebre ibérica tras el salto inter-especie asociados al ha-MYXV en el año 2018, así como la monitorización de los brotes causados por este virus emergente en las poblaciones de liebre ibérica en España en los últimos años.

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OBJETIVOS/OBJECTIVES

OBJETIVOS

El **objetivo general** de la presente Tesis Doctoral es ampliar el conocimiento sobre las principales enfermedades víricas (enfermedad hemorrágica del conejo y mixomatosis) que afectan a las poblaciones de lagomorfos silvestres de la Península Ibérica. Para la consecución de este objetivo general, se han desarrollado los siguientes **objetivos específicos**:

1. Monitorizar, mediante un programa de vigilancia pasiva, los brotes de la nueva variante del virus de la enfermedad hemorrágica del conejo en conejos silvestres en Andalucía durante el periodo 2013-2017 **(Capítulo** 1).

2. Determinar, mediante un programa de vigilancia activa, la seroprevalencia, prevalencia de infección, patrones espacio-temporales y factores de riesgo asociados al virus mixoma en ecosistemas mediterráneos del sur de España **(Capítulo 2).**

3. Monitorizar, mediante vigilancia pasiva, los primeros casos de mixomatosis en liebre ibérica causados por el virus mixoma recombinante (ha-MYXV) **(Capítulo 3)**.

4. Describir la evolución espacio-temporal y los principales hallazgos epidemiológicos asociados al ha-MYXV en liebre ibérica en España durante el periodo 2018-2020 **(Capítulo 4).**

OBJECTIVES

The **overall objective** of this PhD thesis is to increase knowledge about the main viral diseases (rabbit haemorrhagic disease and myxomatosis) affecting wild lagomorph populations in the Iberian Peninsula. To achieve this main objective, the following **specific objectives** were developed:

1. To monitor, through a passive surveillance programme, the novel rabbit haemorrhagic disease virus type 2 epidemic in wild rabbits in Andalusia (southern Spain) over the 2013-2017 period **(Chapter 1)**.

2. To determine, through an active surveillance programme, the seroprevalence, prevalence of infection, spatio-temporal patterns and risk factors affecting MYXV circulation in wild rabbit populations in southern Spanish Mediterranean ecosystems **(Chapter 2).**

3. To monitor, through a passive surveillance programme, the first cases of myxomatosis, caused by the recombinant novel myxoma virus (ha-MYXV) in Iberian hares **(Chapter 3)**.

4. To describe the spatio-temporal evolution and main epidemiological findings of the ha-MYXV epidemics in Iberian hares in Spain over the 2018-2020 period **(Chapter 4).**

CAPÍTULOS/CHAPTERS

CAPÍTULO 1

Monitoring of the novel rabbit haemorrhagic disease virus type 2 (GI.2) epidemic in European wild rabbits *(Oryctolagus cuniculus)* in southern Spain, 2013-2017.

Camacho-Sillero, L., Caballero-Gómez, J., Gómez-Guillamón, F., Martínez-Padilla, A., Agüero, M., San Miguel, E., Zorrilla, I., Rayas, E., Talavera, V., García-Bocanegra, I. (2019). Monitoring of the novel rabbit haemorrhagic disease virus type 2 (GI.2) epidemic in European wild rabbits *(Oryctolagus cuniculus*) in southern Spain, 2013-2017. Veterinary Microbiology 237:108361. doi: 10.1016/j.vetmic.2019.07.013.).

This chapter is prepared in the style format of *Veterinary Microbiology*

Resumen

La enfermedad hemorrágica del conejo (EHC) es una enfermedad infecciosa altamente contagiosa que afecta al conejo europeo (*Oryctolagus cuniculus*), producida por un virus que pertenece al género *lagovirus* (EHC; familia *Caliciviridae*). En 2010, un nuevo genotipo de la EHC (RHD2 o RHDVb, actualmente denominado GI.2) surgió en Francia, afectando tanto a conejo doméstico, incluso aquellos vacunados de los genotipos clásicos de la EHC (actualmente denominado GI.1) como silvestre. Debido a esto, el GI.2, fue identificado en otros países europeos. El objetivo de este presente estudio fue la monitorización del GI.2 epidémico en las poblaciones de conejo silvestre en Andalucía (sur de España) durante el periodo 2013-2017.

A comienzos de verano del 2013, se detectaron unas altas mortalidades en las poblaciones de conejo silvestre en el sur de España. Se controlaron un total de 96 zonas cinegéticas o protegidas. El primer brote se observó en junio de 2013. El número de brotes se incrementó rápidamente en 2013 y 2014, con una tendencia decreciente en los años siguientes. La distribución espacial del GI.2 no fue homogénea, puesto que la mayoría de los brotes se concentraron en la zona oeste de Andalucía. El pico de los brotes se produjo durante invierno y primavera en los últimos cinco años consecutivos, lo cual sugiere una circulación endémica del GI.2 en la población de conejo silvestre en España.

Durante el periodo de estudio, se muestrearon un total de 190 conejos muertos procedentes de 87 de las 96 áreas controladas. Esta mortalidad afectó a conejos de diferentes edades, incluyendo gazapos. La RT-PCR confirmó la presencia de ARN del GI.2 en 185 de los 190 (97,4%) hígados de conejo analizados. Además, se realizaron análisis filogenéticos en 11 muestras de diferentes provincias de Andalucía entre 2013 y 2017, mostrando una alta similitud con las cadenas de GI.2 previamente aisladas en España, Francia y Portugal. Los resultados constituyen un un paso importante para el entendimiento de la aparición y distribución del GI.2 en este país, proveyendo de una valiosa información para el desarrollo de programas de vigilancia en Europa.

Summary

Rabbit hemorrhagic disease (RHD) is a highly infectious disease in European rabbits (*Oryctolagus cuniculus*), caused by a virus belonging to the genus *Lagovirus* (RHDV; family *Caliciviridae*). In 2010, a new genotype of RHDV (RHDV2 or RHDVb, currently designated GI.2) emerged in France, affecting both domestic rabbits, even those vaccinated for the classical RHDV genotypes (currently designated GI.1) and wild rabbits. GI.2 was subsequently identified in other European countries. The aim of the present study was to monitor the GI.2 epidemic in wild rabbits in Andalusia (southern Spain) during the period 2013-2017.

At the beginning of summer 2013, high mortalities were detected in wild rabbit populations in southern Spain. A total of 96 affected hunting or protected areas were surveyed. The first outbreak was observed in June 2013. The number of outbreaks sharply increased in 2013 and 2014, with a decreasing trend being observed during the following years. The spatial distribution of GI.2 was not homogeneous, since most of the detected outbreaks were concentrated in the western part of Andalusia. The outbreaks peaked in winter and spring and have been detected in the last five consecutive years, which suggests endemic circulation of GI.2 in wild rabbit populations in Spain.

A total of 190 dead rabbits from 87 of the 96 areas surveyed were collected during the study period. Mortality affected rabbits of different age classes, including kittens. RT-PCR confirmed the presence of GI.2 RNA in the livers of 185 of the 190 (97.4%) rabbits. Phylogenetic analysis performed on eleven samples collected in different provinces of Andalusia between 2013 and 2017, showed high nucleotide identity with GI.2 strains previously detected in Spain, France and Portugal. The results constitute an important step in understanding the emergence and spread of GI.2 in this country and will provide valuable information for the development of surveillance programs in Europe.

Keywords: *Epidemiology, Lagovirus, Emerging Disease, GI.2, European Wild Rabbit.*

Introduction

Rabbit hemorrhagic disease (RHD) is a highly infectious, often fatal disease caused by the rabbit hemorrhagic disease virus (RHDV; genus *Lagovirus*, family *Caliciviridae*), which affects domestic and wild European rabbits (*Oryctolagus cuniculus*). The etiological agent is a non-enveloped, positive-sense, single-stranded RNA virus. Following the recently proposed classification by Le Pendu et al. (2017), RHD viruses are divided into four genotypes: genotype GI.1 (*Lagovirus europaeus*/GI.1a-GI.1d), which comprises pathogenic lagoviruses previously divided into phylogenetic groups G1-G6, the non-pathogenic RHDV-related viruses detected in Europe and Australia, which are classified into genotypes GI.3 and GI.4, and the novel RHDV genotype 2 (*Lagovirus europaeus* /GI.2, previously referred to as RHDV2 or RHDVb).

Pathogenic GI.1 was first described in China in 1984 (Liu et al., 1984) and has become endemic on many continents, including Europe. On the Iberian Peninsula, where the European rabbit is native and constitutes a keystone species in Mediterranean ecosystems (Delibes-Mateos et al., 2007), GI.1 was first identified in 1988 (Argüello-Villares et al., 1988). In the years that followed, RHDV spread rapidly and became endemic, although mortality was significantly lower (close to 30%) than the 55-75% reported during the first epidemic (Villafuerte et al., 1995). Part of the reason for the lower mortality could be the progressive increase in immune animals produced by the constant circulation of the virus in later years (Calvete et al., 2002). In this context, the prevalence of antibodies against the classical GI.1 strains in wild rabbit populations in southern Spain was found to be above 30% during the period 2003 and 2004 (García-Bocanegra et al., 2011).

The novel *Lagovirus europaeus*/GI.2 (henceforth GI.2) emerged in France in 2010, affecting both domestic rabbits, including those vaccinated against the classical GI.1 genotype, and wild rabbits (Le Gall-Reculé et al., 2011, 2013). In the years that followed, GI.2 was identified in other European countries, as well as on other continents including Australia, Africa, America and Oceania (reviewed in Rouco et al., 2019). Differences in pathogenicity between GI.1 and GI.2 lagoviruses were associated with age class (Dalton et al., 2012; Le Gall-Reculé et al., 2013); rabbits less than 5-8 weeks old were not naturally susceptible to GI.1 infection, whereas GI.2 caused disease and death even in kittens as young as 11 days of age (Dalton et al., 2014). Moreover, although hare species are naturally resistant to the classical GI.1 genotype, GI.2 cases have been detected in different hare species, including European brown hares (*Lepus europaeus*) (Bell et al., 2019; Le Gall-Reculé et al., 2017; Velarde et al., 2016), Cape hares (*Lepus capensis subsp.*

mediterraneus) (Puggioni et al., 2013), Italian hares (*Lepus corsicanus*) (Camarda et al., 2014) and mountain hares (*Lepus timidus*) (Neimanis et al., 2018a).

To date, longitudinal survey studies to assess the evolution and spread of GI.2 in wild rabbit populations have only been conducted in Portugal (Rouco et al., 2018). Hence, using passive surveillance, the aim of this study was to monitor the GI.2 epidemic in wild rabbits in Andalusia (southern Spain) during the period 2013-2017.

Material and methods

Sampling and data collection

By the beginning of summer 2013, high mortalities were being detected in wild rabbit populations in Andalusia, southern Spain (36°N–38°60´N, 1°75´W–7°25´W). An emergency health program was launched in this area by the Regional Ministry of the Environment of Andalusia. A total of 96 areas comprising 91 hunting areas and five protected areas in the eight provinces of Andalusia were visited by veterinarians belonging to the Epidemiological Surveillance Program for Wildlife (Fig. 1). Epidemiological information was gathered at each surveyed site by direct interview of gamekeepers using a standardized questionnaire. Data collected included: location, date, clinical signs, date of onset in clinically affected animals, abnormal mortality in Iberian hares (*Lepus granatensis*) (the other lagomorph species present in the study area), rabbit densities before the outbreak and restocking programs.

A total of 190 rabbits found dead were sampled between June 2013 and March 2017 in 87 out of 96 areas surveyed. Individual information, including age and sex, was gathered from each animal whenever possible. Rabbits were classified according to their weight and the presence/absence of the epiphyseal notch at the head of the tibia as kittens (up to 40 days old), juveniles (from 40 days to 8 months) or adults (over 8 months) (Dalton et al., 2012; Watson and Tyndale-Biscoe, 1953). Liver samples were collected and sent to the Central Veterinary Laboratory in Algete (National Reference Laboratory for RHDV, Madrid, Spain) for the diagnosis of RHD. In the present study, the term ‘case’ was defined as a rabbit with both clinical signs and lesions compatible with RHDV infection and the presence of GI.2 RNA confirmed by real-time reverse transcription PCR (RT-PCR). The term ‘outbreak’ was defined as an area surveyed with at least one case.

Laboratory analysis

The BioSprint 96 DNA Blood Kit (Qiagen, Hilden, Germany) was used to extract RNA from 200 µl of liver homogenate (2%) in PBS, using carrier RNA by a magnetic bead robotic system during extraction to increase yield. A GI.2-specific real-time RT-PCR was then performed using the AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems, Foster City, CA, USA) to detect a conserved region of the VP60 capsid protein gene of GI.2 viruses using primers (0.4 µM) sense 5'-TCCAGATGGTTTYCCTGACATG-3' and antisense 5'-GCGGTAGGGARGGTGYTG-3' and probe (0.15 µM) 5'-FAM-CGCTGAAGGGTACAAATG-MGB-3' (Rocha, manuscript in preparation). The thermal profile was 48°C for 25 min, followed by 10 min at 95°C and 40 cycles of 2 sec at 97°C, 45 sec at 55°C. Samples negative for GI.2 RNA were further analyzed by RT-PCR assay to detect the presence of GI.1 RNA, following the protocol described by Ros Bascuñana et al. (1997).

Phylogenetic analysis was performed on partial VP60 gene sequences (from nucleotide (nt) 6227 to nt 6778. Nucleotide position refers to coordinates in RHDVAst 89 (GenBank Accession Number: Z49271)), amplified using RT-PCRs with the following pairs of primers: sense (RHNaV-F) and antisense (RHNaV-R) (Dalton et al., 2015) and sense (REF) and antisense (REB) (Ros Bascuñana et al., 1997). The amplification products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing reactions were carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed with a 3130XL Genetic Analyzer (Applied Biosystems). Clustal was used for nucleotide sequence alignment, using representative VP60 gene sequences of GI.1 and GI.2 from China, the Czech Republic, Germany, France, Italy, Ireland, Malta, Portugal, the United Kingdom, the United States of America and Spain, available in GenBank. An Australian GI.4 strain sequence was also included (GenBank Accession Number: EU871528). A sequence of the European brown hare syndrome virus (EBHSV) (GenBank Accession Number: Z69620), which is a highly related but phylogenetically distinct lagovirus, was used as an outgroup to root the tree. The phylogenetic tree was reconstructed with the maximum likelihood method, using the Kimura two-parameter evolutionary model implemented in MEGA 7 (Kumar et al., 2016). K2 + G was chosen as the best-fit nucleotide substitution model with the lowest BIC (Bayesian information criterion) using jModelTest 2.1.10 (Kimura, 1980; Darriba et al., 2012).

Results

GI.2 outbreaks were confirmed in 86 of the 96 areas surveyed between 2013 and 2017. Two rabbits from one surveyed area showed negative results for both GI.2 and GI.1 by RT-PCR. In addition, although samples from dead rabbits could not be collected in the nine remaining areas, mortality was observed by gamekeepers. The first outbreak was reported on 27 June 2013 on a hunting estate in the province of Jaen (Fig. 1). Sixteen new outbreaks were detected between November and December of the same year. There was a sharp increase in the number of outbreaks in 2014 (50; 58.1% of the total outbreaks confirmed) followed by a decreasing trend in subsequent years, (11 (12.8%) of total outbreaks confirmed in 2015, 7 (8.1%) in 2016, and one (1.2%) in 2017) (Fig. 2).

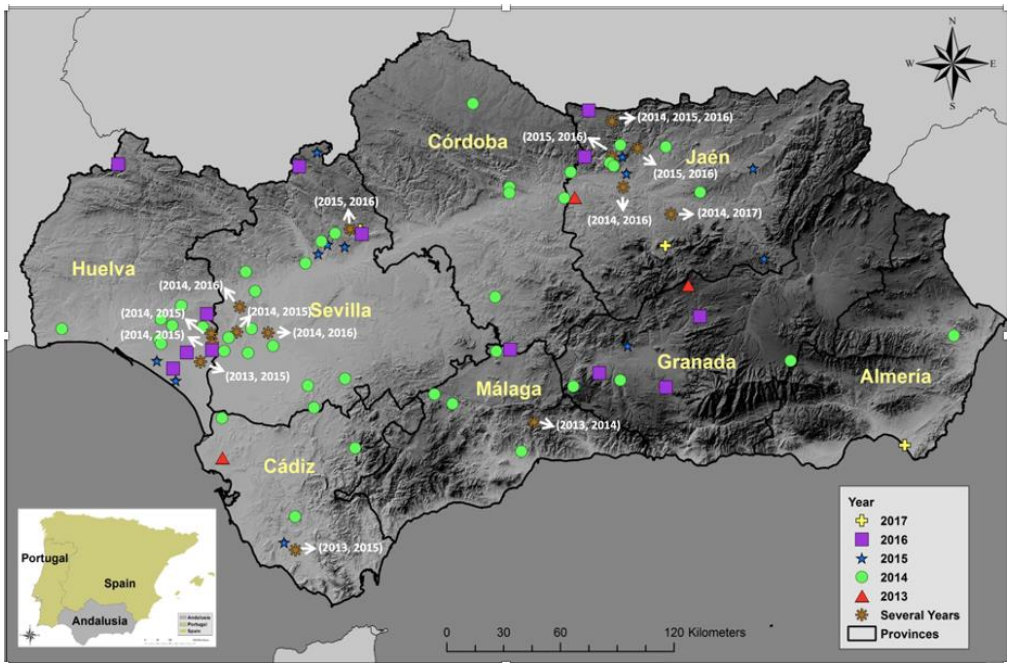

Fig. 1. Spatial distribution of GI.2 outbreaks reported in wild rabbits in Andalusia (southern Spain) between 2013 and 2017.

Whereas GI.2 outbreaks were detected throughout the year, 45 (52.3%) of the 86 GI.2-positive areas surveyed in Andalusia reported outbreaks during the winter, with lower frequencies being noticed in spring (14; 16.3%), autumn (23; 26.7%) and summer (4; 4.7%) (Fig. 2). GI.2 cases were found to be constant throughout the year in seven (8.3%) of the areas surveyed. GI.2 outbreaks were confirmed each year during the study period; furthermore, in 14 of the surveyed areas, GI.2 cases were detected in different years consecutively (Fig. 1). At least one outbreak was confirmed in all eight provinces of Andalusia. Spatial distribution was not homogeneous: Sevilla

(21; 24.4%), Huelva (21; 24.4%) and Jaen (21; 24.4%) were the provinces with the highest number of outbreaks, followed by Granada (7; 8.1%), Cordoba (7; 8.1%), Malaga (5; 5.8%), Cadiz (3; 3.5%), and Almeria (1; 1.2%) (Fig. 1).

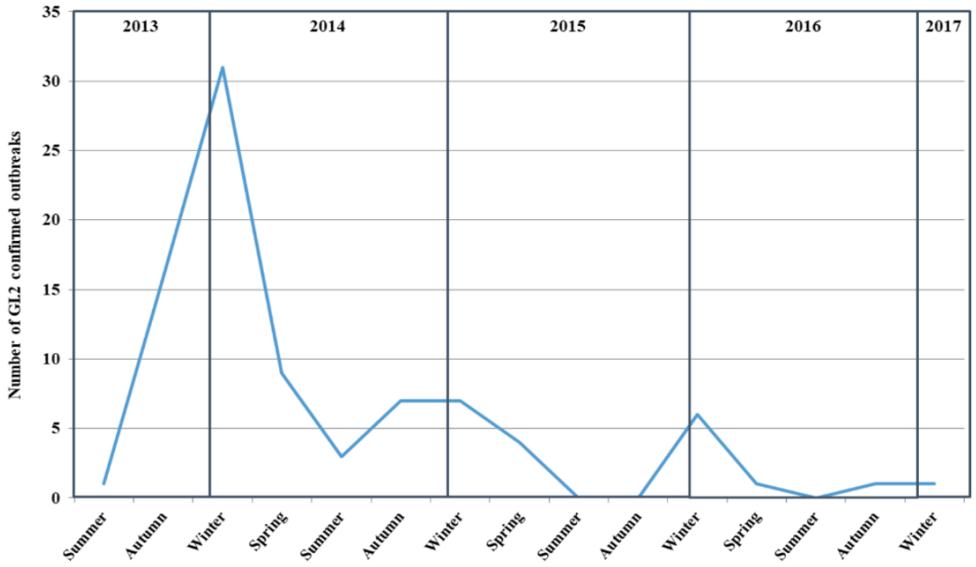

Fig. 2. Temporal evolution (by season) of GI.2 outbreaks in wild rabbits in Andalusia (southern Spain) (2013-2017).

Restocking programs were conducted between six months and two years before the first GI.2 was detected in the restocked hunting area. These programs were performed in 26 of the 86 GI.2-confirmed areas located in the provinces of Cadiz, Cordoba, Huelva, Jaen, Malaga and Seville. All restocked rabbits were captured in other hunting areas in Andalusia. Vaccination was applied to restocked rabbits using single-doses of commercial inactivated vaccine against the classical GI.1 genotype, but not against GI.2. The clinical signs observed by gamekeepers in kittens, juvenile and adult rabbits in the areas surveyed were sudden death (33.3%), opisthotonos (13.8%), convulsion (9.7%), ataxia (4.9%), paralysis (1.3%) and trembling (0.8%). Abnormal mortality was not observed in Iberian hare populations during the study.

GI.2 RNA was detected in 185 out of 190 (97.4%) rabbits analyzed, 26.5% of which were kittens, 39.3% juveniles, and the remaining 34.2% adults. The five GI.2 RNA-negative rabbits were also negative for GI.1 RNA. Phylogenetic analysis was performed on VP60 sequences of eleven samples collected in different provinces of Andalusia during the study period (2013: Malaga (GenBank Accession Number: MK843809) and Granada (MK843810); 2014: Sevilla (MK843807), Huelva (MK843808), Almeria (MK843811); 2015: Huelva (MK843812) and Jaen (MK843813); 2016: Cordoba (MK843814) and Granada (MK843815); 2017: Almeria (MK843817) and Jaen

(MK843816)). The sequences were clustered together and included in a larger clade that comprised the GI.2 sequences from Portugal, and some isolates from Spain and France (Fig. 3). In addition, BLAST analysis showed high nucleotide identity (97-100%) with available GI.2 sequences from Spain and Portugal.

Fig. 3. Maximum likelihood (ML) phylogenetic tree of partial VP60 sequences (n=72 sequences; bootstrap analysis of 1000 replicates) (from nt 6227 to 6778 using Z49271 as reference sequence) based on the nucleotide substitution K2+G model. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. Only bootstrap values ≥70 are shown. The branch including the sequences of GI.2 isolates identified in the present study (in bold) is enlarged.

Discussion

The introduction of the emerging GI.2 in 2011 has led to a substantial decline in wild rabbit populations across the Iberian Peninsula (Monterroso et al., 2016). Their densities also decreased

in the study area after the first GI.2 case was confirmed in 2013 (CMAOT, 2019). The presence of GI.2 outbreaks in at least 86 surveyed areas distributed across all eight provinces of Andalusia indicates the widespread dispersal of this new lagovirus in southern Spain. Nevertheless, the spatial distribution of the GI.2 outbreaks was not homogeneous, since most of them (73.2%) were concentrated in three provinces (Sevilla, Huelva and Jaen). Differences in wild rabbit population densities, variations in the surveillance efforts made to detect cases (which may have been more focused on areas with the presence of endangered species) and differences in habitat or climatic conditions are possible factors implicated in the geographical variation observed. In this context, the distribution and prevalence of RHDV have previously been shown to be associated with environmental factors such as rainfall and temperature (Henzell et al., 2002; García-Bocanegra et al., 2011; Liu et al., 2014). Further study of the spatial distribution of GI.2 in the study area is recommended.

The temporal evolution, as well as the detection of cases in the same areas surveyed in different years, indicate the endemic circulation of GI.2 in wild rabbit populations in southern Spain between 2013 and 2017. This hypothesis is supported by the absence of restocked animals from outside Andalusia during the study period, which decreased the risk of introduction of GI.2 strains from different regions. Following confirmation of the first GI.2 case in southern Spain (Andalusia) in summer 2013, the number of outbreaks increased sharply during 2014, with a decreasing trend in the following years. This uneven temporal distribution could be explained, as was observed for GI.1 (García-Bocanegra et al., 2011) in the study area, and more recently for GI.2 in Portugal (Rouco et al., 2018), by increased population immunity due to natural immunization against the GI.2 lagovirus as a result of contact with wild strains persistently circulating in the field. Further serosurvey studies to assess the immune status of wild rabbit populations in Andalusia would provide valuable information on this point. In addition, the possibility that gamekeepers have reported fewer outbreaks to the Regional Department of Environment in the last few years cannot be ruled out either, in which case, the number of outbreaks reported in the study period may be underestimated. GI.2 outbreaks were detected in consecutive years of the 2013-2017 study period. Although outbreaks were found throughout the year, peak incidence was observed during the coldest months (between November and April), which is consistent with previous observations of GI.1 and GI.2 epidemics elsewhere (Mutze et al., 2002; Rouco et al., 2018; Villafuerte et al., 1995).

Our results show mortality in adults but also in both kittens and juvenile animals, which is consistent with what has previously been reported in domestic and wild rabbits (Dalton et al., 2012, 2014; Neimanis et al., 2018b; Rouco et al., 2018). Clinical signs observed in the present study were compatible with acute and peracute forms associated with GI.1 infections (reviewed in Abrantes et al., 2012) and, as expected, with those previously described in GI.2 infected rabbits (Abade dos Santos et al., 2017; Dalton et al., 2012; Neimanis et al., 2018b). Abnormally high mortality was not found in the Iberian hare populations in Andalusia during the study period. However, because GI.2 cases have been detected previously in European brown hares in Spain (Velarde et al., 2016), monitoring programs should also be implemented to assess the susceptibility of the Iberian hare to GI.2 infection.

Sequence analysis of isolates showed high homology (up to 97-100%) with other GI.2 strains previously isolated in Spain and Portugal. Before the GI.2 lagovirus emerged in Spain, only classical GI.1 strains were known to circulate in domestic and wild rabbits in this country (Müller et al., 2009). However, molecular studies conducted in European countries, including Spain, France, Portugal and Sweden, as well as Australia, have demonstrated that the new GI.2 genotype has replaced the GI.1 strains previously circulating in those countries (Calvete et al., 2014; Dalton et al., 2014; Le Gall-Reculé et al., 2013; Lopes et al., 2015; Mahar et al., 2018; Neimanis et al., 2018c). Our results are in accordance with this hypothesis, and all outbreaks reported between 2013 and 2017 were caused by GI.2, although GI.1 circulation in southern Spain cannot be ruled out. Although most of the restocked rabbits were immunized using commercial vaccines against GI.1, which has been shown to be only partially protective against GI.2 at best (Le Gall-Reculé et al., 2013; Dalton et al., 2014), the number of vaccinated rabbits was too limited to achieve proper population-level immunity. Additional molecular and serological studies are required to elucidate whether GI.1 is still circulating in wild rabbit populations in Spain.

Our study has several limitations that should be taken into account. Because of the difficulties associated with finding dead wild rabbits in the field, the number of outbreaks detected in the present study was probably underestimated. Secondly, although the authors made the same sampling effort during the study period, a bias in spatial distribution associated with fewer notifications of cases by gamekeepers in the last two years cannot be ruled out. Finally, we hypothesized that the temporal distribution could also be influenced by increased natural immunity against the GI.2 lagovirus in wild rabbit populations, although additional active serosurveillance is warranted to support this hypothesis.

In conclusion, our results evidence the widespread distribution of the new GI.2 genotype in wild rabbit populations in southern Spain. The outbreaks consecutively confirmed in the period 2013-2017 suggest active and endemic circulation of this new lagovirus in this region. The results obtained contribute to a better understanding of GI.2 emergence and spread and will provide valuable information for the development of risk-based surveillance programs. Further studies are needed to assess the direct impact of GI.2 on wild rabbit populations, as well as its ecological implications for other sympatric species in Mediterranean ecosystems.

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CAPÍTULO 2

Spatiotemporal monitoring of myxomatosis in European wild rabbit (*Oryctolagus cuniculus*) in Spanish Mediterranean ecosystems.

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Resumen

Con el fin de determinar la seroprevalencia del virus de la mixomatosis (MYXV), la prevalencia de infección y los factores y patrones espacio-temporales asociados con la circulación del MYXV en conejo silvestre (*Oryctolagus cuniculus*) en el ecosistema Mediterráneo español se ha llevado a cabo un programa de vigilancia epidemiológico activo a largo plazo. Se analizaron un total de 2376 animales durante cuatro periodos de estudio: 2009-2012 (P1), 2012-2015 (P2), 2015-2018 (P3) y 2018-2021 (P4). Mediante un test ELISA comercial indirecto se detectaron anticuerpos contra MYXV en 59,9% de los conejos silvestres analizados (1,424/2,376; 95%CI: 58.0-61.9).En 131 de las 136 áreas muestreadas se detectó al menos un ejemplar seropositivo. Se confirmó la infección por MYXV en 94 de 1063 (8.8%; 95%CI: 7.3-10.7) conejos silvestres mediante PCR. La circulación del nuevo MYXV recombinante (ha-MYXV) no se localizó en ninguno de los ejemplares analizados durante el P4. Mediante el modelo de análisis Bernoulli se identificaron cinco clusters espacio-temporales de alta seroprevalencia estadísticamente significativos: uno en el P2 y otros cuatro en el P3. El análisis mediante un modelo mixto lineal generalizado (GLMM) identificó la temporada de muestreo (otoño), edad (adultos y jóvenes), brotes de mixomatosis en el mes anterior al muestreo, la temperatura media anual, y la seropositividad a la enfermedad hemorrágico vírica como factores potencialmente ligados a la seropositividad a MYXV. Igualmente, el análisis GLMM identificó que los brotes de mixomatosis durante el mes anterior al muestreo, la seropositividad a MYXV y la presencia de lesiones compatibles con mixomatosis eran factores asociados a la infección por MYXV. Los resultados indican una alta exposición, distribución amplia aunque heterogénea y una circulación endémica del MYXV en las poblaciones de conejo silvestre del sur de España durante la última década. La prevalencia de anticuerpos contra MYXV muestra fluctuaciones en el año, así como durante todo el periodo de estudio, revelando variaciones en la inmunidad de las poblaciones de conejo silvestre en los ecosistemas mediterráneos que puede incrementar el riesgo de la reaparición del MYXV en poblaciones inmunológicamente libres de la enfermedad. El presente estudio resalta la importancia del seguimiento a largo plazo para poder entender mejor la epidemiología del MYXV en lagomorfos silvestres.

Abstract

A long-term active epidemiological surveillance programme was conducted to determine seroprevalence to myxoma virus (MYXV), infection prevalence and spatiotemporal patterns and factors associated with MYXV circulation in wild rabbits (*Oryctolagus cuniculus*) in Spanish Mediterranean ecosystems. A total of 2,376 animals were sampled over four study periods: 2009-2012 (P1), 2012-2015 (P2), 2015-2018 (P3) and 2018-2021 (P4). Antibodies against MYXV were detected by a commercial indirect ELISA in 59.9% (1,424/2,376; 95%CI: 58.0-61.9) of wild rabbits. At least one seropositive animal was detected on 131 (96.3%) of 136 game estates sampled. MYXV infection was confirmed by PCR in 94 of 1,063 (8.8%; 95%CI: 7.3-10.7) wild rabbits. Circulation of the novel recombinant MYXV (ha-MYXV) was not found in wild rabbits analysed during P4. Five statistically significant spatiotemporal clusters of high seroprevalence were identified using a Bernoulli model: one in P2 and four in P3. A generalized linear mixed model (GLMM) analysis identified sampling season (autumn), age (adult and juvenile), outbreaks of myxomatosis in the month prior to sampling, mean annual temperature and seropositivity to rabbit haemorrhagic disease virus as factors potentially linked with MYXV seropositivity. GLMM analysis identified outbreaks of myxomatosis in the month prior to sampling, MYXV seropositivity and presence of lesions compatible with myxomatosis as factors associated with MYXV infection. The results indicate high exposure, widespread but non-homogeneous distribution, and endemic circulation of MYXV in wild rabbit populations in southern Spain during the last decade. Prevalence of antibodies against MYXV showed fluctuations both within the year and over the study periods, revealing variations in the immunity of wild rabbit populations in Mediterranean ecosystems that could increase the risk of MYXV re-emergence in immunologically naïve populations. The present study highlights the importance of long-term surveillance to better understand the epidemiology of MYXV in wild lagomorphs.

Keywords: Epidemiology, myxoma virus, Spain, surveillance, Wild rabbit.

1. Introduction

Myxoma virus (MYXV) is a double-stranded DNA virus, and a member of the genus *Leporipoxvirus* (family *Poxviridae*). MYXV causes myxomatosis, a highly infectious disease that affects domestic and wild lagomorphs (Murphy et al., 1995). This virus is mainly transmitted by biting arthropods or direct contact with infected animals. In European wild rabbits (*Oryctolagus cuniculus*), MYXV infection usually results in severe and often fatal disease, characterised by blepharoconjunctivitis, respiratory disorders, cephalic and anogenital oedema, and cutaneous pseudotumours called myxomas

MYXV was first introduced into Europe in the 1950s in an attempt to control pest damages caused by European wild rabbitsh (Fenner & Fantini, 1999). The virus then spread rapidly across the continent, with high initial mortality rates (>90%) (Fenner & Ratcliffe, 1965). Over time, the virulence of the strains was attenuated and rabbit resistance grew, offering a remarkable research model for long-term host-pathogen coevolution (Best & Kerr, 2000; Kerr, 2012). In the Iberian Peninsula, the native range of the European wild rabbit Monnerot et al., 1994; Thompson & King, 1994), MYXV is currently considered endemic (Villafuerte et al., 2017), and is, together with rabbit haemorrhagic disease (RHD), one of the main factors explaining the decline of their populations (Calvete et al., 2002). Indeed, the International Union for Conservation of Nature (IUCN) Red List recently listed this lagomorph species as “Near Threatened” across its entire native range, with a trend of declining populations (Villafuerte & Delibes Mateos, 2019). In connis, a cross-species jump of MYXV to the Iberian hare (Lepus granaten*sis), the most im*portant hare species in terms of population and hunting interest on the Iberian Peninsula, was confirmed in 2018, when a natural recombinant MYXV (ha-MYXV) emerged in the region, leading to epidemic outbreaks in this wild lagomorph species (Águeda-Pinto et al., 2019; García-Bocanegra et al., 2019). Since its appearance, the novel ha-MYXV has had a significant impact on the health status of Iberian hare populations in Spain, causing mortality of more than 50% in affected areas (García-Bocanegra et al., 2020).

Even though MYXV and its coevolution with the European wild rabbit are well-studied topics (Cameron et al., 1999; Best and Kerr, 2000; Alves et al., 2019), information on the epidemiology of myxomatosis in this species remains limited. Indeed, what we know about the epidemiology of myxomatosis in the European wild rabbit comes mostly from local or cross-sectional serological studies (Calvete et al., 2002; Fouchet et al., 2006, 2008; García-Bocanegra et al., 2010; Marchandeau et al., 2004; Santoro et al., 2014; Villafuerte et al., 2017), and does not

take into account spatial and/or temporal patterns. Long-term studies are essential to provide a better understanding of how epidemiological systems respond to individual, population and environmental changes (Barroso et al., 2020). Given the high adaptability of European wild rabbit populations to MYXV (Kerr et al., 2015), continuous monitoring is essential to understand the spatiotemporal trends of myxomatosis. Knowledge from long-term and large-scale surveillance is key for the implementation of more accurate and targeted control measures. Hence, the aim of the present study was to determine the seroprevalence, prevalence of infection, spatiotemporal patterns and factors related to MYXV circulation in wild rabbit populations in southern Spanish Mediterranean ecosystems.

2. Material and methods

2.1. Study area

An active epidemiological surveillance programme, coordinated by the Regional Government of Andalusia, was carried out in southern Spain (36° N-38° 60' N, 1° 75' W-7° 25' W) between 2009 and 2021. Andalusia has a Mediterranean climate, characterized by warm to hot and dry summers and mild to cool and wet winters; the average annual temperature is 16°C and the average annual precipitation is 590 mm (CMAOT, 2009). The Regional Government of Andalusia divides this region of 87,597km^2 into 23 hunting areas (HA) based on biological, physical, and environmental features, and on epidemiological criteria concerning the presence and abundance of large and small game species communities. A total of 14 of the 23 HAs were selected, based on the distribution and density (>8 individuals/km^2) of wild rabbits in the study area (CAPMA, 2013).

2.2. Study design and sample collection

Sample collection was divided into four consecutive periods: August 2009-July 2012 (P1), August 2012-July 2015 (P2), August 2015-July 2018 (P3) and August 2018-July 2021 (P4). Animals were sampled for three consecutive years per period. The minimum number of wild rabbits sampled per HA (n = 60) in each period was chosen to ensure a 95% probability of detecting at least one positive animal, assuming a minimum prevalence of 5% within each sampling HA. Animals were legally hunted on a total of 136 game estates located in the 14 selected HAs. Whenever possible, a minimum of 10 individuals were randomly selected from each game estate. A total of 2,376 wild rabbits were sampled: 645 in P1, 614 in P2, 612 in P3 and 505 in P4.

Blood samples from all animals were taken from the heart or thoracic cavity. Samples were placed in sterile tubes without anticoagulant (5.0 ml) and transported to the laboratory under refrigeration. Samples were centrifuged at 400 *g* for 15 min for serum extraction. To confirm active MYXV infection, a subset of 1,063 blood samples, collected from 119 of the 136 game estates during P1 (n= 633) and P4 (n= 430), were also placed into sterile tubes with anticoagulant (2.5 ml). Serum and blood samples were stored at -20°C until serological and molecular analysis, respectively.

Wherever possible, individual information, including age, sex, bodyweight, flea and tick infestations and macroscopic lesions compatible with myxomatosis, was recorded for each animal. Tibial epiphyseal line and bodyweight were used as indicators of age (Watson & Tyndale-Biscoe, 1953). Three age groups were considered: young (< 40 days old), juvenile (from 40 days to 8 months) and adult (over 8 months). During sampling, epidemiological data concerning the game estates was also gathered through personal interviews with gamekeepers using a standardized questionnaire (Supplementary material). The information generated included data on the characteristics of the game estates, the health status of the lagomorph population (particularly regarding myxomatosis and RHD), disease control measures, management practices and the presence of carnivores or other wild and domestic mammals. Additionally, climatic data (mean and maximum annual temperatures (°C), humidity (%), and mean annual rainfall (L/m^2)) recorded at weather stations in the proximity of the sampling game estates was obtained from Spain's National Meteorological Institute (Ministry of Ecological Transition and Demographic Challenge).

2.3. Laboratory analysis

Serum samples were tested for antibodies against MYXV using a commercial indirect enzyme-linked immunosorbent assay (ELISA; INgezim MIXOMATOSIS R.17.MIX.K1, Eurofins Technologies Ingenasa; Madrid, Spain). Sensitivity and specificity values were 98% and 99%, respectively, as specified by the manufacturer. A commercial indirect ELISA (Ingezim RHDV 17. RHD.K.1, Eurofins Technologies Ingenasa; Madrid, Spain) was also used to evaluate serological status for RHD virus (RHDV; considered an explanatory variable). Test sensitivity and specificity provided by the manufacturer were 99% and 86%, respectively. Both ELISAs were used according to the manufacturer's recommendations.

Total DNA from blood samples of the selected 1,063 rabbits was extracted with the commercial G-spin™ Total DNA Extraction Mini Kit (iNtRON Biotechnology, Seongnam, Korea)), following the manufacturer's instructions, and stored at -20 °C until analysis. A conserved region

of the M071L gene was amplified by conventional PCR (T100 Thermal Cycler, Biorad, Hercules, CA, USA) using MyTaq™ Red DNA Polymerase (Bioline) and forward and reverse primers 5'-ACCCGCCAAGAACCACAGTAGT-3' and 5'-TAACGCGAGGAATATCCTGTACCA-3', as previously described (Cavadini et al., 2010). All DNA-positive samples collected during P4 were also tested by conventional PCR to identify whether the viral strain present was the classic rabbit strain or the recombinant ha-MYXV, recently detected in the Iberian hare (*Lepus granatensis*) and wild rabbit in the Iberian Peninsula (Abade dos Santos et al., 2020; García-Bocanegra et al., 2020). The same polymerase was used, although the forward and reverse primers in this case were M009L-F (5'-CGCAGGTCCACGTATAAACC-3') and M009L-R (5'-CGAACGTATCATTAGACAATG-3') (Dalton et al., 2019). The amplicons of both molecular assays were examined on 1.5% agarose gel stained with RedSafe™ Nucleic Acid Staining solution (iNtRON Biotechnology, Seongnam, Korea).

2.4. Statistical analysis

Individual seroprevalence and prevalence of MYXV infection were estimated from the proportion of the number of positives (for the presence of antibodies and MYXV DNA, respectively) to the total number of animals examined, with a 95% confidence interval (95%CI). A spatiotemporal scan statistical analysis was applied using a Bernoulli model to detect significant clusters of high MYXV seroprevalence at game estate and time period level, using SaTScan v.9.6 software (Kulldorff et al., 2006). The number of Monte Carlo simulations was set to 1,000 for the cluster scan statistic. Clusters were considered to be significant at p-value < 0.05.

Associations between MYXV seroprevalence/prevalence and explanatory variables were analysed using the Pearson's chi-square test or Fisher's exact test, as appropriate. All variables with a p-value < 0.10 in this bivariate analysis were selected as possible associated factors (Tables 1 and 2). Collinearity between pairs of variables was tested by Cramer's V coefficient. The effect of variables selected from the bivariate analysis was investigated using a Generalized Linear Mixed Model (GLMM). The number of seropositive/positive rabbits was assumed to follow a binomial distribution and a logit link function was used. Game estate and sampling year were included as random effect factors. A forward stepwise procedure based on Akaike's information criterion (AIC) (Burnham and Anderson, 2002) was used. GLMMs were built using R library lme4, version 1.1-21 in R software, version 4.0.2 (Bates et al., 2015).

3. Results

The overall individual prevalence of antibodies against MYXV was 59.9% (1,424/2,376; 95%CI: 58.0-61.9). Seropositive wild rabbits were detected in 100% of the 14 HAs with seroprevalence values ranging between 29.6% and 91.9%. At least one seropositive animal was found on 131 (96.3%) of 136 game estates sampled. MYXV DNA was detected in 94 of 1,063 (8.8%; 95%CI: 7.3-10.7) wild rabbits, 51 out of 633 (8.1%) in P1, and 43 out of 430 (10.0%) in P4. Active infection was found on 44 (37.0%) of 119 game estates sampled in 13 (92.9%) of 14 HAs (Figure 1). ha-MYXV DNA was not found in any of the wild rabbits tested.

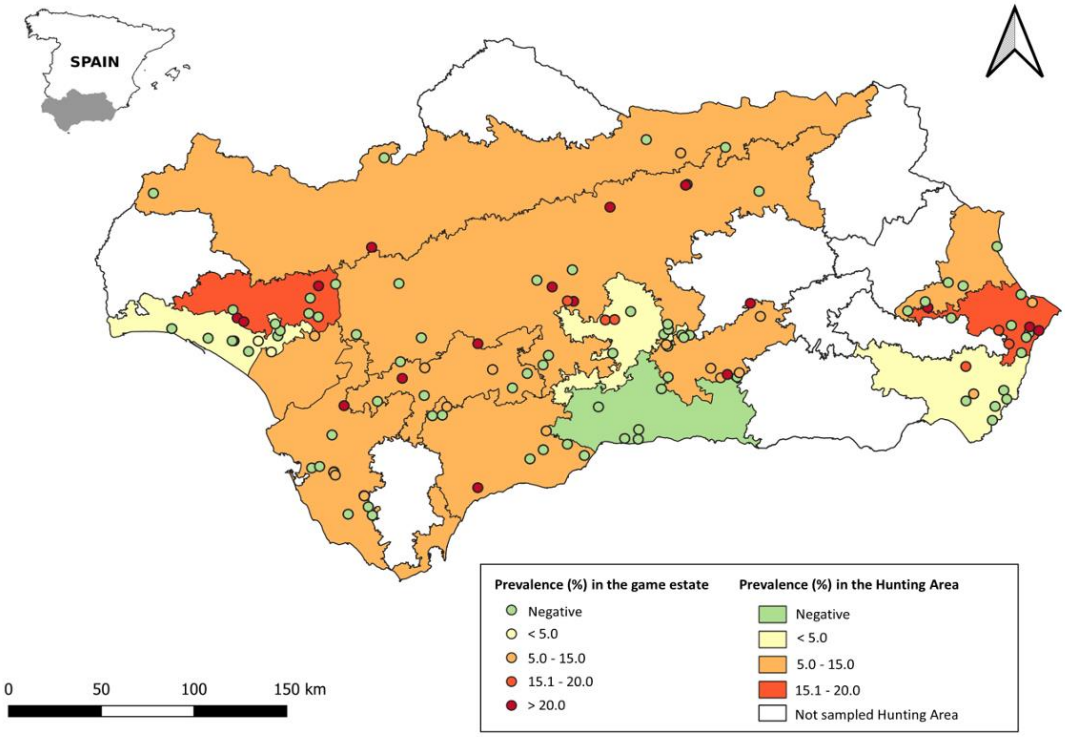

Figure 1. Spatial distribution of the prevalence of MYXV infection in European wild rabbits (*Oryctolagus cuniculus*) in southern Spain.

Seroprevalence fluctuated over time, with a slightly decreasing tendency and cyclical peak variation (Figure 2). Spatiotemporal analysis identified five statistically significant clusters of seropositivity in the study area (Figure 3). The first cluster included 15 game estates, was located in western Andalusia, and occurred during P2 (Relative Risk (RR) = 1.5). The remaining clusters occurred in P3. The second cluster included seven game estates and was restricted to the province of Cadiz in the southwestern part of Andalusia (RR = 1.7). Cluster 3 included 17 game estates located in central south Andalusia (RR = 1.5). The fourth cluster included 12 game estates from

southern Andalusia (RR = 1.5). Finally, the fifth cluster, located in the north of the study region, included five game estates (RR = 1.7). All clusters were statistically significant with *p*-value < 0.001.

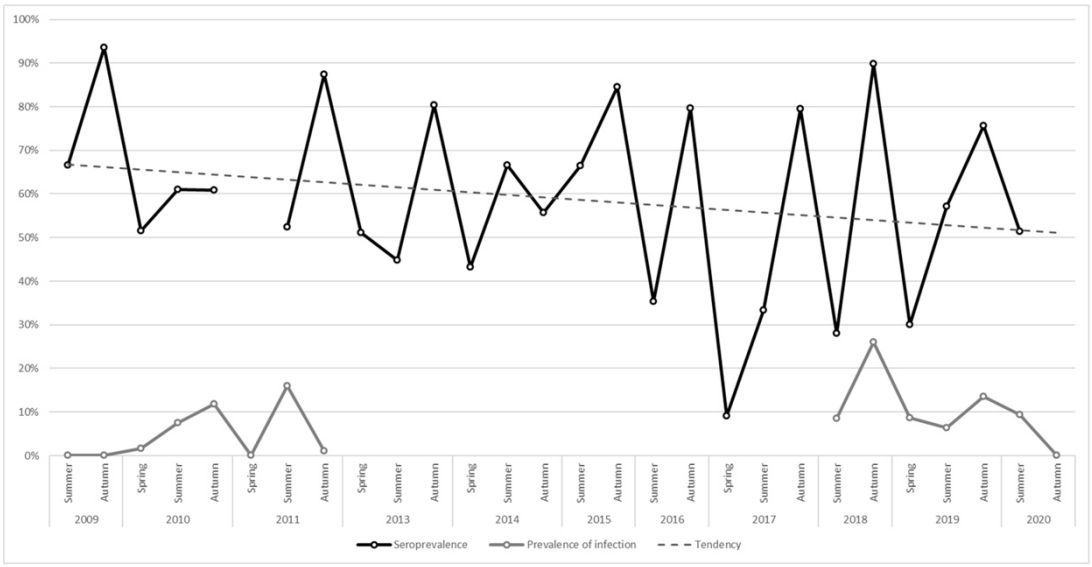

Figure 2. Seroprevalence and prevalence of MYXV in European wild rabbits (*Oryctolagus cuniculus*) inhabiting Mediterranean ecosystems throughout the study period (2009-2020).

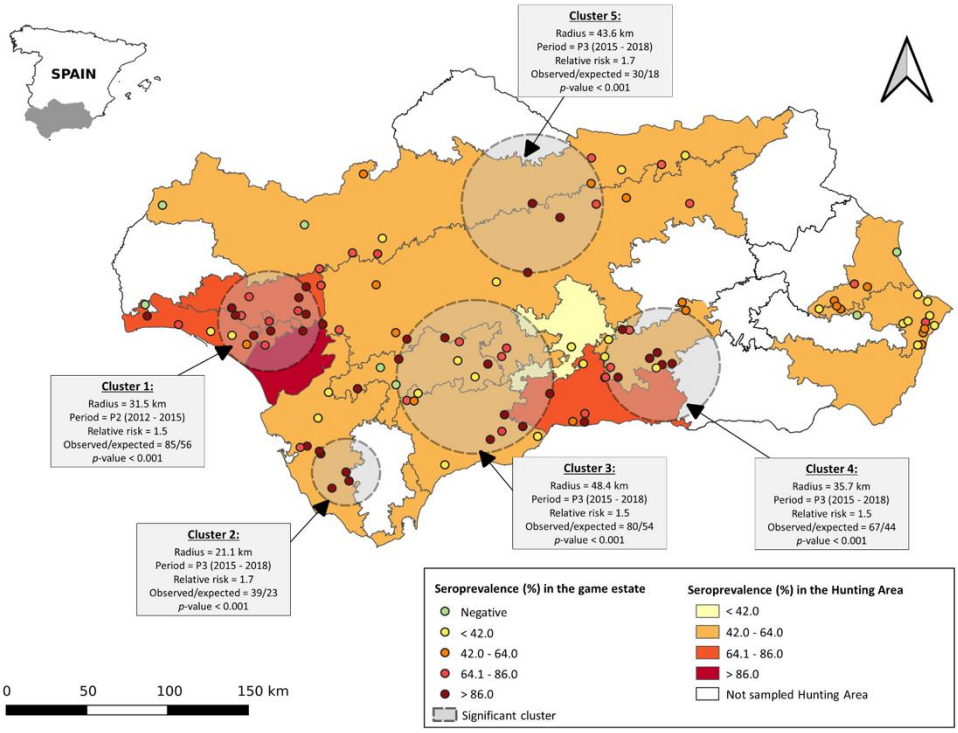

Figure 3. Spatial distribution of MYXV seroprevalence in European wild rabbits (*Oryctolagus cuniculus*) in southern Spain. Grey circles represent significant clusters of seropositivity identified by statistical analysis.

Explanatory variables obtained from the epidemiological questionnaire, and the results of the bivariate analyses of seropositivity and prevalence of infection are summarised in Tables 1 and 2, respectively. A total of 16 and 10 explanatory variables associated ($p < 0.10$) with MYXV seropositivity and infection, respectively, were selected after data exploration and bivariate analyses.

Table 1. Distribution of the prevalence of antibodies against MYXV in European wild rabbits (*Oryctolagus cuniculus*) in southern Spain, by category and bivariate analysis results.

Variable	**Category**	**No. positives/ total** [†]	**Seroprevalence (95%CI)**	***p*-value**
Age	Young	36/159	22.6 (16.8 – 29.8)	< 0.001
	Juveniles	201/470	42.8 (38.4 – 47.3)	
	Adults	1172/1725	67.9 (65.7 – 70.1)	
Sex	Female	732/1281	57.1 (54.4 – 59.8)	0.004
	Male	669/1069	62.6 (59.6 – 65.4)	
Time periods	P1	411/645	63.7 (60.0 – 67.3)	< 0.001
	P2	324/614	52.8 (48.8 – 56.7)	
	P3	422/612	69.0 (65.2 – 72.5)	
	P4	267/505	52.9 (48.5 – 57.2)	
Season	Spring	106/236	44.9 (38.7 – 51.3)	< 0.001
	Summer	701/1332	52.6 (49.9 – 55.3)	
	Autumn	617/808	76.4 (73.3 – 79.2)	
Repopulation in the last two years	No	881/1535	57.4 (54.9 – 59.8)	0.002
	Yes	522/820	63.7 (60.3 – 6.9)	

Outbreaks of myxomatosis during the month previous to sampling	No	710/1276	55.6 (52.9 – 58.3)	< 0.001
	Yes	714/1100	64.9 (62.0 – 67.7)	
Seropositivity to RHDV	Negative	664/1275	52.1 (49.3 – 54.8)	< 0.001
	Positive	759/1098	69.1 (66.3 – 71.8)	
Density of mosquitos	Low	298/541	55.1 (50.9 – 59.2)	0.003
	Moderate	375/638	58.8 (54.9 – 62.5)	
	High	738/1180	62.5 (59.7 – 65.3)	
Presence of ticks in the game estate	No	498/864	57.6 (54.3 – 60.9)	0.019
	Yes	926/1512	61.2 (58.8 – 63.7)	
Presence of Iberian lynx	No	1168/2017	57.9 (55.7 – 60.1)	< 0.001
	Yes	256/359	71.3 (66.4 – 75.7)	
Presence of domestic cats	No	334/515	64.9 (60.6 – 68.9)	0.009
	Yes	1084/1854	58.5 (56.2 – 60.7)	
Presence of sheep	No	916/1482	61.8 (59.3 – 64.3)	0.016
	Yes	508/894	56.8 (53.6 – 60.0)	
Presence of rabbit feeders	No	941/1621	58.1 (55.6 – 60.4)	0.006
	Yes	483/755	64.0 (60.5 – 67.3)	
Presence of ponds	No	851/1474	57.7 (55.2 – 60.2)	0.003
	Yes	573/902	63.5 (60.3 – 66.6)	
Mean annual temperature (°C)	11.0 – 16.0	248/469	52.9 (48.3 – 57.5)	< 0.001
	16.1 – 18.0	782/1364	57.3 (54.7 – 59.9)	
	> 18.0	385/530	72.6 (68.7 – 76.3)	

Mean annual rainfall (L/m2)	264.0 – 573.0	445/836	53.2 (49.8 – 56.6)	0.017
	573.1 – 629.0	481/732	65.7 (62.2 – 69.1)	
	> 629.0	489/795	61.5 (58.1 – 64.8)	

† Missing values omitted.

Table 2. Distribution of the prevalence of MYXV infection in European wild rabbits (*Oryctolagus cuniculus*) in southern Spain, by category and bivariate analysis results.

Variable	**Category**	**No. positives/ total** †	**Prevalence (95%CI)**	***p*-value**
Sex	Female	55/545	10.1 (7.8 – 12.9)	0.025
	Male	38/497	7.6 (5.6 – 10.3)	
Presence of myxomatosis compatible lesions	No	73/1019	7.2 (5.7 – 8.9)	< 0.001
	Yes	21/44	47.7 (33.8 – 62.1)	
Outbreaks of myxomatosis during the month previous to sampling	No	19/575	3.3 (2.2 – 5.1)	< 0.001
	Yes	75/488	15.4 (12.4 – 18.8)	
Seropositivity to MYXV	Negative	18/444	4.1 (2.6 – 6.3)	0.002
	Positive	76/619	12.3 (9.9 – 15.1)	
Outbreaks of RHD during the month previous to sampling	No	85/872	9.7 (8.0 – 11.9)	0.011
	Yes	9/191	4.7 (2.5 – 8.7)	
Age structure of the population	> % adults	26/212	12.3 (8.5 – 17.4)	0.009
	> % young	41/443	9.3 (6.9 – 12.3)	
	Similar %	27/408	6.6 (4.6 – 9.5)	

Density of mosquitos	Low	15/214	7.0 (4.3 – 11.2)	0.094
	Moderate	39/432	9.0 (6.7 – 12.1)	
	High	40/417	9.6 (7.1 – 12.8)	
Vaccination the year previous to sampling	No	83/894	9.3 (7.6 – 11.4)	0.098
	Yes	11/169	6.5 (3.7 – 11.3)	
Maximum annual temperature (°C)	17.0 – 22.0	23/227	10.1 (6.9 – 14.7)	0.031
	22.1 – 24.0	38/549	6.9 (5.1 – 9.4)	
	> 24.0	32/281	11.4 (8.2 – 15.6)	
Mean humidity (%)	30.0 – 60.0	34/254	13.4 (9.7 – 18.1)	0.007
	60.1 – 74.0	49/567	8.6 (6.6 – 11.2)	
	> 74.0	10/236	4.2 (2.3 – 7.6)	

[†] Missing values omitted.

The final GLMM identified the following factors as potentially associated with seropositivity to MYXV: sampling season, age, outbreaks of myxomatosis in the month prior to sampling, mean annual temperature, and seropositivity to RHDV (Table 3). Seropositivity was significantly higher in autumn (76.4%) compared to summer (52.6%) and spring (44.9%). Significantly higher seropositivity was found in juveniles (42.8%) and adults (67.9%) than in young (22.6%) animals. The prevalence of anti-MYXV antibodies increased significantly in animals sampled in areas where myxomatosis outbreaks had been reported in the month before sampling (64.9%) compared to those in sampling areas where no myxomatosis cases had occurred in the same period (55.6%). Seropositivity was highest (72.6%) in areas where mean annual temperatures were above 18°C, decreasing in mean temperature ranges of 16.1-18.0°C and 11.0-16.0°C (57.3% and 52.9%, respectively). MYXV seropositivity was significantly higher in RHDV-seropositive rabbits (69.1%) compared to seronegative rabbits (52.1%). In the final GLMM for MYXV infection, outbreaks of myxomatosis in the month prior to sampling, seropositivity to MYXV, and presence of lesions compatible with myxomatosis were retained as associated variables (Table 4). Sampling game

estates that reported myxomatosis outbreaks in the month prior to sampling had a significantly higher frequency of active infection (15.4%) than sampling game estates without outbreaks (3.3%). Finally, seropositivity to MYXV and the presence of lesions compatible with myxomatosis were significantly higher in infected (80.9 and 22.3%, respectively) versus non-infected (56.0 and 2.4%, respectively) individuals.

Table 3. Results of the generalized linear mixed model (binomial distribution, identity link function) constructed to explain exposure to MYXV in European wild rabbits (*Oryctolagus cuniculus*).

Variables [†]	**Estimate ± Standard Error)**	**Odds ratio (95%CI)**	***p*-value**
Age	Juveniles: 0.94 ± 0.27 Adults: 1.87 ± 0.26	Juveniles: 2.55 (1.51 – 4.34) Adults: 6.49 (3.89 – 10.80)	< 0.001
Outbreaks of myxomatosis during the month previous to sampling	0.84 ± 0.15	2.32 (1.73 – 3.10)	< 0.001
Seropositivity to RHDV	1.05 ± 0.12	2.86 (2.26 – 3.62)	< 0.001
Sampling season	Summer: -0.04 ± 0.23 Autumn: 0.89 ± 0.25	Summer: 0.96 (0.61 – 1.51) Autumn: 2.44 (1.49 – 3.97)	< 0.001
Mean annual temperature	16.1 – 18.0: 0.46 ± 0.26 > 18.0: 1.32 ± 0.30	16.1 – 18.0: 1.58 (0.95 – 2.64) > 18.0: 3.74 (2.08 – 6.74)	< 0.001

[†] Reference categories considered for parameter estimates calculations were “young” for age, “no” for outbreaks of myxomatosis during the month previous to sampling and “negative” for seropositivity to RHDV, “spring” for sampling season and “11.0 – 16.0” for mean annual temperature.

Table 4. Results of the generalized linear mixed model (binomial distribution, identity link function) constructed to explain MYXV infection in European wild rabbits (*Oryctolagus cuniculus*).

Variables †	**Estimate ± Standard Error)**	**Odds ratio (95%CI)**	***p*-value**
Outbreaks of myxomatosis during the month previous to sampling	*1.72* **±** 0.44	*5.58 (2.36 – 13.22)*	*< 0.001*
Seropositivity to MYXV	*1.18* **±** 0.33	*3.25 (1.70 – 6.21)*	*< 0.001*
Presence of myxomatosis compatible lesions	*1.77* **±** 0.44	*5.87 (2.48 – 13.91)*	*< 0.001*

† Reference categories considered for parameter estimates calculations were "no" for outbreaks of myxomatosis during the month previous to sampling and presence of myxomatosis compatible lesions, and "negative" seropositivity to MYXV.

4. Discussion

To the best of the author's knowledge, this is the first long-term survey study on MYXV conducted in wild rabbits in the world. The overall individual seroprevalence obtained (59.9%) indicates high exposure to this virus in wild rabbit populations in the Mediterranean ecosystems of southern Spain. Our result is consistent with those reported in a local study conducted in Cordoba province (within our study area) between 2003 and 2004 (56.4%) (García-Bocanegra et al., 2010). A similar prevalence of anti-MYXV antibodies (53.0%) was also found in a nationwide cross-sectional serosurvey carried out in Spain between 2003 and 2009 (Villafuerte et al., 2017). Lower seroprevalence levels (0.0-24.0%) have been detected in Scotland (Boag et al., 2013) and the Canary Islands (Spain) (27.0%) (Foronda et al., 2005), while higher seropositivity values have been observed in north-east Spain (82.4%) (Calvete et al., 2002) and France (71.2%) (Marchandeau et al., 1998). Variations between studies may reflect differences in strain virulence, the presence and abundance of competent vector species, or environmental characteristics. Nonetheless, comparisons should be made with caution, given the differences in the number of samples examined, diagnostic methods, study designs and epidemiological contexts.

Molecular analysis revealed active MYXV infection in 8.8% of the rabbits tested. MYXV-infected animals were detected in most years and hunting areas sampled, which indicates that MYXV circulated in the study area during P1 and P4. To the best of our knowledge, only one previous study has assessed MYXV infection through active surveillance, a cross-sectional survey carried out during 1993-1995. In that study, using radial immunodiffusion assay, Simón et al. (1998) found a high prevalence of MYXV (22.7%) in wild rabbit populations sampled in northern Spain. It should also be pointed out that the novel ha-MYXV was first reported in Iberian hare populations in our study area (García-Bocanegra et al., 2019) in P4. In connection with this, ha-MYXV infection was not found in any wild rabbits tested in our study in that study period. Nevertheless, several game estates sampled during P4 reported outbreaks of myxomatosis in Iberian hares (ha-MYXV infection confirmed) in the same time period, including game estates where wild rabbits were found to be positive for the classic form of MYXV. Further surveillance programs are warranted to assess the circulation of the emerging ha-MYXV in wild rabbit populations in the study area, as has already been reported in other regions of Spain (García-Bocanegra et al., 2020) and Portugal (Abade dos Santos et al., 2020).

Our results showed a wide spatial distribution and endemic circulation of MYXV in wild rabbit populations in southern Spain during the last decade. Spatial clustering revealed that circulation of this virus in the populations of this lagomorph species was heterogeneous. Five significant clusters of high seroprevalence were identified in all parts of the study area except for the easternmost part. It is worth noting that the first spatiotemporal cluster was found in western Andalusia in P2 (2012-2015), while the remaining four clusters were detected in the central region during P3 (2015-2018). Differences in seroprevalence values throughout the study periods, with higher rates in P1 and P3, and lower rates in P2 and P4, provide evidence of variations in MYXV circulation in Spanish Mediterranean ecosystems over the last decade. We hypothesize that the establishment of high population immunity after epidemic outbreaks of myxomatosis in P1 and P3 may help reduce viral circulation, with the proportion of protected individuals decreasing in the following periods (P2 and P4, respectively). This, together with the natural replacement of seropositive adult animals by seronegative young individuals, would increase the risk of MYXV re-emergence in immunologically naïve wild rabbit populations.

Seroprevalence was markedly seasonal throughout the study periods, as shown by statistical modelling. The prevalence of anti-MYXV antibodies (76.4%) was significantly higher in wild rabbits sampled in autumn than in those tested in spring and summer (44.9% and 52.6%,

respectively). Similar seasonal variations have been reported previously in wild rabbit populations in Spain (García-Bocanegra et al., 2010; Simón et al., 1998), as well as in other European countries (Boag et al., 2013; Marchandeau et al., 1998). The annual pattern of seropositivity to MYXV follows the marked seasonality of outbreaks of myxomatosis observed in this species, which peak in late summer (Calvete et al., 2002; Merchant et al., 2003; Farrell et al., 2020). In relation to this, rabbits from game estates where outbreaks of myxomatosis were observed in the month prior to sampling were 2.3 times more likely to be seropositive and 5.6 times more likely to be infected with MYXV than those from game estates with no outbreaks during that time.

A significant association was found between the presence of lesions compatible with myxomatosis and seropositivity, as well as with the presence of MYXV DNA in blood. These findings were expected, since viraemia, presence of external lesions of the classic form of myxomatosis, such as myxomas, blepharoconjunctivitis and cephalic and anogenital oedema and detectability of anti-MYXV antibodies, have been shown to have overlapping time windows in wild rabbits (Fenner & Woodroofe, 1953). It should be noted that due to the limited viraemia of MXYV (< 10 days post infection, Fenner & Woodroofe, 1953), the prevalence of infection in the present study may be underestimated, as is suggested by the proportion of individuals with clinical signs. Ideally, further active surveillance studies should include tissues with a priori longer persistence of virus (e.g., eyelid skin, Kerr et al., 2015) to detect MXYV infection in wild rabbit populations.

The prevalence of MYXV antibodies in wild rabbits was age-related, as previously reported (Calvete et al., 2002; Ferreira et al., 2009; García-Bocanegra et al., 2010; Villafuerte et al., 2017). Significantly higher seroprevalence values were found in adults (67.9%) and juveniles (42.8%) compared to young individuals (22.6%), which could reflect the cumulative likelihood of exposure to the virus over the lifetime of the rabbit and/or the persistence of antibodies over time (Fouchet et al., 2008; Santoro et al., 2014). Juvenile rabbits are more susceptible to MYXV infection (Villafuerte et al., 2017) and the severity of myxomatosis depends mainly on the presence of maternal antibodies (Fouchet et al., 2006, 2008). The proportion of younger animals in a population and the timing of the reproductive season are key factors for the persistence and impact of this virus (Fouchet et al., 2006, 2008; Villafuerte et al., 2017). In Mediterranean ecosystems, outbreaks occur mainly in the summer months, when the number of naïve juveniles in wild rabbit populations is usually higher (Tablado et al., 2009). This implies that a significant part of the population may be susceptible to MYXV infection during this period, increasing the impact of outbreaks (Farrell et al., 2020; Fouchet et al., 2006; Villafuerte et al., 2017).

The occurrence of myxomatosis outbreaks is also closely related to the activity of arthropod vector species, which also increases in the warmer months (Osácar et al., 2001). In this context, MYXV transmission through vectors may depend on the mean annual temperature, another factor identified in the present study. This climatic factor is key to the biological cycle of competent vector species (fleas, mosquitos, and possibly ticks) of MYXV (Eritja et al., 2005; García-Pereira et al., 2021; Osácar et al., 2001). Consequently, the presence and abundance of MYXV-transmitting arthropods may be higher in locations where the mean annual temperatures are higher, resulting in higher rates of exposure. Further research is required to fully understand the extent to which different vector species are involved in MYXV transmission in wild lagomorphs in Iberian Mediterranean ecosystems.

MYXV seropositivity was also significantly associated with exposure to RHDV. Rabbits that were seropositive to RHDV had significantly higher seropositivity to MYXV (69.1%) than those that were RHDV-seronegative (52.1%). This association was first reported by Marchandeau et al. (2004) after observing that the probability of a rabbit being seropositive to both viruses was more than 5 times that of a rabbit being MYXV-seronegative and RHDV-seropositive. A similar association was also observed by García-Bocanegra et al. (2010) in our study area. The interaction between MYXV and RHDV may be a result of the immunosuppressive effect of MYXV (Jeklova et al., 2008; Santoro et al., 2014) and/or the fact that both viruses are endemic in Mediterranean ecosystems, have annual epizootic cycles and share the same transmission route (Marchandeau et al., 2004).

The serological and molecular results obtained in the present study provide evidence of the widespread but not homogeneous spatiotemporal distribution of MYXV in European wild rabbit populations in southern Spain over the last decade. MYXV seroprevalence showed an annual cyclical pattern, and also over longer periods of time, highlighting temporal fluctuations in the immune status of wild rabbit populations inhabiting Mediterranean ecosystems. The spatial clusters of high seropositivity that have been identified should be prioritized for future risk-based surveillance and control efforts. MYXV seropositivity was related to host-related factors and environmental conditions, whereas MYXV infection was mainly associated with factors related to disease pathogenesis. The present study highlights the importance of long-term surveillance for obtaining a better understanding of the spatiotemporal pattern of MYXV in Iberian Mediterranean ecosystems.

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Ethics Statement

No ethical approval was required since no animals were killed specifically for this study. Samples were collected from wild rabbits legally hunted in complete agreement with Andalusian and Spanish regulations. No ethical approval by an Institutional Animal Care and Use Committee was deemed necessary.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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CAPÍTULO 3

First outbreak of myxomatosis in Iberian hares (*Lepus granatensis*)

García-Bocanegra, I., Camacho-Sillero, L., Risalde, M.A., Dalton, K.P., Caballero-Gómez, J., Agüero, M., Zorrilla, I., Gómez-Guillamón, F. (2019). First outbreak of myxomatosis in Iberian hares *(Lepus granatensis).* Transboundary and Emerging Diseases 66(6): 2204-2208. doi:10.1111/tbed.13289).

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Resumen

La mixomatosis es una enfermedad infecciosa causada por el virus de la mixomatosis (MYXV; género *Leporipoxvirus*), que afecta al conejo silvestre europeo (*Oryctolagus cuniculus*) y, esporádicamente, a la liebre parda (*Lepus europaeus*). Aquí, describimos el primer brote de mixomatosis en liebre ibérica (*Lepus granatensis*). Entre mediados de julio y finales de septiembre de 2018, alrededor de 530 liebres ibéricas se localizaron muertas en las poblaciones del sur de España. La aparente mortalidad media fue de un 56,7% y se estimó que la tasa de letalidad media fue de un 69,2%. Los resultados histopatológicos así como moleculares confirmaron la infección por MYXV en todas las liebres analizadas. Según el conocimiento de los autores, este es el primer brote de mixomatosis que causa una alta mortalidad y la primera descripción detallada de dicho brote de mixomatosis en liebre ibérica. La ausencia de casos en la población simpátrica de conejos silvestres sugiere diferencias en la susceptibilidad entre ambas especies de lagomorfos a la cadena del virus implicada en este brote. Después de este primer caso, el número de zonas afectadas incrementó rápidamente afectando a la mayor parte de las zonas donde la liebre ibérica está presente. Se requieren estudios más profundos para elucidar el origen de la implicación de la cadena del MYXV así como comprobar el impacto de este brote en las poblaciones de liebre ibérica.

Summary

Myxomatosis is an infectious disease caused by Myxoma virus (MYXV; genus *Leporipoxvirus*), which affects the European wild rabbit (*Oryctolagus cuniculus*) and sporadically brown hares (*Lepus europaeus*). Here, we describe the first outbreak of myxomatosis in Iberian hares (*Lepus granatensis*). Between mid-July and the end of September 2018, around 530 dead animals were detected in Iberian hare populations in southern Spain. The apparent mean mortality rate was 56.7% and the estimated mean case-fatality rate was 69.2%. Histopathological and molecular results confirmed MYXV infections in all hares analysed. To the authors' knowledge, this is the first myxomatosis outbreak causing a high mortality in hares and the first detailed characterisation of a myxomatosis outbreak in the Iberian hare. The absence of cases in sympatric wild rabbits suggests differences in the susceptibility between both lagomorph species to the virus strain implicated in the outbreak. After the first case, the number of affected areas increased sharply affecting most of the Iberian Peninsula where the Iberian hare is present. Further studies are required to elucidate the origin of the implicated MYXV strain as well as to assess the impact of this outbreak on the Iberian hare populations.

Keywords: *Epidemiology, Iberian hare, myxoma virus, high mortality, Spain.*

Introduction

Myxomatosis is an infectious disease caused by Myxoma virus (MYXV), a member of the genus *Leporipoxvirus* (family *Poxviridae*) mainly transmitted by biting arthropod vectors or through direct contact with infected animals. MYXV was described for the first time in Uruguay in 1896 in domestic European rabbits (*Oryctolagus cuniculus*). The South American forest rabbit (*Sylvilagus brasiliensis*) is the natural host of MYXV and shows subclinical infection or localized cutaneous fibromas. In contrast, the virus causes systemic and often fatal myxomatosis in domestic and European wild rabbits. The clinical disease is characterized by blepharoconjunctivitis, respiratory disorders, cephalic and anogenital oedema, as well as cutaneous pseudotumors termed 'myxomas' (Fenner & Ratcliffe, 1965; Best & Kerr, 2000).

MYXV is considered a classic example of host-pathogen co-evolution following a species jump (Kerr et al., 2015). The virus was introduced illegally into France in 1952 and spread rapidly throughout Europe. Mortality rates of around 90% were initially reported in wild rabbit populations, but substantially declined during the following decades due to an increase in genetic resistance, the development of acquired immunity and by contact with MYXV strains that underwent a progressive attenuation in virulence (Fenner & Fantini, 1999). At present, myxomatosis has become endemic in most European countries, but epizootic outbreaks with high mortality are frequently reported in susceptible rabbits, particularly those from high density localized populations. MYXV infections have been reported sporadically in free-living brown hares (*Lepus europaeus*) and usually associated with high prevalence in sympatric wild rabbit populations (Wibbelt & Frölich, 2005). MYXV infections were confirmed in brown hares in France and Ireland during the 1950s (Lucas, Bouley, Quincon, & Tocas, 1953; Collins, 1955) and, more recently, in Great Britain (Barlow et al., 2014). The aim of this study was to describe the first outbreak of myxomatosis in the Iberian hare (*Lepus granatensis*), the most relevant hare species in terms of population and hunting interests on the Iberian Peninsula.

Material and methods

Between mid-July and the end of September 2018, high mortalities were detected in Iberian hare populations in 12 geographically close hunting areas located in the province of Córdoba (Andalusia, southern Spain) (4° 31' W, 37° 25' N) (Figure 1). An emergency health program was launched by the Epidemiological Surveillance Program for Wildlife (ESPW) of the Regional Government of Andalusia. Epidemiological information was gathered both through an on-site interview of the gamekeepers and by veterinarians belonging to the ESPW by visiting the

affected hunting areas. Data collected included: location, date of onset in clinically affected animals, presence of wild rabbits, myxomatosis in wild rabbit during the outbreak and in the previous year, estimated number of clinically affected animals and mortality, estimated number of hares found dead, hare densities before and after the outbreak. The apparent mortality rate was calculated as the number of deaths associated to myxomatosis divided by the estimated average number of Iberian hares in each hunting area. Case fatality rate at hunting area level was expressed as the proportion of hares found dead by the total number of clinically affected animals.

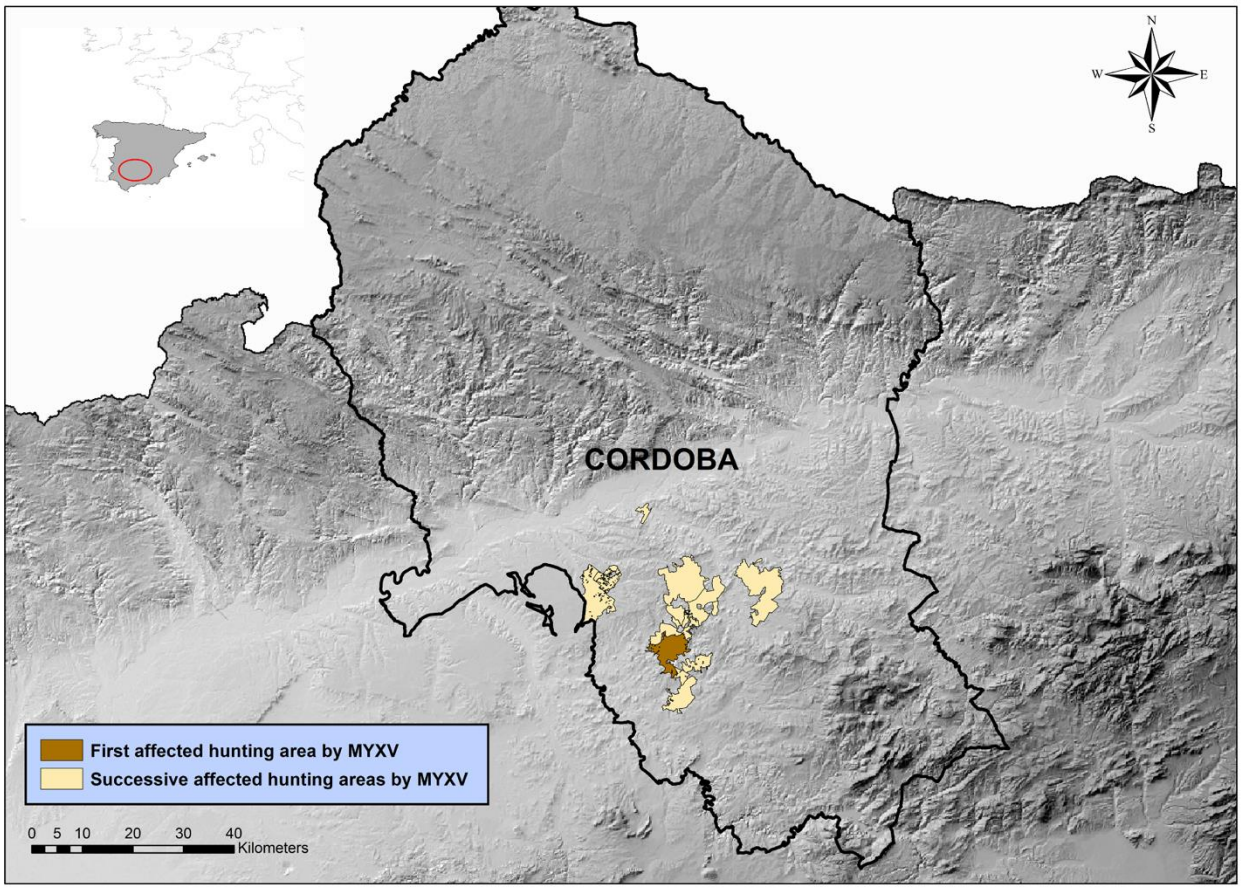

Figure 1. Spatial distribution of the first myxomatosis outbreak in Iberian hares (*Lepus granatensis*) in southern Spain, 2018.

Eighteen dead Iberian hares from the affected hunting areas were sent to the Diagnosis and Analysis Center for Wildlife (Regional Laboratory of Andalusia, Andalusia) and the Animal Health laboratory (University of Cordoba, Spain) for postmortem examination, histopathological (formalin-fixed tissues embedded in paraffin for haematoxylin and eosin staining), microbiological (strain culture, isolation and identification), parasitological (flotation, MacMaster and sedimentation methods) and toxicological (gas chromatography-mass spectrometry (GC-MS/MS) or ultra-performance liquid chromatography mass spectrometry (UHPLC-MS/MS)) analyses. In

parallel to these analyses, eyelid, liver and spleen samples were also sent to the Central Veterinary Laboratory in Algete (Spanish National Reference Animal Health Laboratory) for the diagnosis of myxomatosis using PCR (Cavadini, Botti, Barbieri, Lavazza, & Capucci, 2010), rabbit haemorrhagic disease (RHD) and European brown hare syndrome (EBHS) by real time RT-PCR (Ros Bascuñana, Nowotny, & Bélak, 1997; Dalton et al., 2015; Velarde et al., 2016) and tularemia using culture (OIE, 2018) and PCR (Versage, Severin, Chu, & Petersen, 2003).

Results and discussion

The first cases were detected on the 10th July and the last dead hares were observed at the end of September. During the study period, around 530 hares were found dead with lesions compatible with MYXV infection. The apparent mean mortality rate was 56.7% (20-80%) and the estimated mean case-fatality rate was 69.2%. The spatial distribution was homogeneous throughout the affected hunting areas.

The main external macroscopic lesions observed in the analysed hares included blepharitis, blepharoconjunctivitis, epistaxis and inflammation and oedema around the nasal, oral (Figure 2A), anal and genital orifices, as well as rectal bleeding in some cases. Myxomas were not found at the base of ears, eyelid or other areas of the skin. Internal organs showed a severe and generalized congestion, which was also observed in the subcutaneous tissue. Other vascular lesions were present such as a severe alveolar oedema and haemorrhages in several organs and body cavities. Histopathological examination revealed that the epidermis with predominant hyperkeratosis was generally hyperplastic and invading the dermis at eyelid, where neutrophil infiltrates with bacterial colonies were occasionally observed. In this tissue, the keratinocytes showed widespread hydropic degeneration and contained eosinophilic cytoplasmic inclusion bodies surrounded by a clear halo (Figure 2B, inset). The dermis was characterized in most animals by a loosely arranged slightly basophilic myxoid matrix admixed with edematous areas, and with the presence of inflammatory infiltrates of mixed type (macrophages, lymphocytes and polymorphonuclear cells). The vascular histopathological findings agreed with the macroscopic lesions, a severe congestion and hyperemia of the organs being observed, especially in lungs (Figure 2C) where alveolar oedema and hemorrhages were present (Figure 2D). A severe depletion of lymphocytes was noted in the spleen.

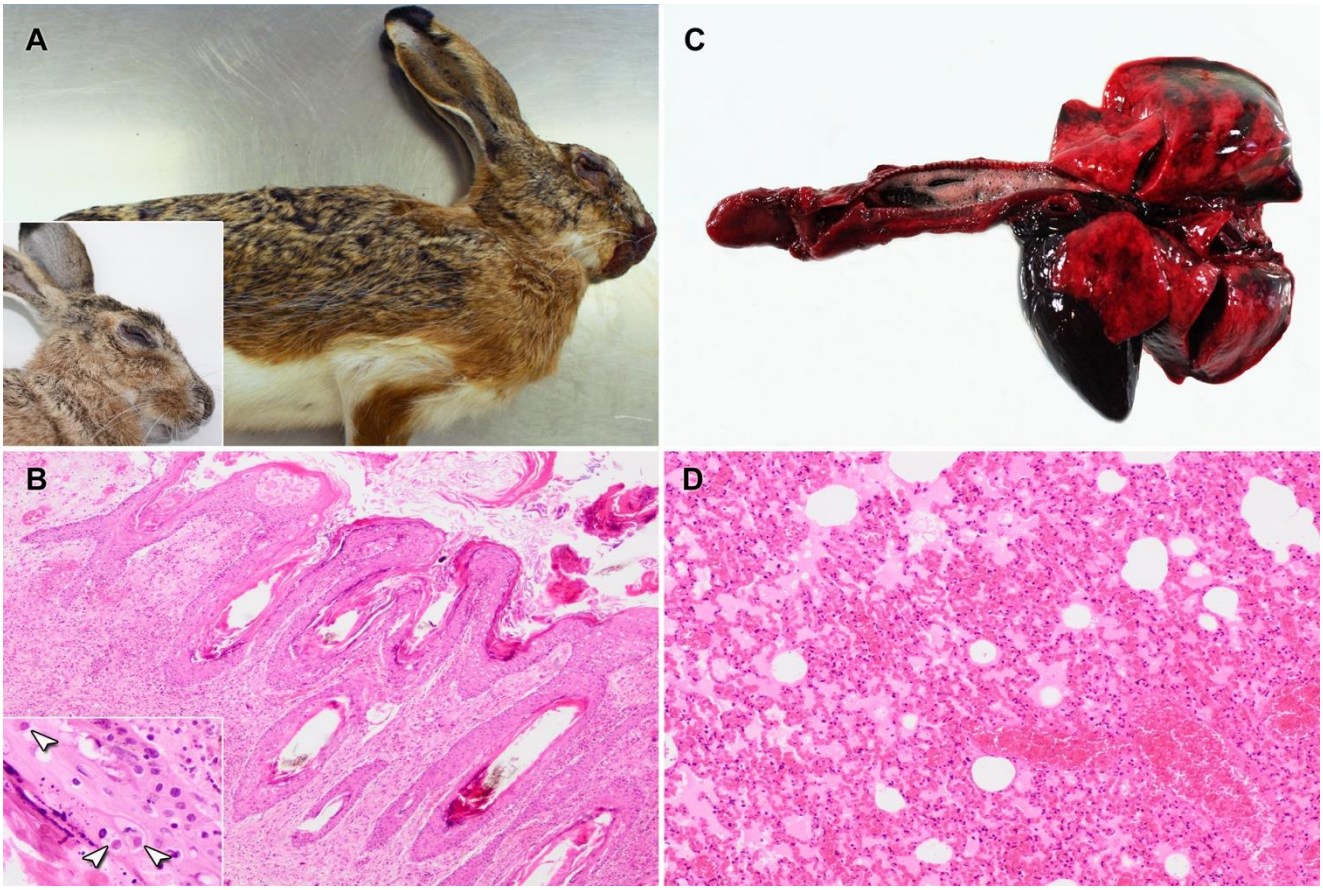

Figure 2. Myxomatosis in Iberian hare (*Lepus granatensis*). A) Epistaxis and blepharitis, which were accompanied by inflammation and oedema around the nasal and oral orifices (inset). B) Hyperkeratosis and hyperplasia of the epidermis (particularly surrounding follicles) in the eyelid, with lack of normal stratification and ballooning degeneration of the epidermal cells, as well as intracytoplasmic viral inclusions that peripheralized the nucleus (arrowheads in the inset). C) Severe congestion of the respiratory tract and heart, as well as pulmonary haemorrhages and oedema. D) Severe congestion and alveolar oedema in the lung, together with mild haemorrhages.

Staphylococcus aureus and *Pasteurella multocida* were isolated in skin lesions and lung in four and two hares, respectively. Parasitological analyses showed *Eimeria* spp. in five hares with the number of oocysts ranging between 1600 and 35000 per gram of faeces. A total of 100 and 1950 eggs per gram of faeces of *Trichostrongylus* spp. were also found in two hares. Toxicological analyses showed negative results for 318 pesticide and rodenticide compounds analysed. All hares showed negative results for RHD virus, EBHS virus and *Francisella tularensis*. MYXV DNA was detected in the 18 animals tested.

Histopathological and molecular results demonstrated MYXV infections in all Iberian hares analysed. Pathological findings were compatible with an acute or hyperacute presentation of the

amyxomatous forms of the disease (atypical myxomatosis). This form is characterized by intense vascular changes and reduced cutaneous clinical signs with myxomas replaced by diffuse swelling of the eyelids (oedematous blepharitis) and sometimes of the cephalic and anogenital areas (Joubert, Duclos, & Tuaillon, 1982; Marlier, Mainil, Linde, & Vindevogel, 2000a). Moreover, the atypical form is usually characterized by intense respiratory distress (Marlier et al., 2000a,b), coinciding with the intense pulmonary oedema and hemorrhages observed in the Iberian hares. These findings contrast with the previously reported in European hare, in which subclinical (Collins, 1955) or myxomatous forms had been described (Barlow et al., 2014), and even its potential role as carrier of the virus had been suggested.

On the Iberian Peninsula, where the European wild rabbit is a keystone species in Mediterranean ecosystems (Delibes-Mateos, Redpath, Angulo, Ferreras, & Villafuerte, 2007), myxomatosis caused significant changes in their populations with reduction of densities close to the extinction in some areas (Calvete, Estrada, Villafuerte, Osácar, & Lucientes, 2002). In the affected area, MYXV is currently endemic with seropositivity higher than 50% in wild rabbit populations and epizootic outbreaks reported during summer and autumn (García-Bocanegra et al., 2010). Interestingly, even though the temporal evolution of the outbreak in Iberian hares was similar to that reported in wild rabbits in previous years, myxomatosis cases were not observed in this species during the study period. Additional research is required to determine the origin of the MYXV strain implicated in the outbreak as well as to elucidate the high susceptibility of the Iberian hare and the apparent resistance of the European wild rabbit to this strain.

In conclusion, histopathological and molecular results confirmed MYXV infection in all hares analysed. To the best of our knowledge, this is the first myxomatosis outbreak causing a high mortality in hares and the first detailed description of myxomatosis in Iberian hare. After the first cases were confirmed, the number of affected areas increased sharply affecting most of the Iberian Peninsula where the Iberian hare is present (RASVE, 2019). The absence of myxomatosis cases in sympatric wild rabbits suggests differences in the susceptibility to the MYXV strain implicated in the outbreak between these lagomorph species. Surveillance programs should be also implemented to assess the impact of the outbreak in the Iberian hare populations and for the early detection of MYXV in this species on the Iberian Peninsula.

Conflict of interest statement

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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CAPÍTULO 4

Monitoring of emerging myxoma virus epidemics in Iberian hares (*Lepus granatensis*) in Spain, 2018-2020

García-Bocanegra, I., Camacho-Sillero, L., Caballero-Gómez, J., Agüero, M., Gómez-Guillamón, F., Ruiz-Casas, J.M., Díaz-Cao, J.M., García, E., Ruano, M.J., de la Haza, R. (2020). Monitoring of emerging myxoma virus epidemics in Iberian hares *(Lepus granatensis)* in Spain, 2018-2020.

This chapter is prepared in the style format of *Transboundary and Emerging Diseases*

Resumen

La mixomatosis es una enfermedad infecciosa causada por el mixoma virus (MYXV), que ha causado altos grados de mortalidad en la población de conejo silvestre europeo (Oryctolagus cuniculus). A pesar de que se han detectado algunos casos esporádicos de mixomatosis en algunas especies de liebres, se considera que estos lagomorfos tienen una baja susceptibilidad a la infección por MYXV. En el presente estudio, describimos la evolución espacio-temporal y los principales hallazgos epidemiológicos del nuevo virus de la mixomatosis de liebre (ha-MYXV o MYXV-Tol) epidémico en liebre ibérica (*Lepus granatensis*) en España. Durante el periodo entre 2018-2020, se confirmó mediante PCR la infección por MYXV en un total de 487 liebres de 372 zonas. Los brotes por ha-MYXV fueron detectados en la mayor parte de las regiones de España donde la liebre ibérica está presente. La distribución espacial no fue homogénea, con una mayoría de brotes concentrada en las partes del centro y sur de España. Los consecutivos brotes comunicados en los últimos dos años sugieren una circulación endémica de este virus emergente en España. Un estudio retrospectivo llevado a cabo justo después del primer periodo epidémico (2018-2019) reveló que el virus podía haber estado circulando desde junio de 2018. El número de brotes empezó a crecer en julio, con un pico durante la primera mitad de agosto y octubre y un descenso acusado hasta enero de 2019. La mortalidad media aparente fue de un 55,4% (mediana: 70%). Los resultados indicaron una alta susceptibilidad de la liebre ibérica a la infección por ha-MYXV pero una aparente resistencia de las especies simpátricas en España y una menor infectividad en el conejo silvestre. El nuevo ha-MYXV está teniendo unas consecuencias significativas en el estatus sanitario de las poblaciones de liebre ibérica en España, en lo concerniente a salud y conservación de la especie. El presente estudio contribuye a un mejer entendimiento del emergente ha-MYXV y da información valiosa sobre el desarrollo de estrategias de control. Son necesarias investigaciones más profundas para estableces el impacto de este virus emergente en las poblaciones de lagomorfos silvestres y así poder elucidar las implicaciones ecológicas que conllevan en los ecosistemas mediterráneos ibéricos.

Abstract

Myxomatosis is an infectious disease caused by the myxoma virus (MYXV), which has very high mortality rates in European wild rabbits (*Oryctolagus cuniculus*). While sporadic cases of myxomatosis have also been reported in some hare species, these lagomorphs are considered to have a low susceptibility to MYXV infection. In the present study, we describe the spatio-temporal evolution and main epidemiological findings of novel hare MYXV (ha-MYXV or MYXV-Tol) epidemics in Iberian hares (*Lepus granatensis*) in Spain. In the period 2018-2020, a total of 487 hares from 372 affected areas were confirmed to be MYXV-infected by PCR. ha-MYXV outbreaks were detected in most of the Spanish regions where the Iberian hare is present. The spatial distribution was not homogeneous, with most outbreaks concentrated in the southern and central parts of Spain. Consecutive outbreaks reported in the last two years suggest endemic circulation in Spain of this emerging virus. A retrospective study carried out just after the first epidemic period (2018-2019) revealed that the virus could have been circulating since June 2018. The number of outbreaks started to rise in July, peaked during the first half of August and October and then decreased sharply until January 2019. The apparent mean mortality rate was 55.4% (median: 70%). The results indicated high susceptibility of the Iberian hare to ha-MYXV infection, but apparent resistance in the sympatric hare species present in Spain and less infectivity in European rabbits. The novel ha-MYXV has had significant consequences on the health status of Iberian hare populations in Spain, which is of animal health and conservation concern. The present study contributes to a better understanding of ha-MYXV emergence and will provide valuable information for the development of control strategies. Further research is warranted to assess the impact of this emerging virus on wild lagomorph populations and to elucidate its ecological implications for Iberian Mediterranean ecosystems.

Keywords: Myxomatosis; ha-MYXV; Iberian hare; Epidemic; Spain

Introduction

The Iberian hare (*Lepus granatensis*) is an endemic species in the Iberian Peninsula and one of the most representative wild lagomorphs in terms of abundance and hunting interest. This species plays a key role in the ecology of Iberian Mediterranean ecosystems, being the staple prey of a large number of predators, including endangered species such as the Iberian lynx (*Lynx pardinus*), Iberian wolf (*Canis lupus signatus*) and the Spanish imperial eagle (*Aquila adalberti*) (Purroy, 2011). The Iberian hare is also among the main small game species, with about 930,000 animals harvested annually in Spain (MAPA, 2020). Although the information about the population densities of Iberian hares in Spain is limited, in some regions their densities have remained stable at local sites, whereas in others, there has been a decreasing trend in population size in recent decades (Ballesteros, Benito, & González-Quirós, 1996; Carro & Soriguer, 2017). Conservation of the Iberian hare is threatened by different natural and anthropogenic factors, including predators and hunting pressure, fragmentation and loss of habitat, use of herbicides and pesticides, weather conditions, roadkill and disease (Duarte, 2000; García-Bocanegra et al., 2019; Sánchez-García et al., 2012). This species has been shown to be susceptible to different infectious and parasitic diseases (Fernández-Aguilar et al., 2013; Ruiz-Fons, Ferroglio, & Gortázar, 2013; Sánchez-García et al., 2012). On the other hand, several pathogens that affect other hare species (Wibbelt & Frölich, 2005) have not been detected in the Iberian hare, although the available information about its health status is still very scarce.

Myxomatosis is an infectious disease caused by the myxoma virus (MYXV; family *Poxviridae*; Genus *Leporipoxvirus*), which is mainly transmitted by biting arthropods or direct contact with infected animals (Kerr, 2012). MYXV is considered a classic example of host-pathogen coevolution following a species jump (Alves et al., 2019; Kerr et al., 2015). MYXV infection induces benign cutaneous fibromas in its natural host, the South American forest rabbit (*Sylvilagus brasiliensis*), while it causes severe and often fatal disease in European rabbits (*Oryctolagus cuniculus*) (Bertagnoli & Marchandeau, 2015). After MYXV was illegally introduced into France in 1952, the virus spread rapidly throughout Europe causing mortality rates of up to 90% in wild rabbit populations (Fenner & Ratcliffe, 1965). Myxomatosis is currently endemic in most European countries, including Spain, with annual epizootic cycles causing high mortality in susceptible domestic and wild rabbit populations (Villafuerte et al., 2017). Even though hare species are considered to be mostly resistant to MYXV infection, sporadic cases of myxomatosis have been reported in the European brown hare (*Lepus europaeus*) and mountain hare (*Lepus timidus*)

(reviewed in Kerr et al., 2015). Between mid-July and the end of October 2018, high mortalities associated with a novel recombinant MYXV strain (ha-MYXV or MYXV-Tol) were detected in Iberian hare populations in Spain and Portugal (García-Bocanegra et al., 2019; OIE, 2018). Molecular studies revealed that an insertion or recombination event with respect to the MYXV Lausanne reference strain may have been involved in the cross-species jump and increased virulence in its new host (Águeda-Pinto et al., 2019; Dalton et al., 2019). The main aim of the present study was to describe the spatio-temporal evolution and main epidemiological findings of the ha-MYXV epidemics in Iberian hares in Spain in the period 2018-2020.

Materials and Methods

After the first outbreaks of the novel ha-MYXV isolate in an Iberian hare population in Andalusia (southern Spain) were notified on 10 July 2018 (García-Bocanegra et al., 2019), a national passive surveillance program, coordinated by the Spanish Ministry of Agriculture, Fisheries and Food, was launched across Spain. Between July 2018 and April 2020, a total of 372 hunting estates and protected areas that reported cases compatible with myxomatosis in Iberian hares in this country were investigated/surveyed. The study period was divided into two consecutive epidemic periods: P1, between July 2018 and April 2019, and P2, between May 2019 and April 2020).

Whenever possible, between one and 16 (mean = 3) full carcasses or eyelid samples were collected from clinically affected Iberian hares and wild rabbits found dead in each investigated area. Only samples from animals with the presence of lesions compatible with myxomatosis were collected. Clinically affected hunted hares were also included, although most of the samples (92%) were from animals that were found dead. During the study period, a total of 1404 and 47 samples of Iberian hares and wild rabbits, respectively, were obtained by the authors, generally in collaboration with the gamekeepers, and sent to the Animal Health laboratory (University of Cordoba, Spain) (93 fresh full carcasses of Iberian hares) for postmortem examination and sampling of carcasses, or directly to the Central Veterinary Laboratory in Algete (Spanish National Reference Animal Health Laboratory) for molecular analysis. Total DNA was extracted with the MagAttract® 96 Cador® Pathogen Kit (QIAGEN, Germany) following the manufacturer's instructions. For the detection of DNA of both the classical MYXV strains and the novel ha-MYXV isolate, a conserved region of the M071L or M005L/R gene was amplified by PCR or real time PCR, respectively, as previously described (Cavadini, Botti, Barbieri, Lavazza, & Capucci, 2010; Dalton et al., 2019; Duarte et al., 2014, 2015). Using TaKaRa LA Taq DNA polymerase (TaKaRa, Japan), a

specific ha-MYXV PCR was carried out with forward and reverse primers M009L-F (5'-CGCAGGTCCACGTATAAACC -3') and M009L-R (5'- CGAACGTATCATTAGACAATG -3') (Dalton et al., 2019). Data on location and date of sampling were also gathered from each surveyed area.

Additional epidemiological information was gathered in P1 through on-site interviews with gamekeepers at the investigated areas, using a standardized questionnaire. In most cases, this information was also verified by the authors during the visit. For each investigated area, the following data were recorded: location data, date of onset and end of clinically affected animals, clinical signs observed, clinical cases of myxomatosis in sympatric wild rabbits during or before the outbreak, estimated number of affected or dead Iberian hares, estimated number of clinically affected animals, estimated mortality rate, hare densities before the outbreak and the presence of other affected hare species.

In the present study, an outbreak was defined as an investigated area with at least one Iberian hare infected by ha-MYXV and confirmed by PCR. Investigated areas where Iberian hare mortality compatible with myxomatosis was observed but could not be confirmed by laboratory analysis due to the absence of samples were considered suspected areas.

Results

Between July 2018 and April 2020, a total of 487 Iberian hares from 372 investigated areas were shown to be positive for ha-MYXV infection by PCR. The classical MYXV strain infection was not detected in the Iberian hares analyzed. In the 372 ha-MYXV-confirmed areas, 210 outbreaks were detected in P1, 162 in P2, and ha-MYXV outbreaks were detected in 16 of these positive areas in both periods. ha-MYXV outbreaks were confirmed in 141 localities in 11 of 17 Spanish regions. A total of 78 and 63 ha-MYXV- positive localities were detected in P1 and P2, respectively, and in 35 of these positive localities, ha-MYXV outbreaks were successively reported in both study periods (Figure 1). The spatial distribution of ha-MYXV was not homogeneous across Spain; the highest numbers of outbreaks were reported in southern and central regions.

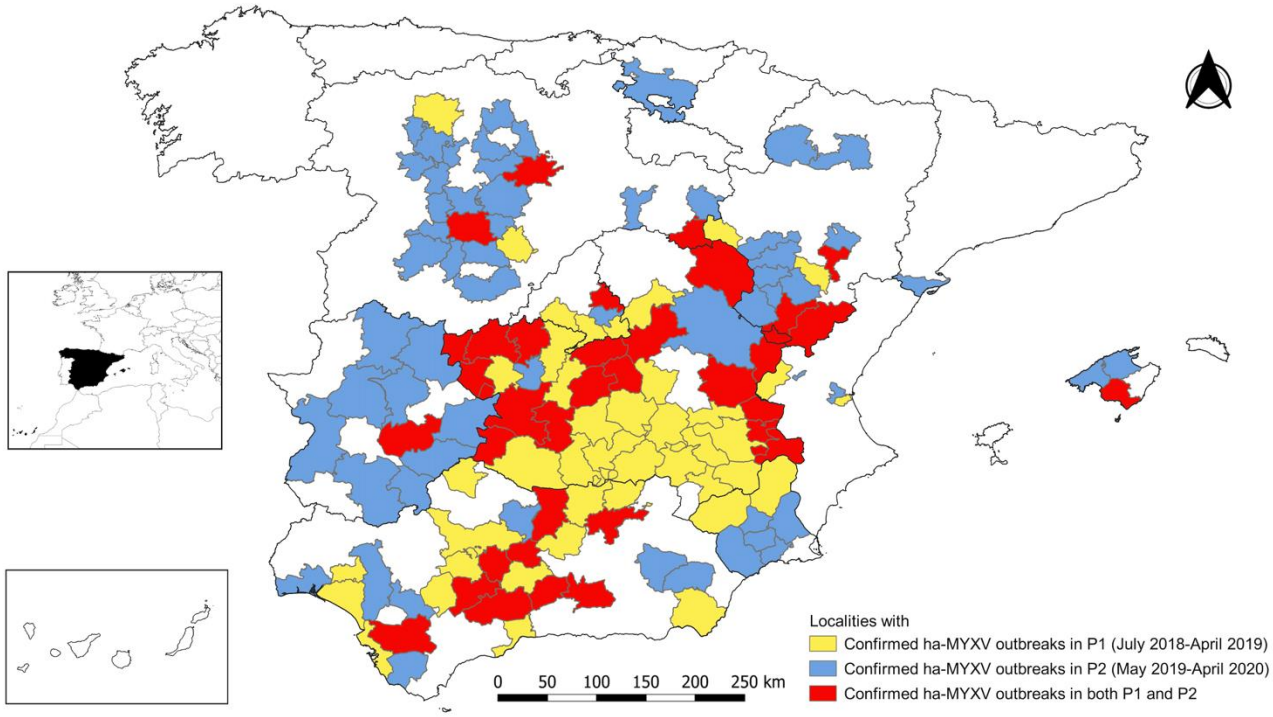

Figure 1. Spatiotemporal distribution of ha-MYXV outbreaks at regional level in Spain during the period 2018-2020.

During P1, epidemiological information was obtained by questionnaire in 312 investigated areas: 176 were confirmed as ha-MYXV-positive areas and the remaining 136 were considered as suspected areas. The first clinically affected Iberian hares were observed on 20 June 2018 on a hunting estate in the province of Cuenca (central Spain). The number of outbreaks started to rise from July, peaked during the first half of August and October and then decreased sharply until January 2019. The last sick animals were observed in early March 2019 on a hunting estate located in Valladolid province (northwest Spain) (Figure 2). In most of the surveyed areas (74.7%), the first clinically affected hares were observed between mid-July and mid-October 2018. In 58.9% of investigated areas in P1, the maximum number of cases was reported between early September and mid-October of the same year (Figure 3). In the surveyed areas, the mean interval between the first and maximum number of cases was 31.3 days and ranged between 0 and 73 days. The mean duration of outbreaks in P1 was 115 days (ranging between 3 and 406 days). A decreasing trend in mean duration of the outbreaks was observed, falling from 167 days in July 2018 to 30 days in January 2019 (Figure 4).

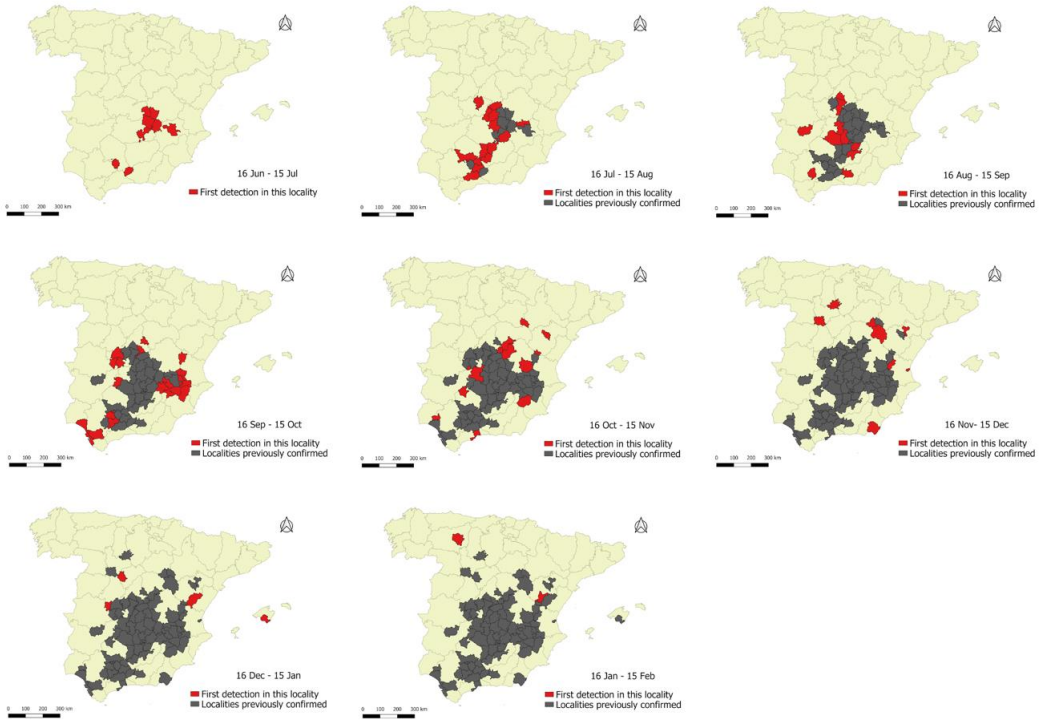

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Figure 2. Spatio-temporal evolution of ha-MYXV outbreaks at regional level in Iberian hares in Spain during P1, 2018-2019.

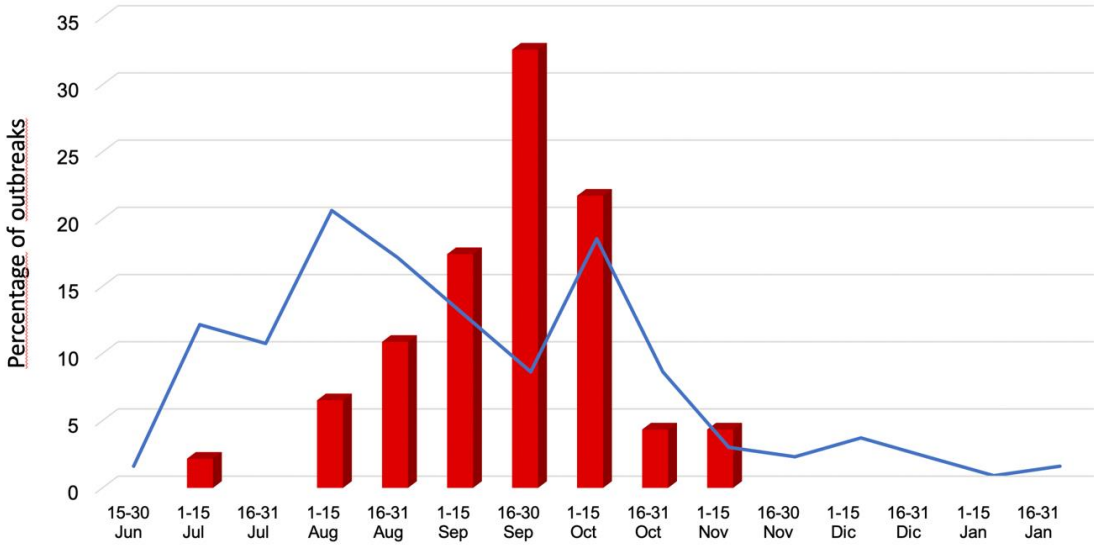

Figure 3. Temporal evolution (by fortnight) of the first (line) and maximum number (bars) of ha-MYXV outbreaks in Iberian hares in Spain during P1, 2018-2019.

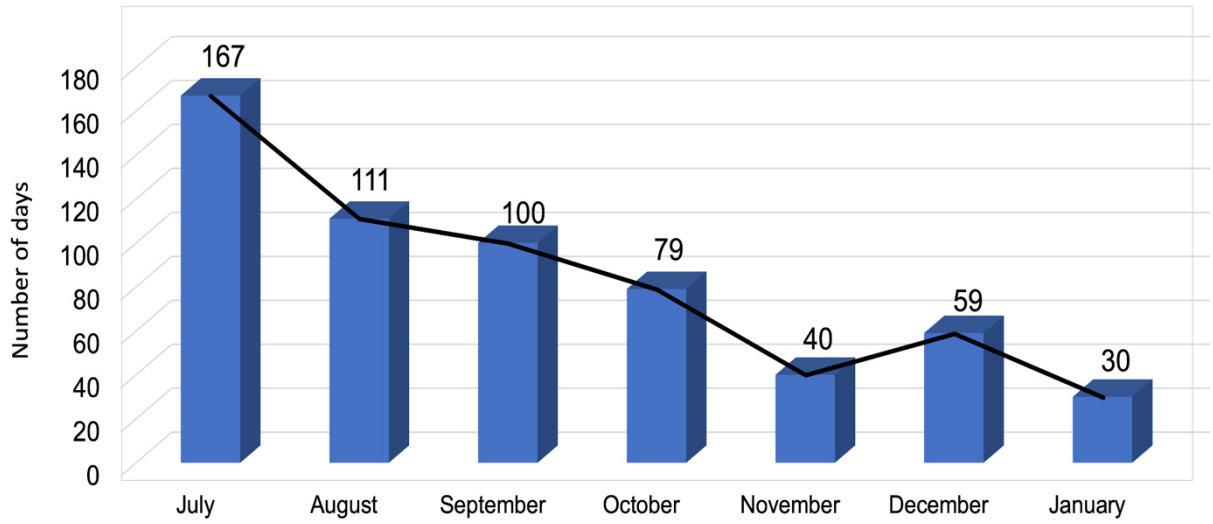

Figure 4. Mean duration (in days) of ha-MYXV outbreaks according to month of onset of the first myxomatosis case in Iberian hares in Spain during P1, 2018-2019.

The frequency of clinical signs associated with ha-MYXV infection in Iberian hares in Spain during P1 is shown in Figure 5. The clinical signs most commonly observed by the interviewed gamekeepers were conjunctivitis (63.4%), myxomas (33.5%), blepharitis (32.9%) and anogenital swelling (20.2%). Cachexia, epistaxis, prostration, dyspnea, convulsions, paralysis, diarrhea, sudden death and opisthotonos were also observed. The number of animals with clinical signs compatible with myxomatosis was similar between males and females in most of the surveyed areas (77.6%). In 53.8% of surveyed areas, adult animals were found to be more frequently affected than juveniles, in 36.5% of affected areas, the distribution across age classes was similar, whereas in the remaining 9.6% of areas, the gamekeepers interviewed indicated that juvenile Iberian hares were the most frequently affected animals.

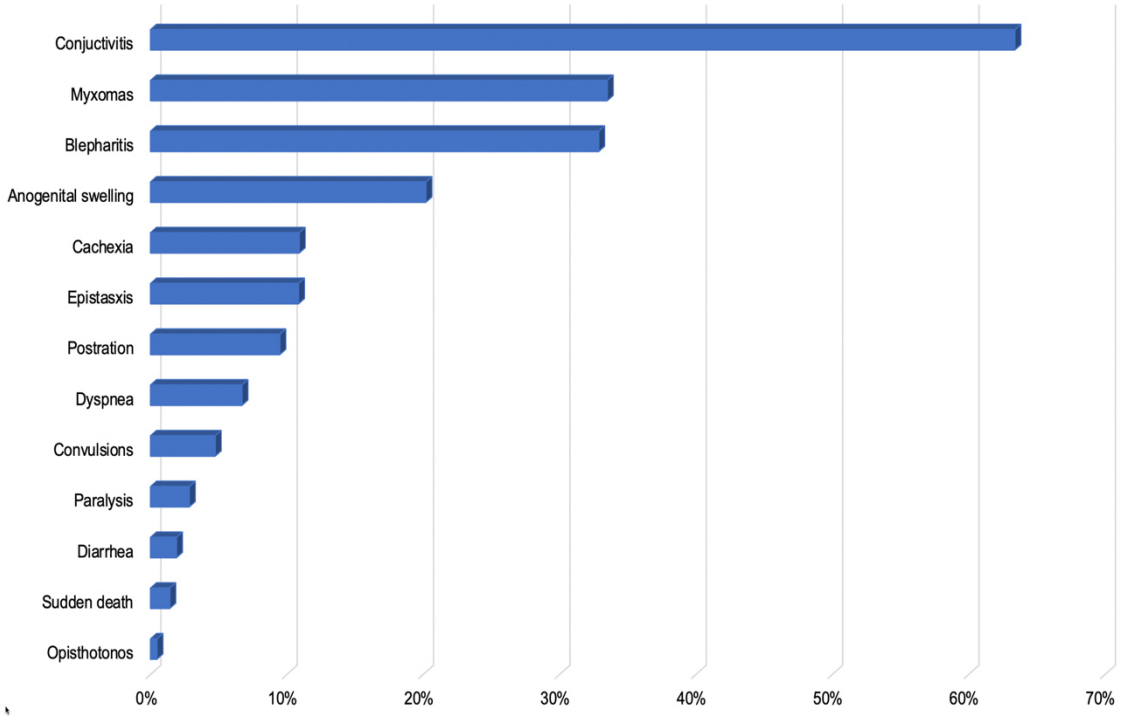

Figure 5. Percentage of clinical signs associated with ha-MYXV outbreaks in Iberian hares in Spain during P1, 2018-2019.

During P1 and P2, neither clinical cases nor mortality compatible with myxomatosis were reported in the other two hare species present in Spain: the European brown hare and the Broom hare (*L. castroviejoi*). Even though most of the surveyed areas (75.7%) had myxomatosis outbreaks in wild rabbits in the two years preceding P1, cases of disease were only observed in this species in 27% of areas investigated in this first epidemic period. A total of 47 clinically affected wild rabbits (seven sampled in P1 and 40 in P2) from localities with ha-MYXV cases in Iberian hares were also analyzed by PCR to detect MYXV DNA. Classical MYXV strain infection was detected in 20 rabbits, whereas ha-MYXV DNA was confirmed in two animals. ha-MYXV-infected rabbits were sampled in August 2018 and November 2019 in the provinces of Toledo and Cuenca (both central Spain), respectively.

During P1, a total of 10,297 Iberian hares (mean: 40.4 hares/affected area) were estimated to have been found dead in the field by interviewed gamekeepers. The estimated number of sick live hares was 900 (mean: 3.5 hares/affected area). The estimated mean mortality rate was 55.4% (ranging between 0 and 100%). Apparent mortality rates were above 70% in 38% of the surveyed areas, between 15 and 70% in 38% of areas, and below 15% in the remaining 24% of areas. The

spatial distribution of cases within the affected areas was homogeneous in most of the areas surveyed (83.5%).

Discussion

In the present large-scale study, we describe the evolution and main findings of the first myxomatosis epidemic causing high mortality in hares worldwide. Between July 2018 and April 2020, a total of 372 ha-MYXV outbreaks were confirmed in Iberian hares, providing evidence of the cross-species transmission event by MYXV (Águeda-Pinto et al., 2019; Dalton et al., 2019). In spite of the fact that the number of cases may be underestimated as a result of the difficulties of finding dead hares in the field, the detection of ha-MYXV-infected hares in most of the Spanish regions where this species is present further highlights the widespread dispersal of this novel virus in Spain. The occurrence of outbreaks was not homogeneous, since most were concentrated in southern and central parts of Spain. Differences in hare population densities, climatic and environmental conditions or surveillance efforts are possible factors accounting for the geographical variations observed (Villafuerte et al., 2017).

The high number of outbreaks reported in P1 and P2 as well as the cases detected in both periods consecutively in 35 localities (Figure 1) suggests endemic circulation of ha-MYXV in Iberian hare populations in Spain in the last two years. This finding is consistent with the endemic occurrence of myxomatosis in wild rabbit populations in recent decades (Calvete, Estrada, Villafuerte, Osácar, & Lucientes, 2002; Villafuerte et al., 2017). While the first confirmed outbreak was notified in July 2018 on a hunting estate in Andalusia (southern Spain) (García-Bocanegra et al., 2019), the information obtained from the areas investigated in the present study suggests that the virus could have been circulating in other Spanish regions at least one month before that, since mortality compatible with myxomatosis was observed in June 2018 in two affected areas in the provinces of Cuenca and Toledo (both central Spain).

Peak incidence was observed in summer and autumn, which is consistent with the temporal distribution of myxomatosis in wild rabbits (Calvete et al., 2002; Ferreira et al., 2009; Villafuerte et al., 2017). This temporal evolution as well as a decreasing trend in mean outbreak duration during P1 may be related to the greater abundance of competent vectors during the summer season. It has been shown that *Xenopsylla cunicularis,* a potential myxomatosis vector, is the most abundant flea species in wild rabbits in Spain, with the highest abundance index detected during the summer months (Osácar et al., 2001). Nevertheless, the high spatiotemporal dissemination of the ha-MYXV outbreaks suggests that other competent vectors may also be

responsible for long distance spread to isolated populations. In this context, the role of *Culicidae* in MYXV transmission has previously been documented (Fenner & Racliffe, 1965; Merchant et al., 2003; Ross & Tittensor, 1986), since some species are able to travel long distances and keep the MYXV active for long periods (Fouchet, Guitton, Marchandeau, & Pontier, 2008). High mosquito density has been shown to be a risk factor for MYXV exposure in wild rabbits in southern Spain (García-Bocanegra et al., 2010). Interestingly, ha-MYXV outbreaks were reported in the Balearic Islands in both P1 and P2. Taking into account the distance between that region and mainland Spain (more than 200 km), it seems unlikely that ha-MYXV was introduced by infected mosquitoes carried on the wind. Transportation of infected vectors by ship, aircraft or fomites, and restocking with infected hares from mainland Spain for hunting purposes are possible hypotheses for ha-MYXV introduction into these islands. In any case, since the reason for the widespread distribution of this virus remains uncertain, so that entomological surveillance programs and molecular analyses of potential competent vector species of ha-MYXV are needed to elucidate sources of transmission in the Iberian hare populations.

Clinical signs observed in the affected hares were similar to those found previously in this species during the first ha-MYXV outbreaks in Spain and Portugal (Águeda-Pinto et al., 2019; Carvalho et al., 2020; García-Bocanegra et al., 2019) and also to classic rabbit myxomatosis (Calvete et al., 2002; Rosell et al., 2019), but contrast with those reported in the European hare, in which subclinical or mild myxomatosis has been described (Barlow et al., 2014; Collins, 1955). The detection of clinical ha-MYXV infection exclusively in Iberian hares indicates apparent resistance among other hare species present in mainland Spain, which is consistent with previous reports (Barlow et al., 2014; Kerr et al., 2015). It should be noted that in some affected areas in northern Spain, the Iberian hare and European hare are sympatric species (Gortázar et al., 2007). The hypothesis about differences in ha-MYXV susceptibility between lagomorphs is also supported by the limited number of myxomatosis cases observed in wild rabbits in the surveyed areas. Consistent with our results, ha-MYXV infections have been detected in Iberian hares but not wild rabbits also in Portugal (Carvalho et al., 2020; OIE, 2019). Nevertheless, the susceptibility of the wild rabbit to this novel virus cannot be totally ruled out, since ha-MYXV DNA was confirmed in two animals sampled during the study period. Furthermore, an ha-MYXV outbreak causing high mortality was also confirmed on a domestic rabbit farm in Murcia province (southeastern Spain) in October 2019 (MAPA, unpublished data). These findings raise questions on ha-MYXV cross-transmission between Iberian hares and European rabbits. Additional experimental and phylogenetic studies would provide valuable information about the origin and evolution of this

emerging virus, as well as elucidate the direction of interspecies transmission (rabbit-hare *vs* hare-rabbit).

Gamekeeper estimates of mean apparent mortality in P1 (55.4%) were very similar to the 56.7% obtained by García-Bocanegra et al. (2019) during the first outbreaks. Mortality of more than 70% was detected in 38% of the investigated areas, which is consistent with the high mortality rates observed after the introduction of MYXV in wild rabbits in Europe in the early 1950s (Fenner & Racliffe, 1965). The larger number of hares found dead compared to the number of sick animals observed in P1 could be associated with acute or hyperacute forms of myxomatosis in Iberian hare populations, as has been suggested previously (Carvalho et al., 2020). ha-MYXV infections were observed in individuals of different sexes and age classes. Similar ha-MYXV exposure levels between sexes in most of the affected areas have also been previously reported during the epizootics of myxomatosis in wild rabbits in Spain (Calvete et al., 2002; García-Bocanegra et al., 2010). In 53.8% of the surveyed areas, interviewed gamekeepers pointed out that more adults than juvenile hares were affected, which contrasts with the higher resistance to MYXV infection reported in adult European rabbits (Calvete et al., 2002; Fenner & Ross, 1994; Villafuerte et al., 2017). This finding could be explained, at least in part, by the absence of immunity to ha-MYXV infection in Iberian hare populations, as well as the fact that it is more difficult to find juveniles in the field than adult hares.

In conclusion, our results provide evidence of the rapid and widespread distribution of ha-MYXV in Iberian hare populations in Spain, which is of both animal health and conservation concern. The high number of outbreaks detected consecutively in P1 and P2 indicates active endemic circulation of this novel virus in this country in the last two years. The limited number of myxomatosis cases among sympatric wild rabbits, as well as the absence of outbreaks in other hare species, suggests differences in susceptibility to ha-MYXV between lagomorph species. The results obtained contribute to a better understanding of ha-MYXV emergence and provide valuable information for the development of control strategies. Risk-based surveillance programs, captivity breeding, controlled sanitary restocking, specific vaccination programs against ha-MYXV, reduced hunting pressure and elimination of hares found dead in the field, are possible measures that could help limit the circulation of ha-MYXV in Iberian hare populations. Further studies are warranted to assess the impact of this emerging virus on the health status of wild lagomorph species and to elucidate its ecological implications for Iberian Mediterranean ecosystems.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Ethical Statement

Ethical statement is not applicable as samples were collected from dead animals or from animals legally hunted by authorized hunters with the correct permits and license and with the permission of landowners. This study did not involve purposeful killing of animals.

Data Availability Statement

The data that support the findings of this study are available from the authors upon reasonable request

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CONCLUSIONES/CONCLUSIONS

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PRIMERA. El programa de vigilancia pasiva realizado en conejo silvestre evidenció que la nueva variante del virus de la enfermedad hemorrágica del conejo (GI.2) presentó una elevada diseminación en Andalucía tras su aparición en el año 2013. La detección de brotes consecutivos durante el periodo 2013-2017 indica una circulación activa y endémica del GI.2 en las poblaciones de conejos silvestres en esta región ***(Capítulo 1. Camacho-Sillero, L., Caballero-Gómez, J., Gómez-Guillamón, F., Martínez-Padilla, A., Agüero, M., San Miguel, E., Zorrilla, I., Rayas, E., Talavera, V., García-Bocanegra, I. (2019). Monitoring of the novel rabbit haemorrhagic disease virus type 2 (GI.2) epidemic in European wild rabbits (Oryctolagus cuniculus) in southern Spain, 2013-2017. Veterinary Microbiology 237:108361. doi: 10.1016/j.vetmic.2019.07.013.).***

SEGUNDA. El programa de vigilancia activa llevado a cabo en conejo silvestre en Andalucía reveló una elevada circulación (59,9% de seroprevalencia y 8,8% de prevalencia de infección) así como una amplia, pero heterogénea distribución del virus mixoma en las poblaciones de este lagomorfo durante el periodo 2009-2021. Se observaron fluctuaciones estacionales e interanuales en la seroprevalencia, lo que podría incrementar el riesgo de circulación del virus en las poblaciones no inmunizadas ***(Capítulo 2. Camacho-Sillero, L., Cardoso, B., Beato-Benítez, A., Gómez-Guillamón, F., Díaz-Cao, JM., Jiménez Martín, D., Caballero-Gómez, J., Castro-Scholten, S., Cano-Terriza, D., García-Bocanegra, I. (2022). Spatiotemporal monitoring of myxomatosis in European wild rabbit (Oryctolagus cuniculus) in Spanish Mediterranean ecosystems. Transboundary and Emerging Diseases. En revisión).***

TERCERA. Los principales factores de riesgo asociados a la exposición al virus mixoma en conejo silvestre en ecosistemas mediterráneos del sur de España son: estación (otoño), edad (adultos y juveniles), presencia de brotes durante el mes previo al muestreo, temperatura media anual y seropositividad frente al virus de la enfermedad hemorrágica del conejo. Asimismo, la presencia de brotes durante el mes previo al muestreo, la seropositividad al virus mixoma y la presencia de lesiones compatible con mixomatosis fueron los principales factores asociados a la infección por el virus mixoma ***(Capítulo 2).***

CUARTA. El programa de vigilancia pasiva permitió detectar y monitorizar los primeros casos de mixomatosis en liebre ibérica. Los resultados histopatológicos y moleculares confirmaron la infección por virus mixoma en las liebres ibéricas recogidas en diferentes cotos de caza menor de la provincia de Córdoba durante los meses de julio y septiembre de 2018. En las zonas

afectadas, la mortalidad y letalidad media estimadas fueron del 57,6% y del 69,2%, respectivamente ***(Capítulo 3. García-Bocanegra, I., Camacho-Sillero, L., Risalde, M.A., Dalton, K.P., Caballero-Gómez, J., Agüero, M., Zorrilla, I., Gómez-Guillamón, F. (2019). First outbreak of myxomatosis in Iberian hares (Lepus granatensis). Transboundary and Emerging Diseases 66(6): 2204-2208. doi:10.1111/tbed.13289).***

QUINTA. El virus mixoma recombinante presentó una rápida diseminación y una amplia distribución espacial en las poblaciones de liebre ibérica en España. El elevado número de brotes (372) detectados consecutivamente durante los años 2018, 2019 y 2020, indican una circulación endémica de este virus emergente en los últimos años, con un pico epidémico desde mediados de agosto a mediados de octubre ***(Capítulo 4. García-Bocanegra, I., Camacho-Sillero, L., Caballero-Gómez, J., Agüero, M., Gómez-Guillamón, F., Ruiz-Casas, J.M., Díaz-Cao, J.M., García, E., Ruano, M.J., de la Haza, R. (2020). Monitoring of emerging myxoma virus epidemics in Iberian hares (Lepus granatensis) in Spain, 2018-2020. Transboundary and Emerging Diseases 68(3):1275-1282. doi:10.1111/tbed.13781).***

SEXTA. El virus mixoma recombinante ha tenido un importante impacto en las poblaciones de liebre ibérica en España. Los resultados indican una elevada susceptibilidad de la liebre ibérica a la infección por este virus emergente, así como una aparente resistencia de otras especies simpátricas de liebre y una menor infectividad en el conejo silvestre **(*Capítulos 3 y 4*).**

SÉPTIMA. Los resultados obtenidos en la presente Tesis Doctoral ponen de manifiesto la necesidad de establecer programas de vigilancia activa y pasiva para conocer la epidemiología de las principales enfermedades infectocontagiosas endémicas y emergentes que afectan a los lagomorfos silvestres en España, así como para evaluar su impacto en las poblaciones afectadas ***(Capítulos 1, 2, 3 y 4).***

CONCLUSIONS

FIRST. The passive surveillance programme carried out in wild rabbits (*Oryctolagus cuniculus*) showed that the new rabbit haemorrhagic disease virus genotype (GI.2) was widely distributed in Andalusia after the first outbreak was confirmed in 2013. The consecutive outbreaks detected in the 2013-2017 period indicate that circulation of GI.2 in wild rabbit populations in this region was active and endemic ***(Chapter 1. Camacho-Sillero, L., Caballero-Gómez, J., Gómez-Guillamón, F., Martínez-Padilla, A., Agüero, M., San Miguel, E., Zorrilla, I., Rayas, E., Talavera, V., García-Bocanegra, I. (2019). Monitoring of the novel rabbit haemorrhagic disease virus type 2 (GI.2) epidemic in European wild rabbits (Oryctolagus cuniculus) in southern Spain, 2013-2017. Veterinary Microbiology 237:108361. doi: 10.1016/j.vetmic.2019.07.013.).***

SECOND. The active surveillance program carried out in wild rabbits in Andalusia revealed a high circulation (59.9% seroprevalence and 8.8% prevalence of infection), as well as widespread but heterogeneous distribution of myxoma virus in the populations of this wild lagomorph during the period 2009-2021. Both seasonal and interannual fluctuations in seroprevalence were observed, which could increase the risk of MYXV re-emergence in immunologically naïve populations ***(Chapter 2. Camacho-Sillero, L., Cardoso, B., Beato-Benítez, A., Gómez-Guillamón, F., Díaz-Cao, JM., Jiménez Martín, D., Caballero-Gómez, J., Castro-Scholten, S., Cano-Terriza, D., García-Bocanegra, I. (2022). Spatiotemporal monitoring of myxomatosis in European wild rabbit (Oryctolagus cuniculus) in Spanish Mediterranean ecosystems. Transboundary and Emerging Diseases. En revisión).***

THIRD. The main risk factors associated with MYXV exposure were sampling season (autumn), age (adults and juveniles), myxomatosis outbreaks during the month previous to sampling, mean annual temperature and seropositivity to rabbit haemorrhagic disease. Outbreaks of myxomatosis during the month previous to sampling, seropositivity to MYXV, and presence of myxomatosis-compatible lesions were also factors associated with MYXV infection **(*Chapter 2*).**

FOURTH. The passive surveillance program made it possible to detect and monitor the first cases of myxomatosis in the Iberian hare (*Lepus granatensis*). Histopathological and molecular results confirmed MYXV infection in all hares collected in different geographically close hunting areas in the province of Cordoba between mid-July and the end of September 2018. In affected areas, the mean apparent mortality and case-fatality rates were 56.7% and 69.2%, respectively ***(Chapter 3. García-Bocanegra, I., Camacho-Sillero, L., Risalde, M.A., Dalton, K.P., Caballero-***

Gómez, J., Agüero, M., Zorrilla, I., Gómez-Guillamón, F. (2019). First outbreak of myxomatosis in Iberian hares (Lepus granatensis). Transboundary and Emerging Diseases 66(6): 2204-2208. doi:10.1111/tbed.13289).

FIFTH. The recombinant myxoma virus showed a rapid and widespread distribution in Iberian hare populations in Spain. The high number of outbreaks detected consecutively in the period 2018-2020 indicates active endemic circulation of this novel virus in the last few years, reaching an epidemic peak between the first half of August and October ***(Chapter 4. García-Bocanegra, I., Camacho-Sillero, L., Caballero-Gómez, J., Agüero, M., Gómez-Guillamón, F., Ruiz-Casas, J.M., Díaz-Cao, J.M., García, E., Ruano, M.J., de la Haza, R. (2020). Monitoring of emerging myxoma virus epidemics in Iberian hares (Lepus granatensis) in Spain, 2018-2020. Transboundary and Emerging Diseases 68(3):1275-1282. doi:10.1111/tbed.13781).***

SIXTH. The novel ha-MYXV has had major consequences for the health status of Iberian hare populations in Spain. The results indicate high susceptibility of the Iberian hare to ha-MYXV infection, but apparent resistance in sympatric hare species present in Spain, and less infectivity in European wild rabbits **(*Chapter 3 and 4*).**

SEVENTH. The results obtained from the studies carried out for this doctoral thesis highlight the need to establish active and passive surveillance programmes to understand the epidemiology of the main endemic and emerging infectious diseases that affect wild lagomorphs in Spain, and to assess their impact on the affected populations ***(Chapter 1, 2, 3 and 4)***.

ANEXOS

ANEXO I. LISTA DE ABREVIATURAS

°C: Grado Celsius

AC: Área Cinegética

ADN o DNA: Ácido desoxirribonucleico

AEC: Asociación Española de Caza

AMAYA: Agencia de Medio Ambiente y Agua de Andalucía

ARN o RNA: Ácido ribonucleico

BLAST: Basic Local Alignment Search Tool

BOE: Boletín Oficial del Estado

BOJA: Boletín Oficial de la Junta de Andalucía

BRs: Biorregiones

CA: California

CAGPDS: Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible

CAPMA: Consejería de Agricultura, Pesca y Medio Ambiente

CIBERINFEC: Centro de Investigación Biomédica en Red de Enfermedades Infecciosas.

CIBIO/InBio: Centro de Investigaciones em Biodiversidad y Recursos Genéticos

CID: Coagulación Intravascular Diseminada

CMAOT: Consejería de Medio Ambiente y Ordenación del Territorio

CREA: Centro de Recuperación de Especies Amenazadas

CSIC: Centro Superior de Investigaciones Científicas

EBHS: European Brown Hare Syndrom

ECDC: Centro Europeo para la Prevención y Control de Enfermedades

EHC: Enfermedad Hemorrágica del Conejo

EHCV: Virus de la Enfermedad Hemorrágica del Conejo

ELISA: Enzyme-Linked ImmunoSorbent Assay

ESPW: Epidemiological Surveillance Program for Wildlife

FAO: Food and Agriculture Organization

FCT: Fundação para a Ciência e Tecnologia

FEDER: Fondo Europeo de Desarrollo Regional

FPU: Formación de Profesorado Universitario

GC: Gas Chromatography

GI.1: Genotipo 1 de la Enfermedad Hemorrágica del Conejo.

GI.2: Genotipo 2 de la Enfermedad Hemorrágica del Conejo.

GISAZ: Grupo de Investigación en Sanidad Animal y Zoonosis

GLMM: General linear mixed model

HA: Hunting Areas

ha-MYXV: Virus de la mixomatosis de la liebre

hpi: Horas post infección

IC o CI: Intervalo de confianza

Ig: Inmunoglobulina

IHA: Inhibición de la Hematoaglutinación

IMIBIC: Instituto Maimónides de Investigación Biomédica de Córdoba

INVESAGA: Investigación en Sanidad Animal: Galicia

ISO: International Organization for Standardization

ISCIII: Instituto de Salud Carlos III

IUCN: International Union for Conservation of Nature

JCCM: Junta de Comunidades de Castilla-La Mancha

JCR: Journal Citation Report

KDa: KiloDalton

km: Kilómetro

l: Litro

LAGOMED: Improvement of preventive actions to emerging lagoviruses in the Mediterranean basin: development and optimization of methodologies for pathogen detection and control.

LCV: Laboratorio Central de Veterinaria

m^2: Metro cuadrado

MAPA: Ministerio de Agricultura, Pesca y Alimentación

MAPAMA: Ministerio de Agricultura, Pesca, Alimentación y Medio Ambiente

ML: Maximum Likelihood

MS: Mass Spectrometry

MTERD: Ministerio para la Transición Ecológica y el Reto Demográfico

MYXV: Virus de la mixomatosis

OIE: Organización Internacional de Epizootías

OMS: Organización Mundial de la Salud

ONG: Organización no gubernamental

ORF: Open Reading Frame

P1: Período temporal 1

P2: Período temporal 2

P3: Período temporal 3

P4: Período temporal 4

PCR: Reacción en Cadena de la Polimerasa

pH: Potencial de Hidrógeno

PhD: Philosophie Doctor

PRIMA: Partnership for Research and Innovacion in the Mediterranean Area

PVE: Programa de Vigilancia Epidemiológica de la Fauna Silvestre en Andalucía

PVSFS: Programa de Vigilancia Sanitaria en Fauna Silvestre.

RASFAS: Red de Alerta Sanitaria de la Fauna Silvestre.

RASVE: Red de Alerta Sanitaria Veterinaria.

REF: Referencia.

RHD: Rabbit Haemorrhagic Disease.

RHDV: Rabbit Haemorrhagic Disease Virus.

RR: Relative Risk.

RT-PCR: Reverse transcription-polymerase chain reaction.

SaBio: Health & Biotechnology Group.

SANDACH: Subproductos de origen animal no destinados a consumo humano

Serp1-Serp2-Serp3: Inhibidores de la proteina sérica (Serpins)

SLPE: Síndrome de la Liebre Parda Europea

SLPEV: Virus del Síndrome de la Liebre Parda Europea

Sp A COAT: Test de coagulación asociado a la proteína A purificada de *Staphylococcus*

UCO: Universidad de Córdoba

UCLM: Universidad de Castilla-La Mancha

UHPLC: Ultra-performance liquid chromatography

USA: United States of America

VP60: Proteína estructural mayor

VP10: Proteína estructural menor

WAHIS: World Animal Health Information System

ANEXO II: PUBLICACIONES EN CONGRESOS CIENTÍFICOS DERIVADAS DE ESTA TESIS DOCTORAL.

Título del trabajo: Situación, evolución y estudios patológicos de la mixomatosis en liebre ibérica en España

Nombre del congreso: II Jornadas divulgativas sobre gestión sanitaria y cinegética en liebres.

Ciudad de celebración: Toledo, España.

Fecha de celebración: 23 de noviembre de 2021

Entidad organizadora: Fundación Artemisan

Autores: Leonor N. Camacho Sillero

Título del trabajo: Seroepidemiological study of Toxoplasma gondii in wild and domestic lagomorphs in Spain.

Nombre del congreso: 69th WDA/14th EWDA

Ciudad de celebración: Ciudad Real, España.

Fecha de celebración: 31 de agosto a 2 de septiembre de 2021

Entidad organizadora: European Wildlife Disease Association

Autores: Castro-Scholten, S., Cano-Terriza, D., Aguayo-Adán, J., Rouco-Zufiaurre, C., Vázques-Calero, D., Almería, S., Camacho-Sillero, L., Jiménez-Martín, D., Jiménez-Ruix, S., Gómez-Guillamón, F., Dubey, J.P. García-Bocanegra, I.

Título del trabajo: Hallazgos patológicos y distribución tisular del virus de la mixomatosis en liebre ibérica (*Lepus granatensis*).

Nombre del congreso: XXXII Reunión SEAPV

Ciudad de celebración: Online

Fecha de celebración: 01 de octubre de 2021

Entidad organizadora: Sociedad Española de Anatomia Patológica Veterinaria

Autores: Agulló-Ros, I., García-Bocangera, I., Jiménez-Martín, D., Camacho-Sillero, L., Gortázar, C., Capuccie, L., Cano-Terriza, D., Gómez-Guillamón, F., Zorrilla, I., Risalde, M.A.

Título del trabajo: Hepatitis E en conejo silvestre (*Oryctolagus cuniculus*) y liebre ibérica (*Lepus granatensis*): ¿son una fuente de infección zoonósica en el sur de España?

Nombre del congreso: V Congreso Nacional del Grupo de Estudio de las Hepatitis Víricas (GEHEP) de la SEIMC

Ciudad de celebración: Cáceres, España

Fecha de celebración: 26 al 28 de septiembre de 2019.

Entidad organizadora: GEHEP (Grupo de Estudio de las Hepatitis Víricas)

Autores: J. Caballero-Gómez, I. García-Bocanegra, F. Gómez-Guillamón, L. Camacho-Sillero, I. Zorrilla, P. López-López, M. Frías, I. Zafra, C. Ruiz-Rubio y A. Rivero-Juárez .

Título del trabajo: Hepatitis E en conejo silvestre (*Oryctolagus cuniculus*) y liebre ibérica (*Lepus granatensis*): ¿son una fuente de infección zoonósica en el sur de España?

Nombre del congreso: XXI Congreso de la Sociedad Andaluza de Enfermedades Infecciosas.

Ciudad de celebración: Sevilla, España

Fecha de celebración: 21 al 23 de noviembre de 2019

Entidad organizadora: Sociedad Andaluza de Enfermedades Infcciosas

Autores: Caballero-Gómez, J., Rivero-Juarez, A., Gómez-Guillamón, F., Camacho-Sillero, L., Zorrilla, I., López-López, P., Frías, M., Ruíz-Torres, L., Zafra- Soto, I., García-Bocanegra, I.

Título del trabajo: Monitoring of the rabbit hemorrhagic disease virus 2 (RHDV2) epidemics in European wild rabbit (*Oryctolagus cuniculus*) in Andalusia (Spain). 2013-2017

Nombre del congreso: 13th EWDA Conference

Ciudad de celebración: Larissa, Thessaly, Greece

Fecha de celebración: 27 al 31 de agosto de 2018

Entidad organizadora: European Wildlife Disease Association

Autores: **C**amacho-Sillero, L., Gómez-Guillamón, F., Caballero, J., Martínez-Padilla, A., San Miguel Ibañez, E., Agüero García, M., Rocha Roso, A., Rayas, E., Talavera, V., Zorrilla, I., García-Bocanegra, I.

Título del trabajo: Pathologica changes and viral antigen distribution in tissues of Iberian hare (*Lepus granatensis*) infected with myxoma virus

Nombre del congreso: 69th WDA/14th EWDA

Ciudad de celebración: Cuenca (España)

Fecha de celebración: 31 de agosto a 2 de septiembre de 2021

Entidad organizadora: European Wildlife Disease Association

Autores: Agulló-Ros, I., García-Bocanegra, I., Jiménez-Martín, D., Camacho-Sillero, L., Gortázar, C., Capucci, L., Cano-Terriza, D., Gómez-Guillamón, F., Zorrilla, I., Lavazza, A., Risalde, M.A.

Título del trabajo: Brote epidémico de mixomatosis en liebre ibérica (*Lepus granatensis*) en Andalucía

Nombre del congreso: II Congreso Nacional de Sanidad Animal

Ciudad de celebración: Córdoba

Fecha de celebración: del 17 al 18 de octubre de 2018

Entidad organizadora: Consejo General de Colegios de Veterinarios junto con el Colegio Oficial de Veterinarios de Córdoba

Autores: Leonor N. Camacho Sillero, E. Rayas, V. Talavera, I. Zorrilla, I. García-Bocanegra, M. A. Risalde, A. B. Martínez-Padilla, S. Jiménez-Ruiz, M. Agüero, F. Gómez-Guillamón.

ANEXO III: OTRAS PUBLICACIONES EN REVISTAS CIENTÍFICAS DERIVADAS DE LA ACTIVIDAD INVESTIGADORA DURANTE EL DESARROLLO DE ESTA TESIS DOCTORAL.

Autores: J. Caballero- Gómez, I.García- Bocanegra, F. Gómez- Guillamón, L.Camacho- Sillero, I. Zorrilla, P. Lopez- Lopez, D. Cano- Terriza, S. Jiménez_ Ruíz, M. Frias, A. Rivero- Juarez.

Título: Absence de Hepatitis E virus circulation in wil rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*) in Mediterranean ecosystems in Spain.

Revista: Transboundary and Emerging Diseases (2020), 67(4): 1422-1427. doi; 10.1111/tbed.13478

Autores: A. Martínez-Padilla, J. Caballero-Gómez, A. Magnet, F. Gómez- Guillamón, F. Izquierdo, L.Camacho- Sillero, S. Jiménez-Ruiz, C. Del Aguila, I.García- Bocanegra.

Título: Zoonotic Microsporidia in Wild Lagomorphs in Southern Spain

Revista: Animals 10(12). doi: 10.3390/ani10122218

Autores: Remesar, S., Castro-Scholten, S., Jiménez-Martín, D., Camacho-Sillero, L., Morrondo, P., Rouco, C., Gómez-Guillamón, F., Cano-Terriza, D., García-Bocanegra, I.

Título: Spatiotemporal monitoring of *Cysticercus pisiformis* in European wild rabbit (*Oryctolagus cuniculus)* in Mediterranean ecosystems in southern Spain.

Revista: Preventive Veterinary Medicine (2021) 197(1): 105508.

doi: 10.1016/j.prevetmed.2021.105508

Autores: F. Gómez-Guillamón, J. Caballero-Gómez, M. Agüero, L. Camacho-Sillero, M.A. Risalde, I. Zorrilla, R. Villalba, A. Rivero-Juárez, I. García-Bocanegra.

Título: Re-emergence of bluetongue virus serotype 4 in Iberian Ibex (*Capra pyrenaica*) and Sympatric livestock in Spain, 2018-2019

Revista: Transboundary and Emerging Diseases (2021) 68(12). doi:10.1111/tbed.13696

Autores: Félix Gómez-Guillamón, José M. Díaz-Cao, Leonor Camacho-Sillero, David Cano-Terriza, Eva M. Alcaide, Óscar Cabezón, Antonio Arenas, Ignacio García-Bocanegra.

Título: Spatio- temporal monitoring of selected pathogens in Iberian ibex (*Capra pyrenaica*)

Revista: Transboundary and Emerging Diseases (2020) 67(5). doi:10.1111/tbed.13576

Autores: Moroni, B., Angelone, S., Pérez, J., Molinar Min, A.R., Pasquetti, M., Tizzani, P, López-Olvera, J.R., Valldeperes, M., Granados, J.E., Lavín, S., Mentaberre, G., Camacho-Sillero, L., Martínez-Carrasco, C., Oleaga, A., Candela, M., Meneguz, P.G., Rossi, L.

Título: Sarcoptic mange in wild ruminants in Spain: solving the epidemiological enigma using microsatellite markers.

Revista: Parasites & Vectors 14(171). doi: 10.1186/s1307-021-04673-x

Autores: Norin Chai, Jean Louis Pouchelon, Jonathan Bouvard, Leonor Camacho Sillero, Minh Huynh, Vincent Segalini, Lisa Point, Veronica Croce, Goulven Rigaux, Jack Highwood, Valérie Chetboul.

Título: Proposed simple method for electrocardiogram recording in free-ranging asian elephant (*Elephas maximus*).

Revista: Journal of Zoo and Wildlife Medicine 47(1):6-11. doi: 10.1638/2015-0162.1

ANEXO IV: OTRAS PUBLICACIONES EN CONGRESOS CIENTÍFICOS DERIVADAS DE LA ACTIVIDAD INVESTIGADORA DURANTE EL DESARROLLO DE ESTA TESIS DOCTORAL.

Título del trabajo: Genetic diversity andantimicrobial resistence of *Campylobacter* and *Salmonella* strains isolated from decoys and raptors in Andalusia.

Nombre del congreso: 12th Conference of the European Wildlife Disease Association (EWDA).

Ciudad de celebración: Berlín (Alemania)

Fecha de celebración: 27 al 31 de agosto de 2016

Entidad organizadora: Leibniz Institute for Zoo Wildlife Research (IZW)

Autores: Jurado-Tarifa, E., Torralbo, A., Borge, C., Cerdà-Cuéllar, M., Ayats, A., Carbonero, A., Camacho, L., García-Bocanegra, I.

Título del trabajo: Schmallenberg virus exposure in wild ruminants in Spain, 2010-2016

Nombre del congreso: 13th European Wildlife Disease Association Conference

Ciudad de celebración: Larissa (Grecia)

Fecha de celebración: 27 al 31 de agosto de 2018

Entidad organizadora: European Wildlife Disease Association

Autores: Jiménez-Ruiz, S., Rodríguez-Hernández, P., Risalde, M.A., Ruiz-Fons, F., Arnal, M.C., Camacho, L., Lázaro, S., Gens, M.J., Escriabano, F., Domínguez, L., Gortázar, C., Gómez-Guillamón, F., Fernández de Luco, D., Vicente, J., García-Bocanegra, I.

Título del trabajo: Administración de carbetocina antes de la obtención de semen mediante Tumasg en el macho montés (*Capra pyrenaica*).

Nombre del congreso: XIV Congreso de la SECEM.

Ciudad de celebración: Jaca (Huesca)

Fecha de celebración: del 5 al 8 de diciembre de 2019

Entidad organizadora: SECEM (Sociedad Española para la Conservación y Estudio de los Mamíferos)

Autores: Adolfo Toledano-Díaz, Cristina Castaño, Rosario Velázquez, Paula Boveda, Leonor Camacho, Félix Gómez-Guillamón, Ricardo Salas, Rodolfo Ungerfeld, Julián Santiago-Moreno

Título del trabajo: Importancia del tiempo de equilibrado en la efectividad de la congelación ultrarrápida de espermatozoides de muflón (Ovis musimon) y macho montés (Capra pyrenaica)

Nombre del congreso: XV Congreso de la SECEM

Ciudad de celebración: Córdoba

Fecha de celebración: del 4 al 7 de diciembre de 2021

Entidad organizadora: SECEM (Sociedad Española para la Conservación y Estudio de los Mamíferos)

Autores: Adolfo Toledano-Díaz, Cristina Castaño, Félix Gómez-Guillamón, Ricardo Salas, Leonor Camacho, Rafael Guerra y Julián Santiago-Moreno

Título del trabajo: Diferencias morfométricas de la cabeza de espermatozoides eyaculados y epididimarios de rumiantes silvestres.

Nombre del congreso: XIII Congreso de la SECEM

Ciudad de celebración: Guadalajara

Fecha de celebración: del 6 al 8 de diciembre de 2017

Entidad organizadora: SECEM (Sociedad Española para la Conservación y Estudio de los Mamíferos)

Autores: Adolfo Toledano-Díaz, Eva Martínez-Nevado, Ricardo Salas, Félix Gómez-Guillamón, Leonor Camacho, Jaime L. Marcos, Paloma Prieto, Manuel López-Fernández, Cristina Castaño, M. Rosario Velázquez, Lucía Martínez-Fresneda, Paula Bóveda, Emma O'Brien, Milagros Cristina Esteso, Antonio López-Sebastián y Julián Santiago-Moreno.

Título del trabajo: Persistence of sarcoptic mange in Spanish wild ruminants. Solving the epidemiological engima using microsatellite marking.

Nombre del congreso: XI RUSI

Ciudad de celebración: Online

Fecha de celebración: 23 y 24 de octubre de 2020.

Entidad organizadora: RUSI (Reunión de Ungulados Silvestres Ibéricos).

Autores: Barbara Moroni, Samer Angelone, Jesús M. Pérez, Anna Rita Molinar Min, Mario Pasquetti, Paolo Tizzani, Jorge Ramón López-Olvera, Marta Valldeperes, José Enrique Granados, Santiago Lavín, Gregorio Mentaberre, Leonor Camacho-Sillero, Carlos Martínez-Carrasco, Alvaro Oleaga, Mónica Candela, Pier Giuseppe Meneguz y Luca Rossi.

Título del trabajo: Primer caso de ectima contagioso en cabra montés (*Capra pyrenaica hispanica*) en libertad.

Nombre del congreso: 35 GEEFSM

Ciudad de celebración: Cofrentes (Valencia)

Fecha de celebración: del 1 al 4 de junio de 2017

Entidad organizadora: GEEFSM (Groupe d'Etudes sur l'Eco-pathologie de la Fauna Sauvage de Montagne)

Autores: Camacho, L., Gómez-Guillamón, F., Risalde, MA., González, D., Zorrilla, I., García-Bocanegra, I.

Título del trabajo: Resultados del Programa de Vigilancia Epidemiológica del muflón (*Ovis musimon*) en Andalucía (2012-2015)

Nombre del congreso: 34 GEEFSM

Ciudad de celebración: Pont de Camps, Laruns, Vallé d'Ossau, Béarn, France.

Fecha de celebración: del 29 de septiembre al 2 de octubre de 2016.

Entidad organizadora: GEEFSM (Groupe d'Etudes sur l'Eco-pathologie de la Fauna Sauvage de Montagne)

Autores: Leonor Natividad Camacho, Ignacio García-Bocanegra, Elena Rayas, Ventura Talavera, Irene Zorrilla, Paloma Prieto, Félix Gómez-Guillamón.

Título del trabajo: Monitorización espacio-temporal de patógenos en la cabra montés (Capra pyrenaica) en Andalucía (Sur de España)

Nombre del congreso: 37 GEEFSM

Ciudad de celebración: Etroubles, Vallee d'Aoste, Italia

Fecha de celebración: del 13 al 16 de junio de 2019

Entidad organizadora: GEEFSM (Groupe d'Etudes sur l'Eco-pathologie de la Fauna Sauvage de Montagne)

Autores: J.M. Díaz-Cao, I. García-Bocanegra, L. Camacho-Sillero, E. Rayas, V. Talavera, A. Arenas, A. Martínez-Padilla, D. Cano-Terriza, F. Gómez-Guillamón.

Título del trabajo: Vigilancia epidmiológica del jabalí (*Sus scrofa*) como reservorio de *Mycobacterium bovis* en ecosistemas mediterráneos en Andalucía

Nombre del congreso: 33 GEEFSM

Ciudad de celebración: Torino, Italia

Fecha de celebración: del 21 al 24 de mayo de 2015

Entidad organizadora: GEEFSM (Groupe d'Etudes sur l'Eco-pathologie de la Fauna Sauvage de Montagne)

Autores: Aida Miralles, Rosa Sales, Elena Rayas, Leonor N. Camacho, Eva Rodríguez, Irene Zorrilla, Ignacio García-Bocanegra, Félix Gómez-Guillamón, María José Cubero.

Título del trabajo: Programa de Vigilancia epidmiológica en cérvidos en Andalucía (2009-2012)

Nombre del congreso: 33 GEEFSM

Ciudad de celebración: Torino, Italia

Fecha de celebración: del 21 al 24 de mayo de 2015

Entidad organizadora: GEEFSM (Groupe d'Etudes sur l'Eco-pathologie de la Fauna Sauvage de Montagne)

Autores: Ignacio García-Bocanegra, Eva Rodríguez, Leonor N. Camacho, Elena Rayas, Ventura Talavera, Irene Zorrilla, Cristina San José y Félix Gómez-Guillamón.

Título del trabajo: *Samonella* in wild boar in Andalusia (Spain)

Nombre del congreso: 1st Annual Scientific Meeting of One Heatlh European Join Programme on Foodborne Zoonoses, Antimicrobial Resistance and Emerging Threats

Ciudad de celebración: Dublín

Fecha de celebración: del 22 al 24 de mayo de 2019

Entidad organizadora: One Health EJP ASM

Autores: Dr Marta Martínez, Mr Félix Gómez, Mr Ventura Talavera, Mrs Leonor Camacho, Mrs Elena Rayas, Dr Ana De La Torre.

Título del trabajo: Prevalence of *Leishmania infantum* in wild animals from southern Spain

Nombre del congreso: XXXI Congreso SOCEPA

Ciudad de celebración: Pontevedra

Fecha de celebración: del 3 al 5 de julio de 2019

Entidad organizadora: Universidad de Vigo y Sociedad Española de Parasitología

Autores: María Ortuño Gil, María Resa Collados, Ignacio García-Bocanegra, Leonor Camacho-Sillero, Saúl Jiménez-Ruiz, Javier Caballero-Gómez, David Cano-Terriza, Eduardo Berriatua.

ANEXO V: ARTÍCULOS PUBLICADOS QUE DAN LUGAR A LOS CAPÍTULOS DE ESTA TESIS.

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Monitoring of the novel rabbit haemorrhagic disease virus type 2 (GI.2) epidemic in European wild rabbits (*Oryctolagus cuniculus*) in southern Spain, 2013–2017

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ABSTRACT

Rabbit hemorrhagic disease (RHD) is a highly infectious disease in European rabbits (*Oryctolagus cuniculus*), caused by a virus belonging to the genus *Lagovirus* (RHDV; family *Caliciviridae*). In 2010, a new genotype of RHDV (RHDV2 or RHDVb, currently designated GI.2) emerged in France, affecting both domestic rabbits, even those vaccinated for the classical RHDV genotypes (currently designated GI.1) and wild rabbits. GI.2 was subsequently identified in other European countries. The aim of the present study was to monitor the GI.2 epidemic in wild rabbits in Andalusia (southern Spain) during the period 2013–2017.

At the beginning of summer 2013, high mortalities were detected in wild rabbit populations in southern Spain. A total of 96 affected hunting or protected areas were surveyed. The first outbreak was observed on June 2013. The number of outbreaks sharply increased in 2013 and 2014, with a decreasing trend being observed during the following years. The spatial distribution of GI.2 was not homogeneous, since most of the detected outbreaks were concentrated in the western part of Andalusia. The outbreaks peaked in winter and spring and have been detected in the last five consecutive years, which suggests endemic circulation of GI.2 in wild rabbit populations in Spain.

A total of 190 dead rabbits from 87 of the 96 areas surveyed were collected during the study period. Mortality affected rabbits of different age classes, including kittens. RT-PCR confirmed the presence of GI.2 RNA in the livers of 185 of the 190 (97.4%) rabbits. Phylogenetic analysis performed on eleven samples collected in different provinces of Andalusia between 2013 and 2017, showed high nucleotide identity with GI.2 strains Spain, France and Portugal. The results constitute an important step in understanding of the emergence and spread of GI.2 in this country and will provide valuable information for the development of surveillance programs in Europe.

1. Introduction

Rabbit hemorrhagic disease (RHD) is a highly infectious, often fatal disease caused by the rabbit hemorrhagic disease virus (RHDV; genus *Lagovirus*, family *Caliciviridae*), which affects domestic and wild European rabbits (*Oryctolagus cuniculus*). The etiological agent is a non-enveloped, positive-sense, single-stranded RNA virus. Following the recently proposed classification by Le Pendu et al. (2017), RHD viruses are divided into four genotypes: genotype GI.1 (*Lagovirus europaeus*/GI.1a-GI.1d), which comprises pathogenic lagoviruses previously divided into phylogenetic groups G1-G6, the non-pathogenic RHDV-related viruses detected in Europe and Australia, which are classified into genotypes GI.3 and GI.4, and the novel RHDV genotype 2 (*Lagovirus europaeus*/GI.2, previously referred to as RHDV2 or RHDVb).

Pathogenic GI.1 was first described in China in 1984 (Liu et al., 1984) and has become endemic on many continents, including Europe.

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On the Iberian Peninsula, where the European rabbit is native and constitutes a keystone species in Mediterranean ecosystems (Delibes-Mateos et al., 2007), GI.1 was first identified in 1988 (Argüello-Villares et al., 1988). In the years that followed, RHDV spread rapidly and became endemic, although mortality was significantly lower (close to 30%) than the 55–75% reported during the first epidemic (Villafuerte et al., 1995). Part of the reason for the lower mortality could be the progressive increase in immune animals produced by the constant circulation of the virus in later years (Calvete et al., 2002). In this context, the prevalence of antibodies against the classical GI.1 strains in wild rabbit populations in southern Spain was found to be above 30% during the period 2003 and 2004 (García-Bocanegra et al., 2011).

The novel *Lagovirus europaeus*/GI.2 (henceforth GI.2) emerged in France in 2010, affecting both domestic rabbits, including those vaccinated against the classical GI.1 genotype, and wild rabbits (Le Gall-Reculé et al., 2011, 2013). In the years that followed, GI.2 was identified in other European countries, as well as on other continents including Australia, Africa, America and Oceania (reviewed in Rouco et al., 2019). Differences in pathogenicity between GI.1 and GI.2 lagoviruses were associated with age class (Dalton et al., 2012; Le Gall-Reculé et al., 2013); rabbits less than 5-8 weeks old were not naturally susceptible to GI.1 infection, whereas GI.2 caused disease and death even in kittens as young as 11 days of age (Dalton et al., 2014). Moreover, although hare species are naturally resistant to the classical GI.1 genotype, GI.2 cases have been detected in different hare species, including European brown hares (*Lepus europaeus*) (Bell et al., 2019; Le Gall-Reculé et al., 2017; Velarde et al., 2017), Cape hares (*Lepus capensis subsp. mediterraneus*) (Puggioni et al., 2013), Italian hares (*Lepus corsicanus*) (Camarda et al., 2014) and mountain hares (*Lepus timidus*) (Neimanis et al., 2018a).

To date, longitudinal survey studies to assess the evolution and spread of GI.2 in wild rabbit populations have only been conducted in Portugal (Rouco et al., 2016). Hence, using passive surveillance, the aim of this study was to monitor the GI.2 epidemic in wild rabbits in Andalusia (southern Spain) during the period 2013–2017.

2. Material and methods

2.1. Sampling and data collection

By the beginning of summer 2013, high mortalities were being detected in wild rabbit populations in Andalusia, southern Spain (36°N–38°60′N, 1°75′W–7°25′W). An emergency health program was launched in this area by the Regional Ministry of the Environment of Andalusia. A total of 96 areas comprising 91 hunting areas and five protected areas in the eight provinces of Andalusia were visited by veterinarians belonging to the Epidemiological Surveillance Program for Wildlife (Fig. 1). Epidemiological information was gathered at each surveyed site by direct interview of gamekeepers and using a standardized questionnaire. Data collected included: location, date, clinical signs, date of onset in clinically affected animals, abnormal mortality in Iberian hares (*Lepus granatensis*) (the other lagomorph species present in the study area), rabbit densities before the outbreak and restocking programs.

A total of 190 rabbits found dead were sampled between June 2013 and March 2017 in 87 out of 96 areas surveyed. Individual information, including age and sex, was gathered from each animal whenever possible. Rabbits were classified according to their weight and the presence/absence of the epiphyseal notch at the head of the tibia as kittens (up to 40 days old), juveniles (from 40 days to 8 months) or adults (over 8 months) (Dalton et al., 2012; Watson and Tyndale-Biscoe, 1953). Liver samples were collected and sent to the Central Veterinary Laboratory in Algete (National Reference Laboratory for RHDV, Madrid, Spain) for the diagnosis of RHD. In the present study, the term ‘case’ was defined as a rabbit with both clinical signs and lesions compatible with RHDV infection and the presence of GI.2 RNA confirmed by real-time reverse transcription PCR (RT-PCR). The term ‘outbreak’ was defined as an area surveyed with at least one case.

2.2. Laboratory analysis

The BioSprint 96 DNA Blood Kit (Qiagen, Hilden, Germany) was used to extract RNA from 200 µl of liver homogenate (2%) in PBS, using carrier RNA by a magnetic bead robotic system during extraction to increase yield. A GI.2-specific real-time RT-PCR was then performed using the AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems, Foster City, CA, USA) to detect a conserved region of the VP60 capsid protein gene of GI.2 viruses using primers (0.4 µM) sense 5’-TCCAGATGGTTTYCCTGACATG-3’ and antisense 5’-GCGGTAGGGARGGTGYTG-3’ and probe (0.15 µM) 5’-FAM-CGCTGAAGGGTACAAATG-MGB-3’ (Rocha, manuscript in preparation). The thermal profile was 48 °C for 25 min, followed by 10 min at 95 °C and 40 cycles of 2 s at 97 °C, 45 s at 55 °C. Samples negative for GI.2 RNA were further analyzed by RT-PCR assay to detect the presence of GI.1 RNA, following the protocol described by Ros Bascuñana et al. (1997).

Phylogenetic analysis was performed on partial VP60 gene sequences (from nucleotide (nt) 6227 to nt 6778. Nucleotide position refers to coordinates in RHDVAst 89 (GenBank Accession Number: Z49271)), amplified using RT-PCRs with the following pairs of primers: sense (RHNaV-F) and antisense (RHNaV-R) (Dalton et al., 2015) and sense (REF) and antisense (REB) (Ros Bascuñana et al., 1997). The amplification products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing reactions were carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed with a 3130XL Genetic Analyzer (Applied Biosystems). Clustal was used for nucleotide sequence alignment, using representative VP60 gene sequences of GI.1 and GI.2 from China, the Czech Republic, Germany, France, Italy, Ireland, Malta, Portugal, the United Kingdom, the United States of America and Spain, available in GenBank. An Australian GI.4 strain sequence was also included (GenBank Accession Number: EU871528). A sequence of the European brown hare syndrome virus (EBHSV) (GenBank Accession Number: Z69620), which is a highly related but phylogenetically distinct lagovirus, was used as an outgroup to root the tree. The phylogenetic tree was reconstructed with the maximum likelihood method, using the Kimura two-parameter evolutionary model implemented in MEGA 7 (Kumar et al., 2016). K2 + G was chosen as the best-fit nucleotide substitution model with the lowest BIC (Bayesian information criterion) using jModelTest 2.1.10 (Kimura, 1980; Darriba et al., 2012).

3. Results

GI.2 outbreaks were confirmed in 86 of the 96 areas surveyed between 2013 and 2017. Two rabbits from one surveyed area showed negative results for both GI.2 and GI.1 by RT-PCR. In addition, although samples from dead rabbits could not be collected in the nine remaining areas, mortality was observed by gamekeepers. The first outbreak was reported on 27 June 2013 on a hunting estate in the province of Jaen (Fig. 1). Sixteen new outbreaks were detected between November and December of the same year. There was a sharp increase in the number of outbreaks in 2014 (50; 58.1% of the total outbreaks confirmed) followed by a decreasing trend in subsequent years, (11 (12.8%) of total outbreaks confirmed in 2015, 7 (8.1%) in 2016, and one (1.2%) in 2017) (Fig. 2).

Whereas GI.2 outbreaks were detected throughout the year, 45 (52.3%) of the 86 GI.2-positive areas surveyed in Andalusia reported outbreaks during the winter, with lower frequencies being noticed in spring (14; 16.3%), autumn (24; 27.9%) and summer (4; 4.7%) (Fig. 2). GI.2 cases were found to be constant throughout the year in seven (8.3%) of the areas surveyed. GI.2 outbreaks were confirmed each year during the study period; furthermore, in 14 of the surveyed areas, GI.2

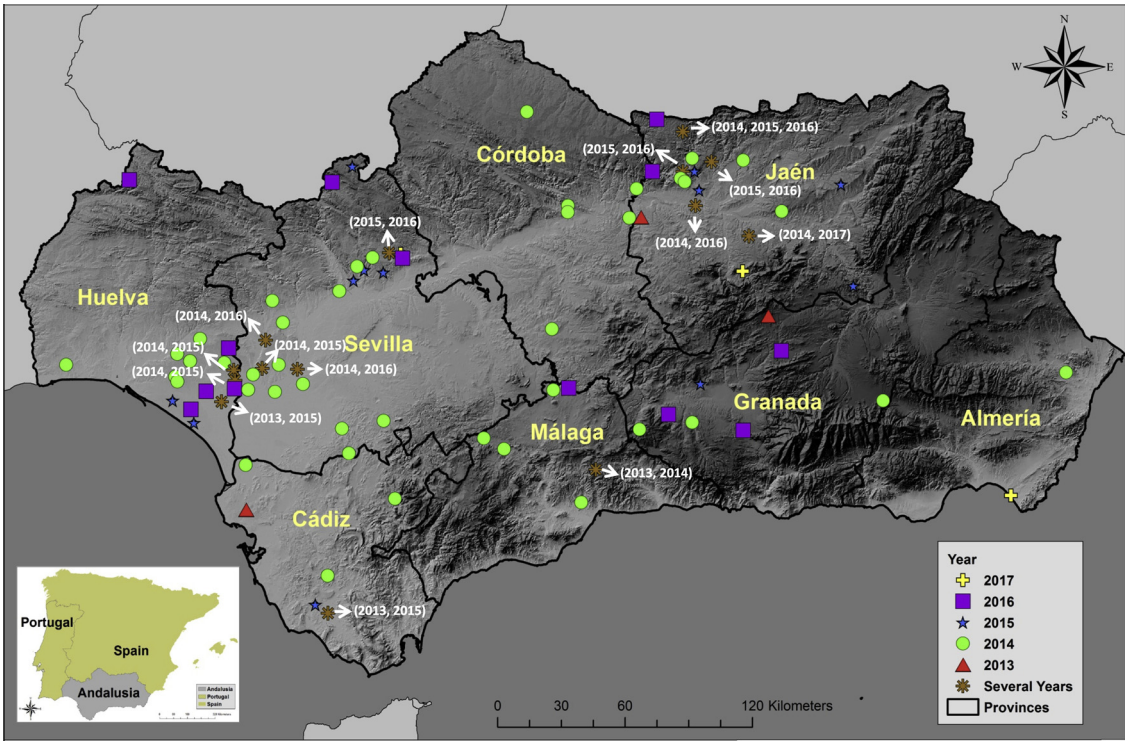

Fig. 1. Spatial distribution of GI.2 outbreaks reported in wild rabbits in Andalusia (southern Spain) between 2013 and 2017.

cases were detected in different years consecutively (Fig. 1). At least one outbreak was confirmed in all eight provinces of Andalusia. Spatial distribution was not homogeneous: Sevilla (21; 24.4%), Huelva (21; 24.4%) and Jaen (21; 24.4%) were the provinces with the highest number of outbreaks, followed by Granada (7; 8.1%), Cordoba (7; 8.1%), Malaga (5; 5.8%), Cadiz (3; 3.5%), and Almeria (1; 1.2%) (Fig. 1).

Restocking programs were conducted between six months and two years before the first GI.2 was detected in the restocked hunting area. These programs were performed in 26 of the 86 GI.2-confirmed areas located in the provinces of Cadiz, Cordoba, Huelva, Jaen, Malaga and Seville. All restocked rabbits were captured in other hunting areas in Andalusia. Vaccination was applied to restocked rabbits using single-doses of commercial inactivated vaccine against the classical GI.1 genotype, but not against GI.2. The clinical signs observed by game-keepers in kittens, juvenile and adult rabbits in the areas surveyed were sudden death (33.3%), opisthotonos (13.8%), convulsion (9.7%), ataxia (4.9%), paralysis (1.3%) and trembling (0.8%). Abnormal mortality was not observed in Iberian hare populations during the study.

GI.2 RNA was detected in 185 out of 190 (97.4%) rabbits analyzed, 26.5% of which were kittens, 39.3% juveniles, and the remaining 34.2% adults. The five GI.2 RNA-negative rabbits were also negative for GI.1 RNA. Phylogenetic analysis was performed on VP60 sequences of eleven samples collected in different provinces of Andalusia during the study period (2013: Malaga (GenBank Accession Number: MK843809) and Granada (MK843810); 2014: Sevilla (MK843807), Huelva

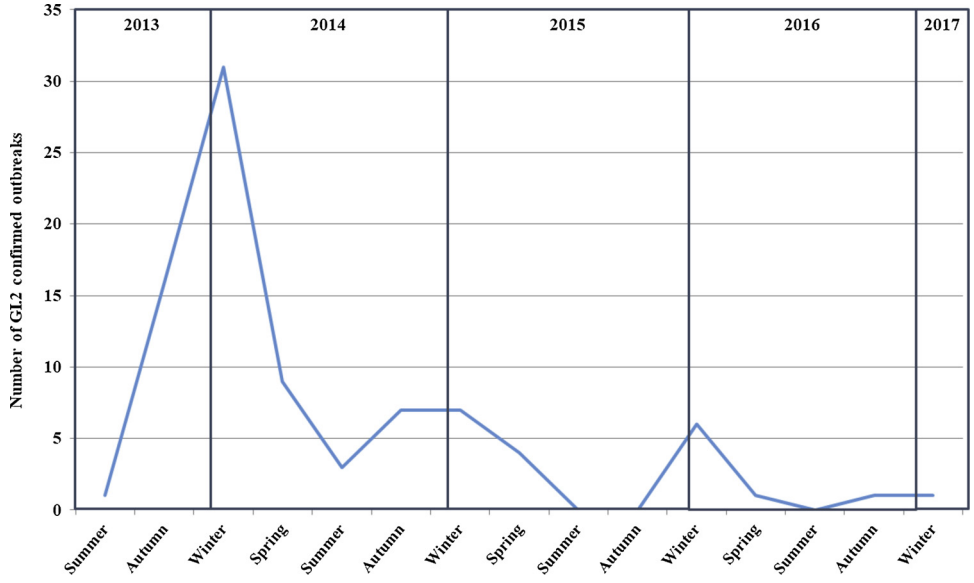

Fig. 2. Temporal evolution (by season) of GI.2 outbreaks in wild rabbits in Andalusia (southern Spain) (2013–2017).

Fig. 3. Maximum likelihood (ML) phylogenetic tree of partial VP60 sequences (n = 72 sequences; bootstrap analysis of 1000 replicates) (from nt 6227–6778 using Z49271 as reference sequence) based on the nucleotide substitution K2 + G model. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. Only bootstrap values ≥70 are shown. The branch including the sequences of GI.2 isolates identified in the present study (in bold) is enlarged.

(MK843808), Almeria (MK843811); 2015: Huelva (MK843812) and Jaen (MK843813); 2016: Cordoba (MK843814) and Granada (MK843815); 2017: Almeria (MK843817) and Jaen (MK843816)). The sequences were clustered together and included in a larger clade that comprised the GI.2 sequences from Portugal, and some isolates from Spain and France (Fig. 3). In addition, BLAST analysis showed high nucleotide identity (97–100%) with available GI.2 sequences from Spain and Portugal.

4. Discussion

The introduction of the emerging GI.2 in 2011 has led to a substantial decline in wild rabbit populations across the Iberian Peninsula (Monterroso et al., 2016). Their densities also decreased in the study area after the first GI.2 case was confirmed in 2013 (CMAOT, 2019). The presence of GI.2 outbreaks in at least 86 surveyed areas distributed across all eight provinces of Andalusia indicates the widespread dispersal of this new lagovirus in southern Spain. Nevertheless, the spatial distribution of the GI.2 outbreaks was not homogeneous, since most of them (73.2%) were concentrated in three provinces (Sevilla, Huelva and Jaen). Differences in wild rabbit population densities, variations in the surveillance efforts made to detect cases (which may have been more focused on areas with the presence of endangered species) and differences in habitat or climatic conditions are possible

factors implicated in the geographical variation observed. In this context, the distribution and prevalence of RHDV have previously been shown to be associated with environmental factors such as rainfall and temperature (Henzell et al., 2002; García-Bocanegra et al., 2011; Liu et al., 2014). Further study of the spatial distribution of GI.2 in the study area is recommended.

The temporal evolution, as well as the detection of cases in the same areas surveyed in different years, indicate the endemic circulation of GI.2 in wild rabbit populations in southern Spain between 2013 and 2017. This hypothesis is supported by the absence of restocked animals from outside Andalusia during the study period, which decreased the risk of introduction of GI.2 strains from different regions. Following confirmation of the first GI.2 case in southern Spain (Andalusia) in summer 2013, the number of outbreaks increased sharply during 2014, with a decreasing trend in the following years. This uneven temporal distribution could be explained, as was observed for GI.1 (García-Bocanegra et al., 2011) in the study area, and more recently for GI.2 in Portugal (Rouco et al., 2016), by increased population immunity due to natural immunization against the GI.2 lagovirus as a result of contact with wild strains persistently circulating in the field. Further serosurvey studies to assess the immune status of wild rabbit populations in Andalusia would provide valuable information on this point. In addition, the possibility that gamekeepers have reported fewer outbreaks to the Regional Department of Environment in the last few years cannot be ruled out either, in which case, the number of outbreaks reported in the study period may be underestimated. GI.2 outbreaks were detected in consecutive years of the 2013–2017 study period. Although outbreaks were found throughout the year, peak incidence was observed during the coldest months (between November and April), which is consistent with previous observations of GI.1 and GI.2 epidemics elsewhere (Mutze et al., 2002; Rouco et al., 2016; Villafuerte et al., 1995).

Our results show mortality in adults but also in both kittens and juvenile animals, which is consistent with what has previously been reported in domestic and wild rabbits (Dalton et al., 2012, 2014; Neimanis et al., 2018b; Rouco et al., 2016). Clinical signs observed in the present study were compatible with acute and peracute forms associated with GI.1 infections (reviewed in Abrantes et al., 2012) and, as expected, with those previously described in GI.2 infected rabbits (Abade dos Santos et al., 2017; Dalton et al., 2012; Neimanis et al., 2018b). Abnormally high mortality was not found in the Iberian hare populations in Andalusia during the study period. However, because GI.2 cases have been detected previously in European brown hares in Spain (Velarde et al., 2017), monitoring programs should also be implemented to assess the susceptibility of the Iberian hare to GI.2 infection.

Sequence analysis of isolates showed high homology (up to 97–100%) with other GI.2 strains previously isolated in Spain and Portugal. Before the GI.2 lagovirus emerged in Spain, only classical GI.1 strains were known to circulate in domestic and wild rabbits in this country (Müller et al., 2009). However, molecular studies conducted in European countries, including Spain, France, Portugal and Sweden, as well as Australia, have demonstrated that the new GI.2 genotype has replaced the GI.1 strains previously circulating in those countries (Calvete et al., 2014; Dalton et al., 2014; Le Gall-Reculé et al., 2013; Lopes et al., 2014; Mahar et al., 2018; Neimanis et al., 2018c). Our results are in accordance with this hypothesis, and all outbreaks reported between 2013 and 2017 were caused by GI.2, although GI.1 circulation in southern Spain cannot be ruled out. Although most of the restocked rabbits were immunized using commercial vaccines against GI.1, which has been shown to be only partially protective against GI.2 at best (Le Gall-Reculé et al., 2013; Dalton et al., 2014), the number of vaccinated rabbits was too limited to achieve proper population-level immunity. Additional molecular and serological studies are required to elucidate whether GI.1 is still circulating in wild rabbit populations in Spain.

Our study has several limitations that should be taken into account. Because of the difficulties associated with finding dead wild rabbits in the field, the number of outbreaks detected in the present study was probably underestimated. Secondly, although the authors made the same sampling effort during the study period, a bias in spatial distribution associated with fewer notifications of cases by gamekeepers in the last two years cannot be ruled out. Finally, we hypothesized that the temporal distribution could also be influenced by increased natural immunity against the GI.2 lagovirus in wild rabbit populations, although additional active serosurveillance is warranted to support this hypothesis.

In conclusion, our results evidence the widespread distribution of the new GI.2 genotype in wild rabbit populations in southern Spain. The outbreaks consecutively confirmed in the period 2013–2017 suggest active and endemic circulation of this new lagovirus in this region. The results obtained contribute to a better understanding of GI.2 emergence and spread and will provide valuable information for the development of risk-based surveillance programs. Further studies are needed to assess the direct impact of GI.2 on wild rabbit populations, as well as its ecological implications for other sympatric species in Mediterranean ecosystems.

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RAPID COMMUNICATION

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First outbreak of myxomatosis in Iberian hares (*Lepus granatensis*)

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Abstract

Myxomatosis is an infectious disease caused by myxoma virus (MYXV; genus *Leporipoxvirus*), which affects the European wild rabbit (*Oryctolagus cuniculus*) and sporadically brown hares (*Lepus europaeus*). Here, we describe the first outbreak of myxomatosis in Iberian hares (*Lepus granatensis*). Between mid-July and the end of September 2018, around 530 dead animals were detected in Iberian hare populations in southern Spain. The apparent mean mortality rate was 56.7%, and the estimated mean case fatality rate was 69.2%. Histopathological and molecular results confirmed MYXV infections in all hares analysed. To the authors' knowledge, this is the first myxomatosis outbreak causing a high mortality in hares and the first detailed characterization of a myxomatosis outbreak in the Iberian hare. The absence of cases in sympatric wild rabbits suggests differences in the susceptibility between both lagomorph species to the virus strain implicated in the outbreak. After the first case, the number of affected areas increased sharply affecting most of the Iberian Peninsula where the Iberian hare is present. Further studies are required to elucidate the origin of the implicated MYXV strain as well as to assess the impact of this outbreak on the Iberian hare populations.

KEYWORDS

Epidemiology, Iberian hare, myxoma virus, high mortality, Spain

1 | INTRODUCTION

Myxomatosis is an infectious disease caused by myxoma virus (MYXV), a member of the genus *Leporipoxvirus* (family *Poxviridae*) mainly transmitted by biting arthropod vectors or through direct contact with infected animals. MYXV was described for the first time in Uruguay in 1896 in domestic European rabbits (*Oryctolagus cuniculus*). The South American forest rabbit (*Sylvilagus brasiliensis*) is the natural host of MYXV and shows subclinical infection or localized cutaneous fibromas. In contrast, the virus causes systemic

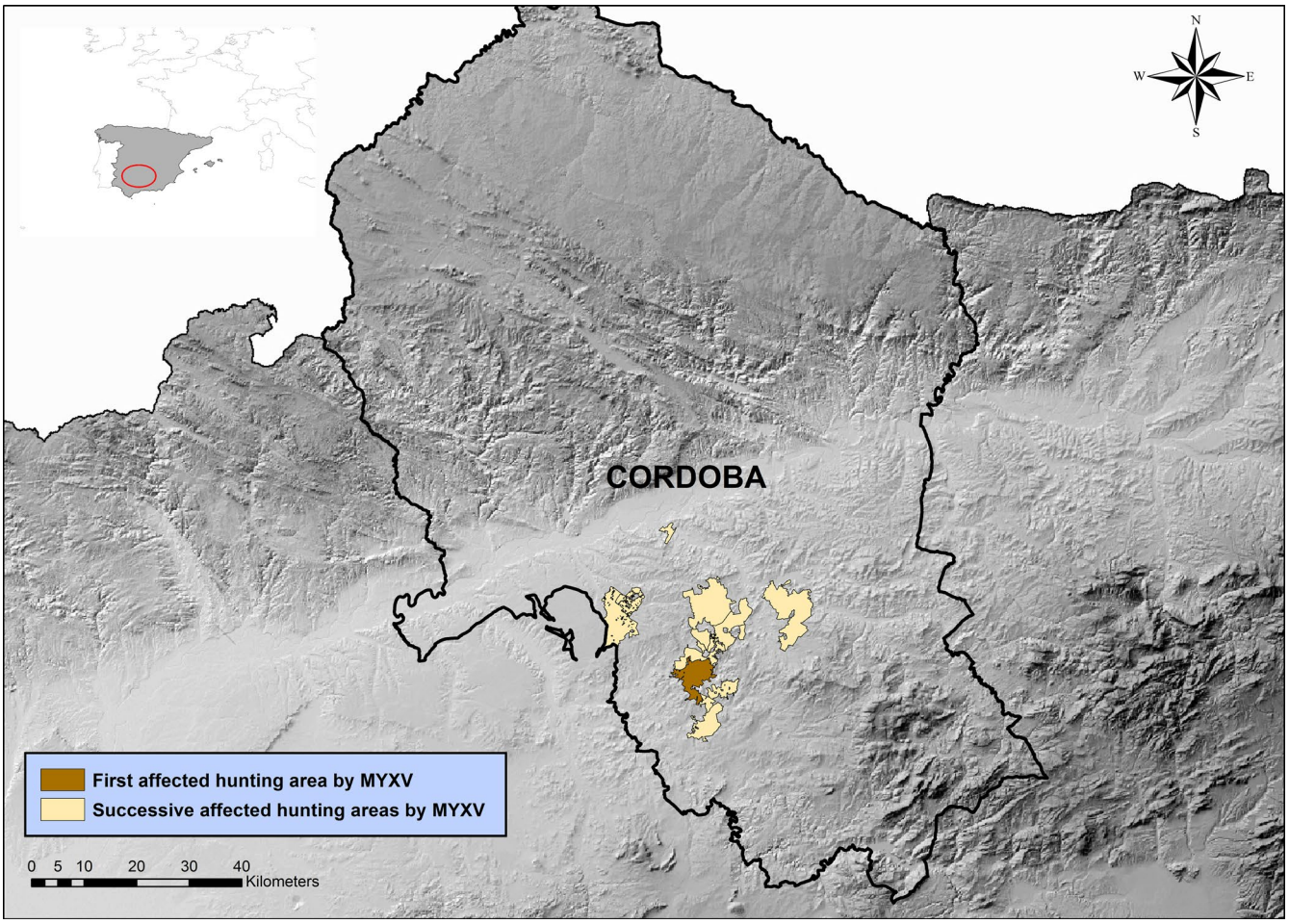

FIGURE 1 Spatial distribution of the first myxomatosis outbreak in Iberian hares (*Lepus granatensis*) in southern Spain, 2018

and often fatal myxomatosis in domestic and European wild rabbits. The clinical disease is characterized by blepharoconjunctivitis, respiratory disorders, cephalic and anogenital oedema, as well as cutaneous pseudotumours termed "myxomas" (Fenner & Ratcliffe, 1965; Best & Kerr, 2000).

MYXV is considered a classic example of host–pathogen co-evolution following a species jump (Kerr et al., 2015). The virus was introduced illegally into France in 1952 and spread rapidly throughout Europe. Mortality rates of around 90% were initially reported in wild rabbit populations, but substantially declined during the following decades due to an increase in genetic resistance, the development of acquired immunity and by contact with MYXV strains that underwent a progressive attenuation in virulence (Fenner & Fantini, 1999). At present, myxomatosis has become endemic in most European countries, but epizootic outbreaks with high mortality are frequently reported in susceptible rabbits, particularly those from high density localized populations. MYXV infections have been reported sporadically in free-living brown hares (*Lepus europaeus*) and usually associated with high prevalence in sympatric wild rabbit populations (Wibbelt & Frölich, 2005). MYXV infections were confirmed in brown hares in France and Ireland during the 1950s (Lucas et al., 1953; Collins, 1955) and, more recently, in Great Britain (Barlow et al., 2014). The aim of this study was to describe the first outbreak of myxomatosis in the Iberian hare (*Lepus granatensis*), the most relevant hare species in terms of population and hunting interests on the Iberian Peninsula.

2 | MATERIAL AND METHODS

Between mid-July and the end of September 2018, high mortalities were detected in Iberian hare populations in 12 geographically close hunting areas located in the province of Córdoba (Andalusia, southern Spain; 4°31′W, 37°25′N; Figure 1). An emergency health programme was launched by the Epidemiological Surveillance Program for Wildlife (ESPW) of the Regional Government of Andalusia. Epidemiological information was gathered both through an on-site interview of the gamekeepers and by veterinarians belonging to the ESPW by visiting the affected hunting areas. Data collected included location, date of onset in clinically affected animals, presence of wild rabbits, myxomatosis in wild rabbit during the outbreak and in the previous year, estimated number of clinically affected animals and mortality, estimated number of hares found dead, hare densities before and after the outbreak. The apparent mortality rate was

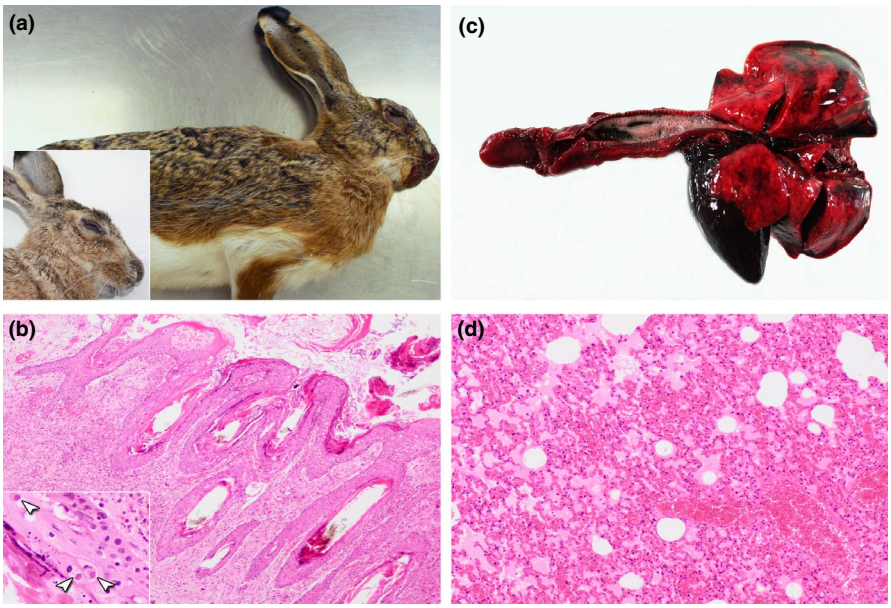

FIGURE 2 Myxomatosis in Iberian hare (*Lepus granatensis*). (a) Epistaxis and blepharitis, which were accompanied by inflammation and oedema around the nasal and oral orifices (inset). (b) Hyperkeratosis and hyperplasia of the epidermis (particularly surrounding follicles) in the eyelid, with lack of normal stratification and ballooning degeneration of the epidermal cells, as well as intracytoplasmic viral inclusions that peripheralized the nucleus (arrowheads in the inset). (c) Severe congestion of the respiratory tract and heart, as well as pulmonary haemorrhages and oedema. (d) Severe congestion and alveolar oedema in the lung, together with mild haemorrhages

calculated as the number of deaths associated with myxomatosis divided by the estimated average number of Iberian hares in each hunting area. Case fatality rate at hunting area level was expressed as the proportion of hares found dead by the total number of clinically affected animals.

Eighteen dead Iberian hares from the affected hunting areas were sent to the Diagnosis and Analysis Center for Wildlife (Regional Laboratory of Andalusia) and the Animal Health laboratory (University of Cordoba, Spain) for postmortem examination, histopathological (formalin-fixed tissues embedded in paraffin for haematoxylin and eosin staining), microbiological (strain culture, isolation and identification), parasitological (flotation, MacMaster and sedimentation methods) and toxicological (gas chromatography–mass spectrometry (GC-MS/MS) or ultra-performance liquid chromatography–mass spectrometry (UHPLC-MS/MS)) analyses. In parallel to these analyses, eyelid, liver and spleen samples were also sent to the Central Veterinary Laboratory in Algete (Spanish National Reference Animal Health Laboratory) for the diagnosis of myxomatosis using PCR (Cavadini, Botti, Barbieri, Lavazza, & Capucci, 2010), rabbit haemorrhagic disease (RHD) and European brown hare syndrome (EBHS) by real-time RT-PCR (Ros Bascuñana et al., 1997; Dalton et al., 2015; Velarde et al., 2017) and tularaemia using culture (OIE, 2018) and PCR (Versage, Severin, Chu, & Petersen, 2003). No live animals were sampled; thus, no animal ethics permit was necessary.

3 | RESULTS AND DISCUSSION

The first cases were detected on the 10th July, and the last dead hares were observed at the end of September. During the study period, around 530 hares were found dead with lesions compatible with MYXV infection. The apparent mean mortality rate was 56.7% (20%–80%), and the estimated mean case fatality rate was 69.2%. The spatial distribution was homogeneous throughout the affected hunting areas.

The main external macroscopic lesions observed in the analysed hares included blepharitis, blepharoconjunctivitis, epistaxis and inflammation and oedema around the nasal, oral (Figure 2a), anal and genital orifices, as well as rectal bleeding in some cases. Myxomas were not found at the base of ears, eyelid or other areas of the skin. Internal organs showed a severe and generalized congestion, which was also observed in the subcutaneous tissue. Other vascular lesions were present such as a severe alveolar oedema and haemorrhages in several organs and body cavities. Histopathological examination revealed that the epidermis with predominant hyperkeratosis was generally hyperplastic and invading the dermis at eyelid, where neutrophil infiltrates with bacterial colonies were occasionally observed. In this tissue, the keratinocytes showed widespread hydropic degeneration and contained eosinophilic cytoplasmic inclusion bodies surrounded by a clear halo (Figure 2b, inset). The dermis was characterized in most animals by a loosely arranged slightly basophilic myxoid matrix admixed with oedematous areas, and with the presence of inflammatory infiltrates of mixed type (macrophages, lymphocytes and polymorphonuclear cells). The vascular histopathological findings agreed with the macroscopic lesions, a severe congestion and hyperaemia of the organs being observed, especially in lungs (Figure 2c) where alveolar oedema and haemorrhages were present (Figure 2d). A severe depletion of lymphocytes was noted in the spleen.

Staphylococcus aureus and *Pasteurella multocida* were isolated in skin lesions and lung in four and two hares, respectively. Parasitological analyses showed *Eimeria* spp. in five hares with the number of oocysts ranging between 1,600 and 35,000 per gram of faeces. A total of 100 and 1,950 eggs per gram of faeces of *Trichostrongylus* spp. were also found in two hares. Toxicological analyses showed negative results for 318 pesticide and rodenticide compounds analysed. All hares showed negative results for RHD

virus, EBHS virus and *Francisella tularensis*. MYXV DNA was detected in the 18 animals tested.

Histopathological and molecular results demonstrated MYXV infections in all Iberian hares analysed. Pathological findings were compatible with an acute or hyperacute presentation of the amyxomatous forms of the disease (atypical myxomatosis). This form is characterized by intense vascular changes and reduced cutaneous clinical signs with myxomas replaced by diffuse swelling of the eyelids (oedematous blepharitis) and sometimes of the cephalic and anogenital areas (Joubert, Duclos, & Tuaillon, 1982; Marlier, Mainil, Linde, & Vindevogel, 2000a). Moreover, the atypical form is usually characterized by intense respiratory distress (Marlier et al., 2000a,2000b), coinciding with the intense pulmonary oedema and haemorrhages observed in the Iberian hares. These findings contrast with the previously reported in European hare, in which subclinical (Collins, 1955) or myxomatous forms had been described (Barlow et al., 2014), and even its potential role as carrier of the virus had been suggested.

On the Iberian Peninsula, where the European wild rabbit is a keystone species in Mediterranean ecosystems (Delibes-Mateos, Redpath, Angulo, Ferreras, & Villafuerte, 2007), myxomatosis caused significant changes in their populations with reduction in densities close to the extinction in some areas (Calvete, Estrada, Villafuerte, Osácar, & Lucientes, 2002). In the affected area, MYXV is currently endemic with seropositivity higher than 50% in wild rabbit populations and epizootic outbreaks reported during summer and autumn (García-Bocanegra et al., 2010). Interestingly, even though the temporal evolution of the outbreak in Iberian hares was similar to that reported in wild rabbits in previous years, myxomatosis cases were not observed in this species during the study period. Additional research is required to determine the origin of the MYXV strain implicated in the outbreak as well as to elucidate the high susceptibility of the Iberian hare and the apparent resistance of the European wild rabbit to this strain.

In conclusion, histopathological and molecular results confirmed MYXV infection in all hares analysed. To the best of our knowledge, this is the first myxomatosis outbreak causing a high mortality in hares and the first detailed description of myxomatosis in Iberian hare. After the first cases were confirmed, the number of affected areas increased sharply affecting most of the Iberian Peninsula where the Iberian hare is present (RASVE, 2019). The absence of myxomatosis cases in sympatric wild rabbits suggests differences in the susceptibility to the MYXV strain implicated in the outbreak between these lagomorph species. Surveillance programmes should be also implemented to assess the impact of the outbreak in the Iberian hare populations and for the early detection of MYXV in this species on the Iberian Peninsula.

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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. All collection of samples was performed following routine procedures before the design of this study, in compliance with the Ethical Principles in Animal Research. Protocols, amendments and other resources were completed according to guidelines approved by each regional autonomous government following the R.D.1337/2013 of the Ministry of Presidency of Spain (1st February 2013, BOE 8th February 2013) (https://www.boe.es/diario_boe/txt.php?xml:id=BOE-A-2013-1337).

CONFLICT OF INTEREST

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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ORIGINAL ARTICLE

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Monitoring of emerging myxoma virus epidemics in Iberian hares (*Lepus granatensis*) in Spain, 2018–2020

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Abstract

Myxomatosis is an infectious disease caused by the myxoma virus (MYXV), which has very high mortality rates in European wild rabbits (*Oryctolagus cuniculus*). While sporadic cases of myxomatosis have also been reported in some hare species, these lagomorphs are considered to have a low susceptibility to MYXV infection. In the present study, we describe the spatiotemporal evolution and main epidemiological findings of novel hare MYXV (ha-MYXV or MYXV-Tol) epidemics in Iberian hares (*Lepus granatensis*) in Spain. In the period 2018–2020, a total of 487 hares from 372 affected areas were confirmed to be MYXV-infected by PCR. ha-MYXV outbreaks were detected in most of the Spanish regions where the Iberian hare is present. The spatial distribution was not homogeneous, with most outbreaks concentrated in the southern and central parts of Spain. Consecutive outbreaks reported in the last two years suggest endemic circulation in Spain of this emerging virus. A retrospective study carried out just after the first epidemic period (2018–2019) revealed that the virus could have been circulating since June 2018. The number of outbreaks started to rise in July, peaked during the first half of August and October and then decreased sharply until January 2019. The apparent mean mortality rate was 55.4% (median: 70%). The results indicated high susceptibility of the Iberian hare to ha-MYXV infection, but apparent resistance in the sympatric hare species present in Spain and less infectivity in European rabbits. The novel ha-MYXV has had significant consequences on the health status of Iberian hare populations in Spain, which is of animal health and conservation concern. The present study contributes to a better understanding of ha-MYXV emergence and will provide valuable information for the development of control strategies. Further research is warranted to assess the impact of this emerging virus on wild lagomorph populations and to elucidate its ecological implications for Iberian Mediterranean ecosystems.

KEYWORDS
epidemic, ha-MYXV, Iberian hare, myxomatosis, Spain

Leonor Camacho-Sillero and Javier Caballero-Gómez contributed equally to this work.

1 | INTRODUCTION

The Iberian hare (*Lepus granatensis*) is an endemic species in the Iberian Peninsula and one of the most representative wild lagomorphs in terms of abundance and hunting interest. This species plays a key role in the ecology of Iberian Mediterranean ecosystems, being the staple prey of a large number of predators, including endangered species such as the Iberian lynx (*Lynx pardinus*), Iberian wolf (*Canis lupus signatus*) and the Spanish imperial eagle (*Aquila adalberti*) (Purroy, 2011). The Iberian hare is also among the main small game species, with about 930,000 animals harvested annually in Spain (MAPA, 2020). Although the information about the population densities of Iberian hares in Spain is limited, in some regions their densities have remained stable at local sites, whereas in others, there has been a decreasing trend in population size in recent decades (Ballesteros, Benito, & González-Quirós, 1996; Carro & Soriguer, 2017). Conservation of the Iberian hare is threatened by different natural and anthropogenic factors, including predators and hunting pressure, fragmentation and loss of habitat, use of herbicides and pesticides, weather conditions, roadkill and disease (Duarte, 2000; García-Bocanegra et al., 2019; Sánchez-García et al., 2012). This species has been shown to be susceptible to different infectious and parasitic diseases (Fernández-Aguilar et al., 2013; Ruiz-Fons, Ferroglio, & Gortázar, 2013; Sánchez-García et al., 2012). On the other hand, several pathogens that affect other hare species (Wibbelt & Frölich, 2005) have not been detected in the Iberian hare, although the available information about its health status is still very scarce.

Myxomatosis is an infectious disease caused by the myxoma virus (MYXV; family *Poxviridae*; Genus *Leporipoxvirus*), which is mainly transmitted by biting arthropods or direct contact with infected animals (Kerr, 2012). MYXV is considered a classic example of host–pathogen coevolution following a species jump (Alves et al., 2019; Kerr et al., 2015). MYXV infection induces benign cutaneous fibromas in its natural host, the South American forest rabbit (*Sylvilagus brasiliensis*), while it causes severe and often fatal disease in European rabbits (*Oryctolagus cuniculus*) (Bertagnoli & Marchandeau, 2015). After MYXV was illegally introduced into France in 1952, the virus spread rapidly throughout Europe causing mortality rates of up to 90% in wild rabbit populations (Fenner & Ratcliffe, 1965). Myxomatosis is currently endemic in most European countries, including Spain, with annual epizootic cycles causing high mortality in susceptible domestic and wild rabbit populations (Villafuerte et al., 2017). Even though hare species are considered to be mostly resistant to MYXV infection, sporadic cases of myxomatosis have been reported in the European brown hare (*Lepus europaeus*) and mountain hare (*Lepus timidus*) (reviewed in Kerr et al., 2015). Between mid-July and the end of October 2018, high mortalities associated with a novel recombinant MYXV strain (ha-MYXV or MYXV-Tol) were detected in Iberian hare populations in Spain and Portugal (García-Bocanegra et al., 2019; OIE, 2018). Molecular studies revealed that an insertion or recombination event with respect to the MYXV Lausanne reference strain may have been involved in the cross-species jump and increased virulence in its new host (Águeda-Pinto et al., 2019; Dalton et al., 2019). The main aim of the present study was to describe the spatiotemporal evolution and main epidemiological findings of the ha-MYXV epidemics in Iberian hares in Spain in the period 2018–2020.

2 | MATERIALS AND METHODS

After the first outbreaks of the novel ha-MYXV isolate in an Iberian hare population in Andalusia (southern Spain) were notified on 10 July 2018 (García-Bocanegra et al., 2019), a national passive surveillance program, coordinated by the Spanish Ministry of Agriculture, Fisheries and Food, was launched across Spain. Between July 2018 and April 2020, a total of 372 hunting estates and protected areas that reported cases compatible with myxomatosis in Iberian hares in this country were investigated/surveyed. The study period was divided into two consecutive epidemic periods: P1, between July 2018 and April 2019, and P2, between May 2019 and April 2020.

Whenever possible, between one and 16 (mean = 3) full carcasses or eyelid samples were collected from clinically affected Iberian hares and wild rabbits found dead in each investigated area. Only samples from animals with the presence of lesions compatible with myxomatosis were collected. Clinically affected hunted hares were also included, although most of the samples (92%) were from animals that were found dead. During the study period, a total of 1,404 and 47 samples of Iberian hares and wild rabbits, respectively, were obtained by the authors, generally in collaboration with the gamekeepers, and sent to the Animal Health laboratory (University of Cordoba, Spain) (93 fresh full carcasses of Iberian hares) for postmortem examination and sampling of carcasses, or directly to the Central Veterinary Laboratory in Algete (Spanish National Reference Animal Health Laboratory) for molecular analysis. Total DNA was extracted with the MagAttract® 96 Cador® Pathogen Kit (QIAGEN, Germany) following the manufacturer's instructions. For the detection of DNA of both the classical MYXV strains and the novel ha-MYXV isolate, a conserved region of the M071L or M005L/R gene was amplified by PCR or real-time PCR, respectively, as previously described (Cavadini, Botti, Barbieri, Lavazza, & Capucci, 2010; Dalton et al., 2019; Duarte et al., 2014, 2015). Using TaKaRa LA Taq DNA polymerase (TaKaRa, Japan), a specific ha-MYXV PCR was carried out with forward and reverse primers M009L-*F* (5′-CGCAGGTCCACGTATAAACC-3′) and M009L-R (5′-CGAACGTATCATTAGACAATG-3′) (Dalton et al., 2019). Data on location and date of sampling were also gathered from each surveyed area.

Additional epidemiological information was gathered in P1 through on-site interviews with gamekeepers at the investigated areas, using a standardized questionnaire. In most cases, this information was also verified by the authors during the visit. For each investigated area, the following data were recorded: location data, date of onset and end of clinically affected animals, clinical signs observed, clinical cases of myxomatosis in sympatric wild rabbits

during or before the outbreak, estimated number of affected or dead Iberian hares, estimated number of clinically affected animals, estimated mortality rate, hare densities before the outbreak and the presence of other affected hare species.

In the present study, an outbreak was defined as an investigated area with at least one Iberian hare infected by ha-MYXV and confirmed by PCR. Investigated areas where Iberian hare mortality compatible with myxomatosis was observed but could not be confirmed by laboratory analysis due to the absence of samples were considered suspected areas.

3 | RESULTS

Between July 2018 and April 2020, a total of 487 Iberian hares from 372 investigated areas were shown to be positive for ha-MYXV infection by PCR. The classical MYXV strain infection was not detected in the Iberian hares analysed. In the 372 ha-MYXV-confirmed areas, 210 outbreaks were detected in P1, 162 in P2, and ha-MYXV outbreaks were detected in 16 of these positive areas in both periods. ha-MYXV outbreaks were confirmed in 141 localities in 11 of 17 Spanish regions. A total of 78 and 63 ha-MYXV-positive localities were detected in P1 and P2, respectively, and in 35 of these positive localities, ha-MYXV outbreaks were successively reported in both study periods (Figure 1). The spatial distribution of ha-MYXV was not homogeneous across Spain; the highest numbers of outbreaks were reported in southern and central regions.

During P1, epidemiological information was obtained by questionnaire in 312 investigated areas: 176 were confirmed as ha-MYXV-positive areas and the remaining 136 were considered as suspected areas. The first clinically affected Iberian hares were observed on 20 June 2018 on a hunting estate in the province of Cuenca (central Spain). The number of outbreaks started to rise from July, peaked during the first half of August and October and then decreased sharply until January 2019. The last sick animals were observed in early March 2019 on a hunting estate located in Valladolid province (northwest Spain) (Figure 2). In most of the surveyed areas (74.7%), the first clinically affected hares were observed between mid-July and mid-October 2018. In 58.9% of investigated areas in P1, the maximum number of cases was reported between early September and mid-October of the same year (Figure 3). In the surveyed areas, the mean interval between the first and maximum number of cases was 31.3 days and ranged between 0 and 73 days. The mean duration of outbreaks in P1 was 115 days (ranging between 3 and 406 days). A decreasing trend in mean duration of the outbreaks was observed, falling from 167 days in July 2018 to 30 days in January 2019 (Figure 4).

The frequency of clinical signs associated with ha-MYXV infection in Iberian hares in Spain during P1 is shown in Figure 5. The clinical signs most commonly observed by the interviewed gamekeepers were conjunctivitis (63.4%), myxomas (33.5%), blepharitis (32.9%) and anogenital swelling (20.2%). Cachexia, epistaxis, prostration, dyspnoea, convulsions, paralysis, diarrhoea, sudden death and opisthotonos were also observed. The number of animals with clinical signs compatible with myxomatosis was similar between males and females in most of the surveyed areas (77.6%). In 53.8% of surveyed areas, adult animals were found to be more frequently affected than juveniles, in 36.5% of affected areas, the distribution across age classes was similar, whereas

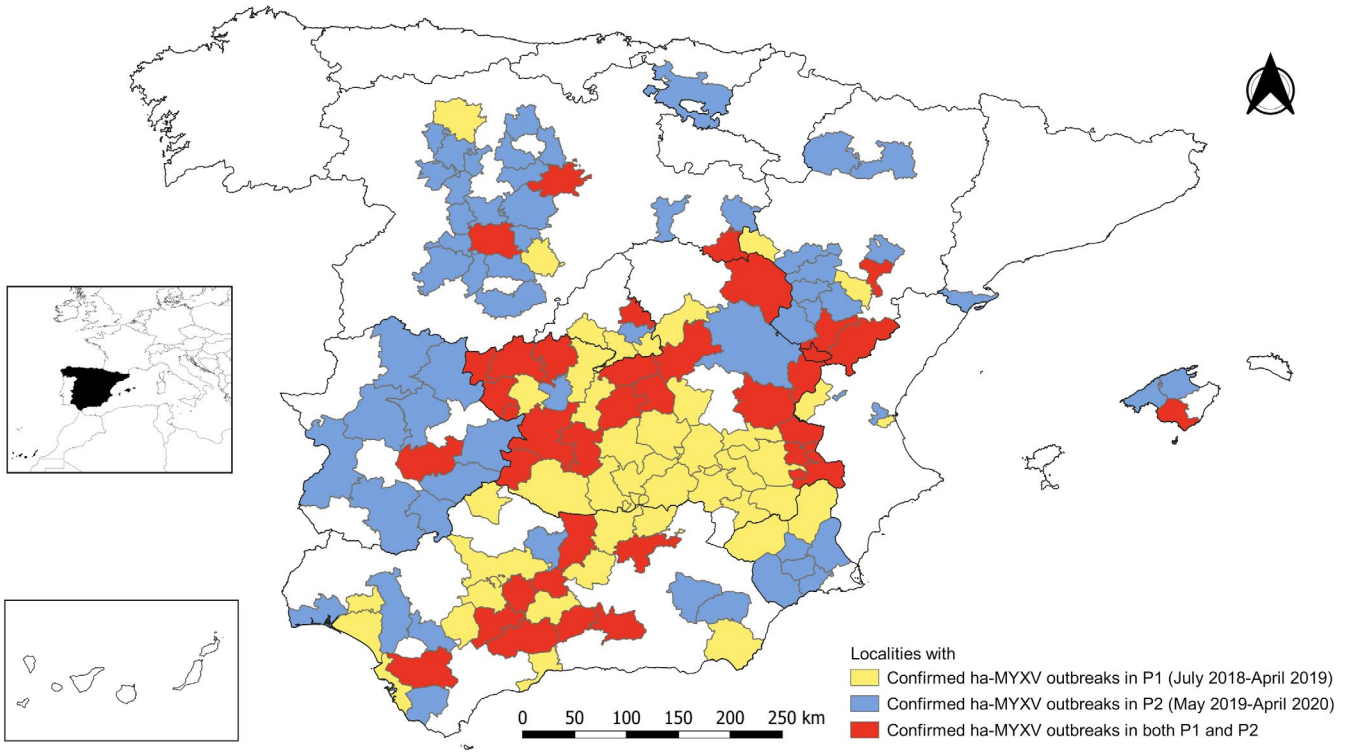

FIGURE 1 Spatiotemporal distribution of ha-MYXV outbreaks at regional level in Spain during the period 2018–2020

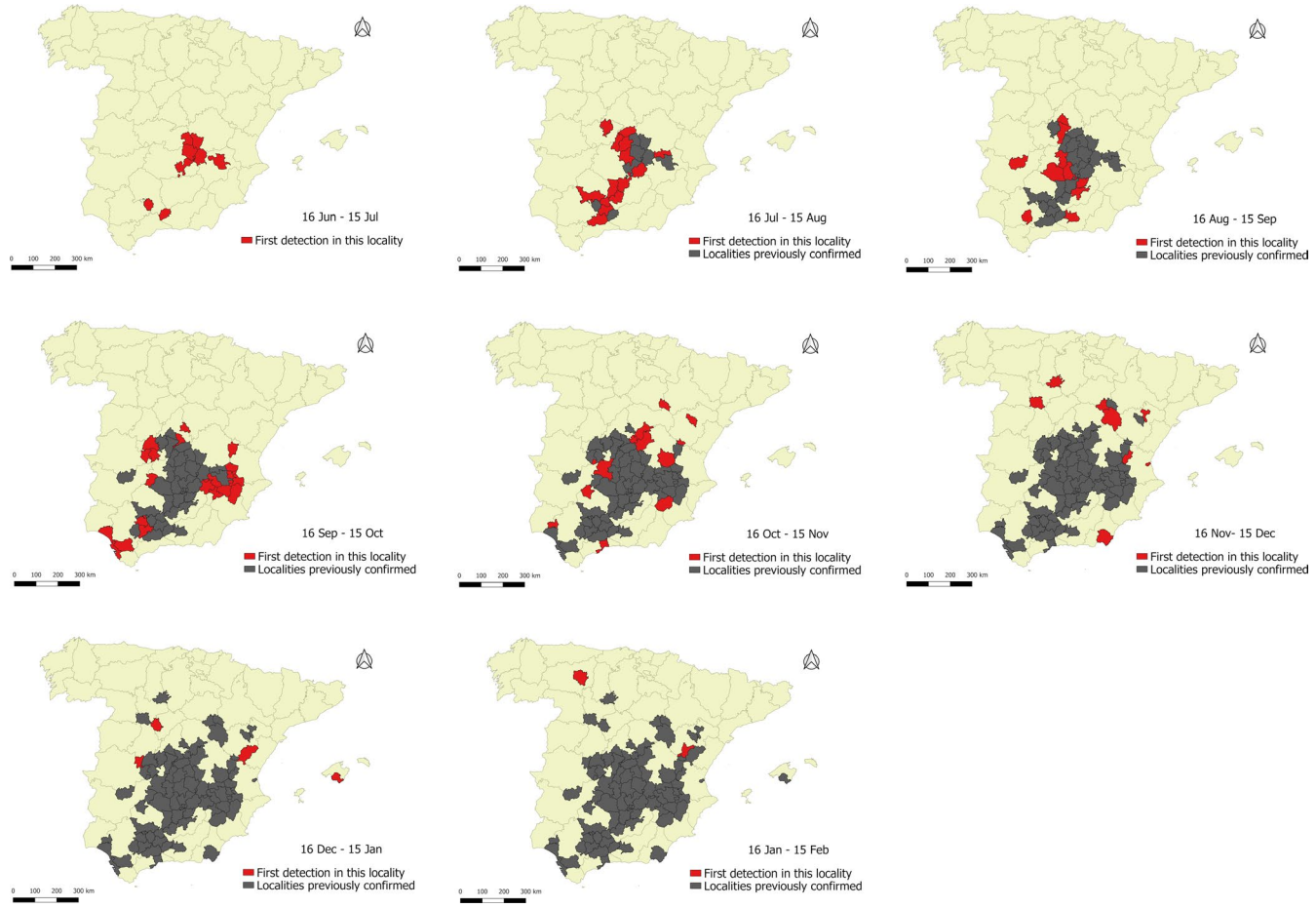

FIGURE 2 Spatiotemporal evolution of ha-MYXV outbreaks at regional level in Iberian hares in Spain during P1, 2018–2019

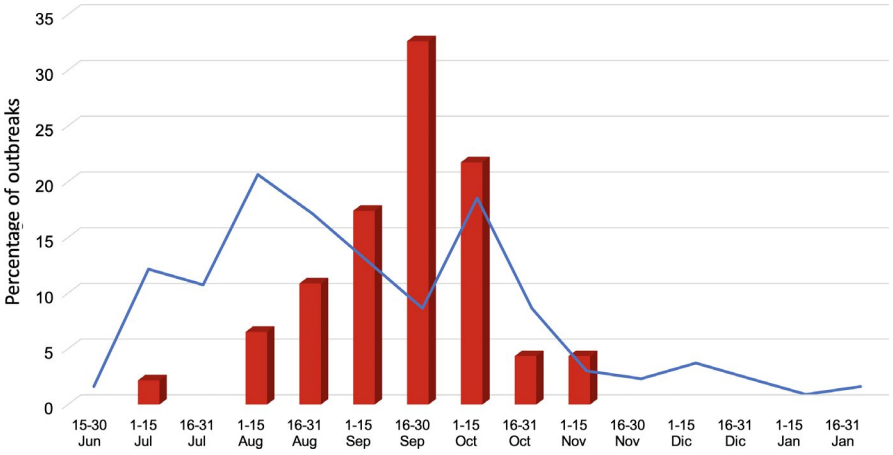

FIGURE 3 Temporal evolution (by fortnight) of the first (line) and maximum number (bars) of ha-MYXV outbreaks in Iberian hares in Spain during P1, 2018–2019

in the remaining 9.6% of areas, the gamekeepers interviewed indicated that juvenile Iberian hares were the most frequently affected animals.

During P1 and P2, neither clinical cases nor mortality compatible with myxomatosis were reported in the other two hare species present in Spain: the European brown hare and the Broom hare (*L. castroviejoi*). Even though most of the surveyed areas (75.7%) had myxomatosis outbreaks in wild rabbits in the two years preceding P1, cases of disease were only observed in this species in 27% of areas investigated in this first epidemic period. A total of 47 clinically affected wild rabbits (seven sampled in P1 and 40 in P2) from localities with ha-MYXV cases in Iberian hares were also analysed by PCR to detect MYXV DNA. Classical MYXV strain infection was detected in 20 rabbits, whereas ha-MYXV DNA was confirmed in two animals. ha-MYXV-infected rabbits were sampled in August 2018 and November 2019 in the provinces of Toledo and Cuenca (both central Spain), respectively.

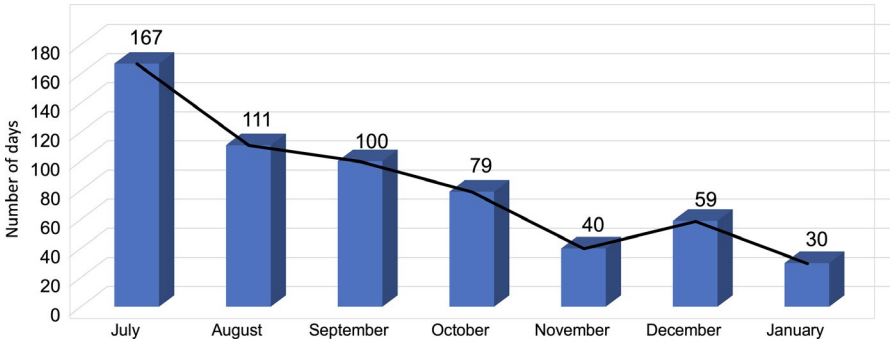

FIGURE 4 Mean duration (in days) of ha-MYXV outbreaks according to month of onset of the first myxomatosis case in Iberian hares in Spain during P1, 2018–2019

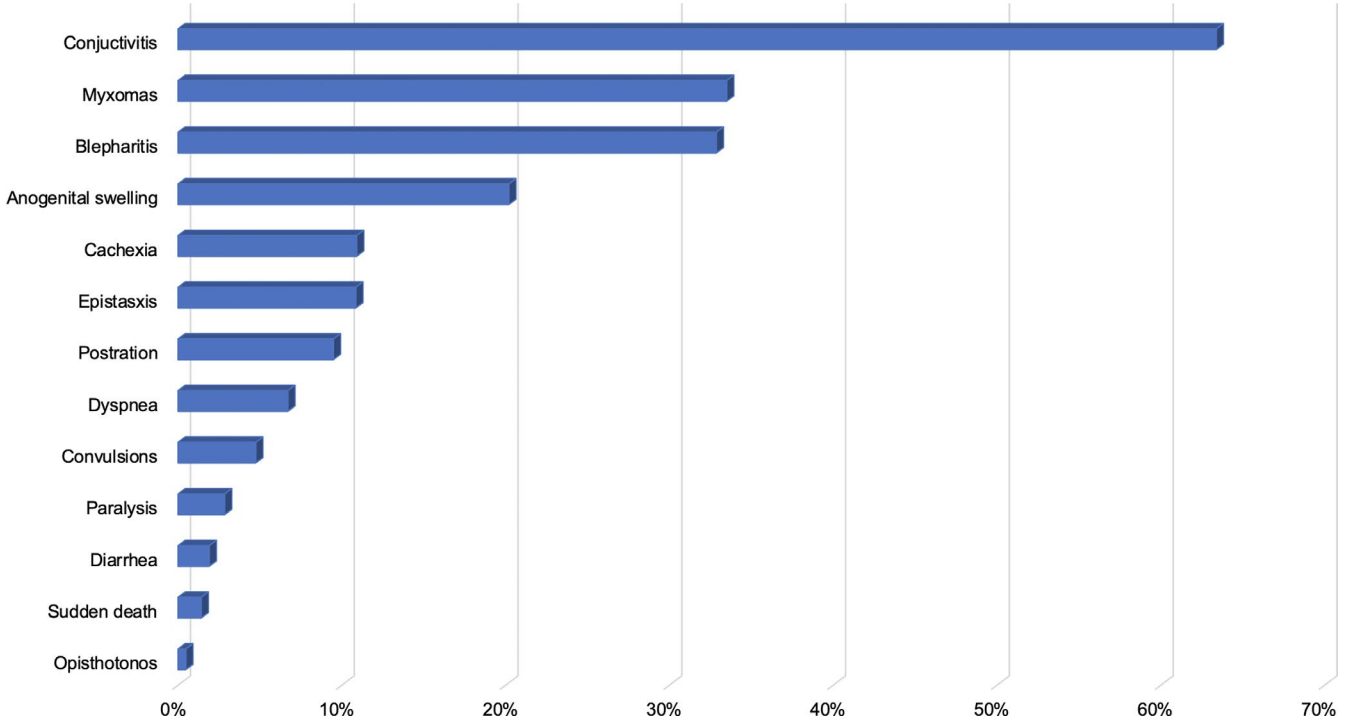

FIGURE 5 Percentage of clinical signs associated with ha-MYXV outbreaks in Iberian hares in Spain during P1, 2018–2019

During P1, a total of 10,297 Iberian hares (mean: 40.4 hares/affected area) were estimated to have been found dead in the field by interviewed gamekeepers. The estimated number of sick live hares was 900 (mean: 3.5 hares/affected area). The estimated mean mortality rate was 55.4% (ranging between 0% and 100%). Apparent mortality rates were above 70% in 38% of the surveyed areas, between 15% and 70% in 38% of areas, and below 15% in the remaining 24% of areas. The spatial distribution of cases within the affected areas was homogeneous in most of the areas surveyed (83.5%).

4 | DISCUSSION

In the present large-scale study, we describe the evolution and main findings of the first myxomatosis epidemic causing high mortality in hares worldwide. Between July 2018 and April 2020, a total of 372 ha-MYXV outbreaks were confirmed in Iberian hares, providing evidence of the cross-species transmission event by MYXV (Águeda-Pinto et al., 2019; Dalton et al., 2019). In spite of the fact that the number of cases may be underestimated as a result of the difficulties of finding dead hares in the field, the detection of ha-MYXV-infected hares in most of the Spanish regions where this species is present further highlights the widespread dispersal of this novel virus in Spain. The occurrence of outbreaks was not homogeneous, since most were concentrated in southern and central parts of Spain. Differences in hare population densities, climatic and environmental conditions or surveillance efforts are possible factors accounting for the geographical variations observed (Villafuerte et al., 2017).

The high number of outbreaks reported in P1 and P2 as well as the cases detected in both periods consecutively in 35 localities (Figure 1) suggests endemic circulation of ha-MYXV in Iberian hare populations in Spain in the last two years. This finding is consistent with the endemic occurrence of myxomatosis in wild rabbit populations in recent decades (Calvete, Estrada, Villafuerte, Osácar, & Lucientes, 2002; Villafuerte et al., 2017).

While the first confirmed outbreak was notified in July 2018 on a hunting estate in Andalusia (southern Spain) (García-Bocanegra et al., 2019), the information obtained from the areas investigated in the present study suggests that the virus could have been circulating in other Spanish regions at least one month before that, since mortality compatible with myxomatosis was observed in June 2018 in two affected areas in the provinces of Cuenca and Toledo (both central Spain).

Peak incidence was observed in summer and autumn, which is consistent with the temporal distribution of myxomatosis in wild rabbits (Calvete et al., 2002; Ferreira et al., 2009; Villafuerte et al., 2017). This temporal evolution as well as a decreasing trend in mean outbreak duration during P1 may be related to the greater abundance of competent vectors during the summer season. It has been shown that *Xenopsylla cunicularis*, a potential myxomatosis vector, is the most abundant flea species in wild rabbits in Spain, with the highest abundance index detected during the summer months (Osácar et al., 2001). Nevertheless, the high spatiotemporal dissemination of the ha-MYXV outbreaks suggests that other competent vectors may also be responsible for long-distance spread to isolated populations. In this context, the role of *Culicidae* in MYXV transmission has previously been documented (Fenner & Ratcliffe, 1965; Merchant et al., 2003; Ross & Tittensor, 1986), since some species are able to travel long distances and keep the MYXV active for long periods (Fouchet, Guitton, Marchandeau, & Pontier, 2008). High mosquito density has been shown to be a risk factor for MYXV exposure in wild rabbits in southern Spain (García-Bocanegra et al., 2010). Interestingly, ha-MYXV outbreaks were reported in the Balearic Islands in both P1 and P2. Taking into account the distance between that region and mainland Spain (more than 200 km), it seems unlikely that ha-MYXV was introduced by infected mosquitoes carried on the wind. Transportation of infected vectors by ship, aircraft or fomites, and restocking with infected hares from mainland Spain for hunting purposes are possible hypotheses for ha-MYXV introduction into these islands. In any case, since the reason for the widespread distribution of this virus remains uncertain, so that entomological surveillance programs and molecular analyses of potential competent vector species of ha-MYXV are needed to elucidate sources of transmission in the Iberian hare populations.

Clinical signs observed in the affected hares were similar to those found previously in this species during the first ha-MYXV outbreaks in Spain and Portugal (Águeda-Pinto et al., 2019; Carvalho et al., 2020; García-Bocanegra et al., 2019) and also to classic rabbit myxomatosis (Calvete et al., 2002; Rosell et al., 2019), but contrast with those reported in the European hare, in which subclinical or mild myxomatosis has been described (Barlow et al., 2014; Collins, 1955). The detection of clinical ha-MYXV infection exclusively in Iberian hares indicates apparent resistance among other hare species present in mainland Spain, which is consistent with previous reports (Barlow et al., 2014; Kerr et al., 2015). It should be noted that in some affected areas in northern Spain, the Iberian hare and European hare are sympatric species (Gortázar et al., 2007). The hypothesis about differences in ha-MYXV susceptibility between lagomorphs is also supported by the limited number of myxomatosis cases observed in wild rabbits in the surveyed areas. Consistent with our results, ha-MYXV infections have been detected in Iberian hares but not wild rabbits also in Portugal (Carvalho et al., 2020; OIE, 2019). Nevertheless, the susceptibility of the wild rabbit to this novel virus cannot be totally ruled out, since ha-MYXV DNA was confirmed in two animals sampled during the study period. Furthermore, an ha-MYXV outbreak causing high mortality was also confirmed on a domestic rabbit farm in Murcia province (southeastern Spain) in October 2019 (MAPA, unpublished data). These findings raise questions on ha-MYXV cross-transmission between Iberian hares and European rabbits. Additional experimental and phylogenetic studies would provide valuable information about the origin and evolution of this emerging virus, as well as elucidate the direction of interspecies transmission (rabbit–hare vs. hare–rabbit).

Gamekeeper estimates of mean apparent mortality in P1 (55.4%) were very similar to the 56.7% obtained by García-Bocanegra et al. (2019) during the first outbreaks. Mortality of more than 70% was detected in 38% of the investigated areas, which is consistent with the high mortality rates observed after the introduction of MYXV in wild rabbits in Europe in the early 1950s (Fenner & Racliffe, 1965). The larger number of hares found dead compared to the number of sick animals observed in P1 could be associated with acute or hyperacute forms of myxomatosis in Iberian hare populations, as has been suggested previously (Carvalho et al., 2020). ha-MYXV infections were observed in individuals of different sexes and age classes. Similar ha-MYXV exposure levels between sexes in most of the affected areas have also been previously reported during the epizootics of myxomatosis in wild rabbits in Spain (Calvete et al., 2002; García-Bocanegra et al., 2010). In 53.8% of the surveyed areas, interviewed gamekeepers pointed out that more adults than juvenile hares were affected, which contrasts with the higher resistance to MYXV infection reported in adult European rabbits (Calvete et al., 2002; Fenner & Ross, 1994; Villafuerte et al., 2017). This finding could be explained, at least in part, by the absence of immunity to ha-MYXV infection in Iberian hare populations, as well as the fact that it is more difficult to find juveniles in the field than adult hares.

In conclusion, our results provide evidence of the rapid and widespread distribution of ha-MYXV in Iberian hare populations in Spain, which is of both animal health and conservation concern. The high number of outbreaks detected consecutively in P1 and P2 indicates active endemic circulation of this novel virus in this country in the last two years. The limited number of myxomatosis cases among sympatric wild rabbits, as well as the absence of outbreaks in other hare species, suggests differences in susceptibility to ha-MYXV between lagomorph species. The results obtained contribute to a better understanding of ha-MYXV emergence and provide valuable information for the development of control strategies. Risk-based surveillance programs, captivity breeding, controlled sanitary restocking, specific vaccination programs against ha-MYXV, reduced hunting pressure and elimination of hares found dead in the field, are possible measures that could help limit the circulation of ha-MYXV in Iberian hare populations. Further studies are warranted to assess

the impact of this emerging virus on the health status of wild lagomorph species and to elucidate its ecological implications for Iberian Mediterranean ecosystems.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

Ethical statement is not applicable as samples were collected from dead animals or from animals legally hunted by authorized hunters with the correct permits and license and with the permission of landowners. This study did not involve purposeful killing of animals.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the authors upon reasonable request.

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