

# UNIVERSIDAD DE CÓRDOBA



## Doctoral Thesis

### **Epidemiological study of zoonotic diseases in wild lagomorphs in Spain from a One Health perspective**

Estudio epidemiológico de enfermedades infectocontagiosas en lagomorfos silvestres en España desde una perspectiva One Health

MEMORIA PARA OPTAR AL GRADO DE DOCTOR  
INTERNACIONAL

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*El camino es el regalo;  
en cada instante de esfuerzo está el verdadero éxito.*



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## Summary

The European wild rabbit (*Oryctolagus cuniculus*) and the Iberian hare (*Lepus granatensis*) are the most relevant lagomorph species in terms of distribution, abundance and hunting interest in Spain. In Andalusia, 1.4 million and 200 thousand European wild rabbits and Iberian hares, respectively, are harvested each year, which makes them of great economic importance. Nevertheless, wild lagomorphs' populations have shown a decreasing tendency in recent years, likely attributed to a combination of factors, among which the impact of transmissible diseases stands out. In addition, wild lagomorphs can serve as reservoirs or spillover hosts for various zoonotic pathogens, which, while not directly affecting their populations, can have a significant impact on public health. Nevertheless, scientific information about the role of wild lagomorphs in the epidemiology of zoonotic pathogens remains very limited. Thus, the main general objective of this PhD thesis is to assess the potential role of the European wild rabbit and the Iberian hare in the epidemiology of zoonotic pathogens in southern Spain. For this purpose, several studies were developed and are presented here under seven main headings.

In the first study (Chapter 1.1), exposure of hepatitis E virus (HEV) was assessed in serum and fecal samples of wild lagomorphs. By species, the seroprevalences in wild rabbit and Iberian hare were 6.8% (29/370; 95%CI: 4.2-9.3) and 6.7% (4/60; 95%CI: 0.4-13.0), respectively. Seropositivity was significantly higher in eastern compared to central Andalusia. HEV RNA was not detected in faeces. The results provide evidence of low, widespread and heterogeneous spatial distribution of HEV among wild lagomorphs in Spanish Mediterranean ecosystems, which indicates a limited role of wild lagomorphs in the maintenance of the virus in this region.

The aim of the second study (Chapter 1.2) was to assess exposure to Crimean-Congo hemorrhagic fever virus (CCHFV) in wild lagomorphs in southern Spain and to evaluate the presence of the virus in ticks feeding on these species. None of the 635 (0.0 %; 95 %CI: 0.0-0.6) lagomorphs tested had anti-CCHFV antibodies and CCHFV RNA was not found in any of the 120 (0.0 %; 95%CI: 0.0-3.0) ticks analyzed. This finding suggested that the European wild rabbit and the Iberian hare do not play a significant role in the epidemiology of CCHFV within the Mediterranean ecosystems of southern Spain.



The third study (Chapter 1.3) analyzed the circulation of West Nile virus (WNV) in wild lagomorph's populations of Andalusia. Exposure to WNV was confirmed in 4.8% (19/394; 95%CI: 2.7-6.9) of wild rabbits and 0.7% (1/141; 95%CI: 0.0-2.1) of Iberian hares tested by both ELISA and virus neutralization test. The spatiotemporal distribution of the seropositive animals overlaps with the largest outbreak in equid and humans of WNV in Spain so far, suggesting that these species could act as sentinels in Mediterranean ecosystems of Spain, particularly in years with high circulation of this flavivirus.

The aims of the fourth and fifth studies (Chapters 2.1 and 2.2) were to determine the prevalence, seroprevalence and risk factors associated with *Coxiella burnetii* circulation in wild lagomorphs and to assess the presence of this pathogen in ticks collected from these animals in southern Spain. Anti-*C. burnetii* antibodies were found in 11.3% (53/471; 95%CI: 8.4-14.1) of the wild rabbits and in 2.4% (4/167; 95%CI: 0.1-4.7) of the Iberian hares analyzed. *C. burnetii* DNA was detected in 16.3% (74/453; 95%CI: 12.9-19.7) and 24.0% (29/121; 95%CI: 16.4-31.6) of the European wild rabbits and Iberian hares tested, respectively, and in 33 of the 120 (27.5%; 95%CI: 19.5-35.5) tick pools tested. These studies are the first surveys on *C. burnetii* performed in Iberian hare. Our results indicate widespread endemic circulation of this zoonotic bacterium and highlight the importance of both wild lagomorph species as natural reservoirs of *C. burnetii* in Andalusia.

The sixth study (Chapter 2.3) assessed the occurrence of *Francisella tularensis* in the wild lagomorphs inhabiting southern Spain. None of the 952 (0.0%; 95%CI: 0.0-0.4) European wild rabbits and Iberian hares sampled showed presence of *F. tularensis* DNA. This result indicates absence or very low circulation of the bacterium in wild lagomorph populations in Mediterranean ecosystems of southern Spain, denoting a limiting risk of transmission of this pathogen to other sympatric species, including human beings.

In the seventh and last study (Chapter 3.1), the aims were to determine the seroprevalence and potential risk factors associated with *Sarcoptes scabiei* exposure in European wild rabbits and Iberian hares in Andalusia. The overall apparent individual seroprevalence was 15.9% (95/596; 95%CI: 13.0-18.9). Significantly higher seroprevalence was found in Iberian hares (31.1%) than in European wild rabbits (11.6%). The multivariate analyses identified “age” (adult) and “geographical area” (western Andalusia) as risk factors potentially associated with exposure to this parasite. The results

indicate a widespread, endemic, and heterogeneous circulation of *S. scabiei* among wild lagomorphs in the Mediterranean ecosystems of southern Spain.

This doctoral thesis adds novel knowledge about the epidemiological role of wild lagomorphs to zoonotic pathogens, providing valuable information on risk factors associated. The obtained information will enhance control measures for disease of public health importance and underline the importance of epidemiological surveillance in wildlife.

## Resumen

El conejo silvestre europeo (*Oryctolagus cuniculus*) y la liebre Ibérica (*Lepus granatensis*) son las especies de lagomorfos más relevantes en cuanto a distribución, abundancia e interés cinegético en España. En Andalucía estas especies presentan una gran importancia económica ya que se capturan anualmente 1,4 millones y 200 mil conejos silvestres y liebres Ibéricas, respectivamente. Sin embargo, las poblaciones de lagomorfos silvestres han mostrado una tendencia decreciente en los últimos años. Aunque el declive se atribuye a una combinación de factores, destaca el impacto de las enfermedades transmisibles. Asimismo, los lagomorfos silvestres pueden actuar de reservorios o hospedadores accidentales de diversos patógenos zoonóticos que, aunque no afecten directamente a sus poblaciones, pueden tener un impacto importante en salud pública. Sin embargo, la información científica sobre el papel de los lagomorfos silvestres en la epidemiología de patógenos zoonóticos sigue siendo muy limitada. Por ello, la presente Tesis Doctoral tiene el objetivo principal de evaluar el potencial papel epidemiológico del conejo silvestre europeo y la liebre Ibérica en la transmisión y mantenimiento de patógenos zoonóticos en el sur de España. Para ello se han diseñado siete estudios que abordan cada uno de los objetivos específicos.

En el primero (Capítulo 1.1), se evalúa la exposición al virus de la hepatitis E (VHE) en muestras de suero y heces de lagomorfos silvestres. Por especies, las seroprevalencias en conejo silvestre y liebre Ibérica fueron del 6,8% (29/370; IC 95%: 4,2-9,3) y 6,7% (4/60; IC 95%: 0,4-13,0), respectivamente. La seropositividad fue significativamente mayor en Andalucía oriental que en Andalucía central. No se detectó ARN del VHE en las heces analizadas. Los resultados obtenidos indican una baja y amplia pero heterogénea distribución espacial del VHE en lagomorfos silvestres de los ecosistemas mediterráneos españoles, lo que indica un papel limitado de los lagomorfos silvestres en el mantenimiento del virus en esta región.

El objetivo del segundo estudio (Capítulo 1.2) es determinar la exposición al virus de la fiebre hemorrágica de Crimea-Congo (VFHCC) en lagomorfos silvestres del sur de España y evaluar la presencia del virus en garrapatas recogidas sobre estas especies. Ninguno de los 635 (0,0 %; IC 95 %: 0,0-0,6) lagomorfos analizados presentó anticuerpos anti-VFHCC y no se detectó ARN del virus en ninguna de las 120 (0,0 %; IC 95 %: 0,0-3,0) garrapatas analizadas. Este hallazgo sugiere que el conejo silvestre europeo y la liebre

ibérica no desempeñan un papel significativo en la epidemiología del VFHCC en los ecosistemas mediterráneos del sur de España.

El tercer estudio (Capítulo 1.3) analiza la circulación del West Nile virus (WNV) en poblaciones de lagomorfos silvestres de Andalucía. Se confirmó la exposición al WNV en el 4,8% (19/394; IC 95%: 2,7-6,9) de los conejos silvestres y en el 0,7% (1/141; IC 95%: 0,0-2,1) de las liebres ibéricas analizadas mediante ELISA y test de neutralización vírica. La distribución espacio-temporal de los animales seropositivos se solapa con el mayor brote de WNV en humanos y caballos descritos en España hasta la fecha, sugiriendo que estas especies podrían actuar como centinelas en los ecosistemas mediterráneos de la península Ibérica, particularmente en años con alta circulación de este flavivirus.

Los objetivos de los estudios cuarto y quinto (Capítulos 2.1 y 2.2) son determinar la prevalencia, seroprevalencia y factores de riesgo asociados a la circulación de *Coxiella burnetii* en lagomorfos silvestres y evaluar la presencia de este patógeno en garrapatas recogidas de estos animales en el sur de España. Se encontraron anticuerpos frente a *C. burnetii* en el 11,3% (53/471; IC 95%: 8,4-14,1) de los conejos silvestres y en el 2,4% (4/167; IC 95%: 0,1-4,7) de las liebres ibéricas analizadas. Se detectó ADN de *C. burnetii* en el 16,3% (74/453; IC 95%: 12,9-19,7) y el 24,0% (29/121; IC 95%: 16,4-31,6) de los conejos silvestres europeos y las liebres ibéricas analizados, respectivamente, y en 33 de los 120 (27,5%; IC 95%: 19,5-35,5) pools de garrapatas. Estos estudios son los primeros realizados sobre la exposición de liebres Ibéricas a *C. burnetii*. Nuestros resultados indican una amplia y endémica circulación de este patógeno zoonótico y destacan la importancia de ambas especies como reservorios naturales de *C. burnetii* en Andalucía.

El sexto estudio (Capítulo 2.3) evalúa la presencia de *Francisella tularensis* en lagomorfos silvestres del sur de España. Ninguno de los 952 (0,0%; IC 95%: 0,0-0,4) conejos silvestres europeos y liebres Ibéricas muestreados presentó de ADN de *F. tularensis*. Este resultado indica la ausencia o muy baja circulación de la bacteria en las poblaciones de lagomorfos silvestres de los ecosistemas mediterráneos del sur de España, lo que denota un riesgo limitado en la transmisión de este patógeno a otras especies simpátricas, incluido al ser humano.

En el séptimo y último estudio (Capítulo 3.1), se determinó la seroprevalencia y los posibles factores de riesgo asociados a la exposición a *Sarcoptes scabiei* en conejos

## *Summary/Resumen*

silvestres europeos y liebres Ibéricas en Andalucía. La seroprevalencia global fue del 15,9% (95/596; IC 95%: 13,0-18,9). Las liebres Ibéricas (31,1%) presentaron una seroprevalencia significativamente superior que los conejos silvestres (11,6%). Se identificó la “edad” (adulto) y la “zona geográfica” (Andalucía occidental) como factores de riesgo potencialmente asociados a la exposición por este parásito. Los resultados indican una circulación generalizada, endémica y heterogénea a nivel espacial de *S. scabiei* entre los lagomorfos silvestres de los ecosistemas mediterráneos del sur de España.

Esta Tesis Doctoral aporta nuevos conocimientos acerca del papel epidemiológico de los lagomorfos silvestres frente a patógenos zoonóticos, proporcionando información valiosa sobre los factores de riesgo asociados. La información obtenida permitirá mejorar las medidas de lucha de enfermedades de importancia para la salud pública y subrayan la importancia de la vigilancia epidemiológica en fauna silvestre.

## **List of abbreviations and acronyms**

**ADN:** Ácido desoxirribonucleico

**AEMET:** Agencia Estatal de Meteorología

**ARN:** Ácido ribonucleico

**BAGV:** Bagaza virus

**bELISA:** Blocking enzyme-linked immunosorbent assay

**CAPMA:** Consejería de Agricultura Pesca y Medio Ambiente

**CBUA:** Consorcio de Bibliotecas Universitarias de Andalucía

**CCHF:** Crimean-Congo hemorrhagic fever

**CCHFV:** Crimean-Congo hemorrhagic fever virus

**CDC:** Centers for Disease Control and Prevention

**CIBER:** Centro de Investigación Biomédica en Red

**cm:** centimeters

**CSMA:** Consejería de Sostenibilidad y Medio Ambiente

**DNA:** Deoxyribonucleic acid

**dNTPs:** Deoxynucleotide triphosphates

**EBHSV:** European brown hare syndrome virus

**ECDC:** European Centre for Disease Prevention and Control

**EFSA:** European Food Safety Authority

**ELISA:** Enzyme Linked Immunosorbent Assay

**EU:** European Union

**E%:** ELISA percentage

**FEDER:** Fondo Europeo de Desarrollo Regional

**FIS:** Fundación para la Investigación en Salud

**FPU:** Formación de Profesorado Universitario

**g:** grams

**GEE:** Generalized estimating equation

**HE:** Hepatitis E

**HEV:** Hepatitis E virus

**HEV-3:** Genotype 3

*List of abbreviations and acronyms*

**HEV-4:** Genotype 4

**HEV-7:** Genotype 7

**HI:** Hemagglutination inhibition

**IC 95%:** Intervalos de confianza del 95%

**ICTV:** International Committee on Taxonomy of Viruses

**ISCIII:** Instituto de Salud Carlos III

**IUCN:** International Union for Conservation of Nature

**JCR:** Journal Citation Report

**Kg:** kilograms

**Km:** kilometer

**Km<sup>2</sup>:** Square kilometer

**MAPA:** Ministerio de Agricultura, Pesca y Alimentación

**m<sup>3</sup>:** cubic meter

**micro-VNT:** micro virus neutralization test

**min:** minute

**MITECO:** Ministerio para la transición ecológica y el reto demográfico

**mm:** millimeters

**MYXV:** Myxomatosis virus

**N:** North

**NC:** Negative control

**OD:** Optical density

**OD<sub>PC</sub>:** Optical density of the positive control

**OD<sub>s</sub>:** Sample optical density

**OR:** Odds ratio

**PCR:** Polymerase chain reaction

**PI:** Percentage of inhibition

**PNVSFS:** National Wildlife Health Surveillance Plan

**PVE:** Epidemiological Surveillance Program in Wildlife

**QIC:** Quasi-likelihood Information Criteria

**qPCR:** Quantitative polymerase chain reaction

**RASFAS:** Wildlife Health Alert Network  
**RASVE:** Red de Alerta Sanitaria Veterinaria  
**RENAVE:** Red Nacional de Vigilancia Epidemiológica  
**RHD:** Rabbit haemorrhagic disease  
**RHDV:** Rabbit haemorrhagic disease virus  
**RHDV2:** Rabbit haemorrhagic disease virus GI. 2 genotype  
**RNA:** Ribonucleic acid  
**RNVE:** Red Nacional de Vigilancia Epidemiológica  
**RR:** Relative Risk  
**RT-PCR:** Reverse transcription polymerase chain reaction  
**s:** second  
**SAS:** Servicio Andaluz de Salud  
**SPSS:** Statistical package for the social sciences  
**TMB:** Tetramethylbenzidine  
**UCO:** University of Córdoba  
**USA:** United States of America  
**USUV:** Usutu virus  
**VFHCC:** virus de la fiebre hemorrágica de Crimea-Congo  
**VHE:** Virus de la hepatitis E  
**W:** West  
**WAHIS:** World Animal Health Information System  
**WHO:** World Health Organization  
**WNV:** West Nile virus  
**WOAH:** World Organization for Animal Health  
**95%CI:** 95% confidence intervals  
**°C:** Celsius  
**µl:** Microliter  
**µM:** Micromolar



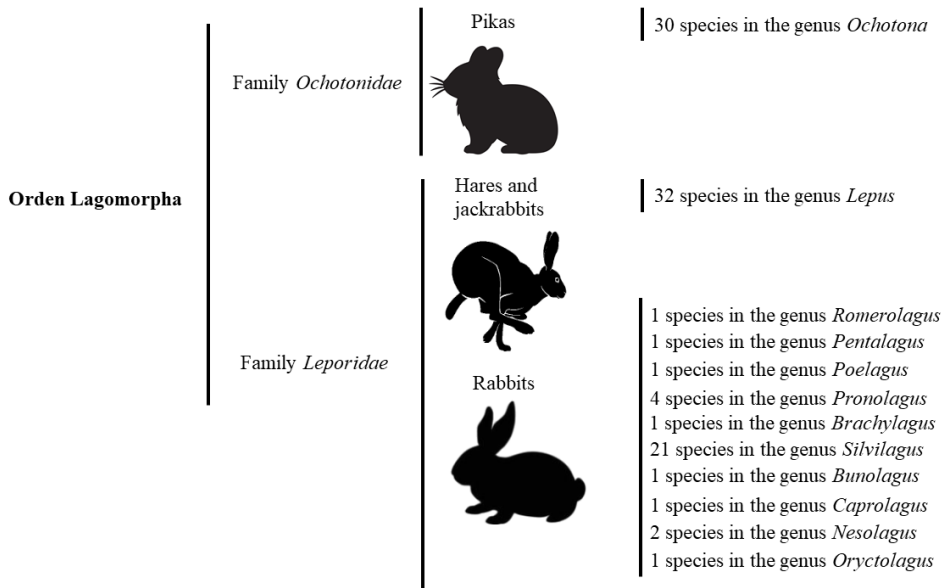


## INTRODUCTION

### 1. The order Lagomorpha

Lagomorpha is a globally distributed (barring Antarctica) mammalian order with a long evolutionary history that can be traced back to 55 million years ago in Mongolia (Asher et al., 2005). The order consists of species across two families: the *Ochotonidae* (pikas) and the *Leporidae* (rabbits, hares, and jackrabbits) (Figure 1). Most species of lagomorphs are present in Asia, America and Africa, while Europe accounts with only eight species of the order, all of them belonging to the *Leporidae* family (Smith et al., 2018). This family is the largest, with 66 species classified into 11 different genera (IUCN, 2024). These species are economically, ecologically and scientifically important, as they are human food resources, game species, pests of agricultural significance, key prey species in the food chain of many predators and model laboratory animals.

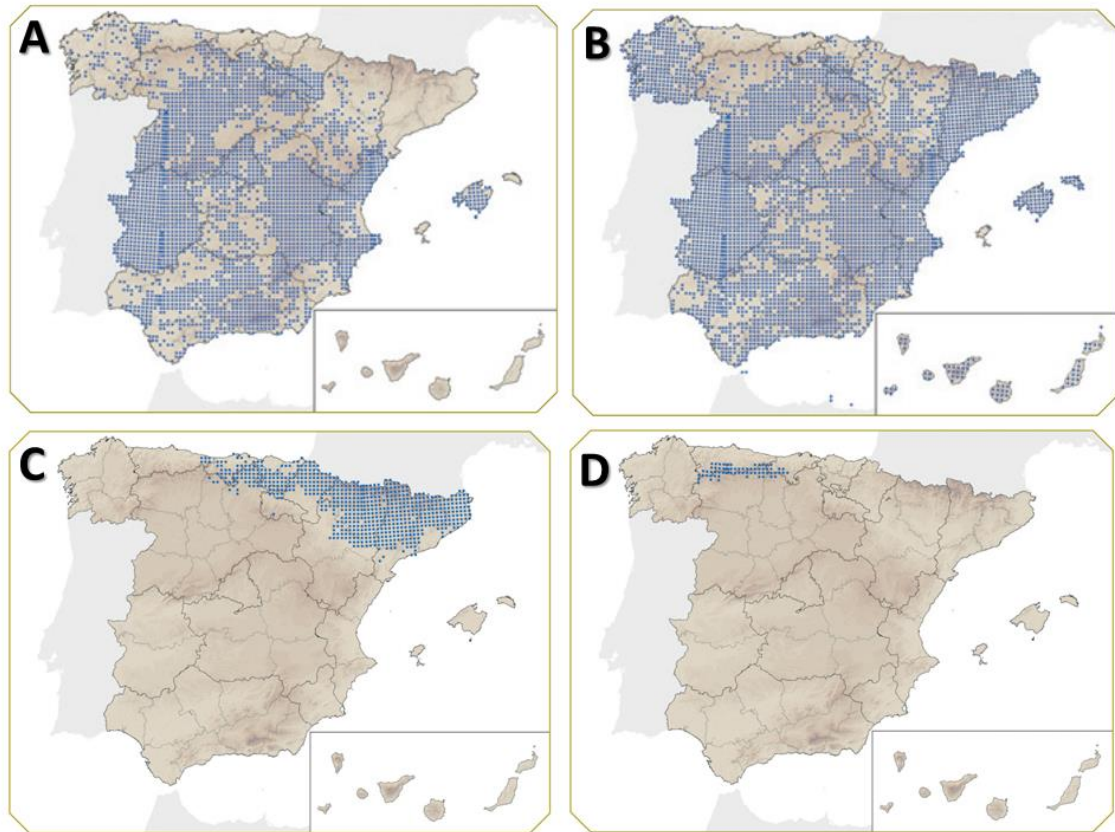
Rabbits are small lagomorphs divided into 10 genera. The European wild rabbit (*Oryctolagus cuniculus*) is the single species in the genus *Oryctolagus* and arguably the best-known lagomorph species (Asin et al., 2024). It is native to the Iberian Peninsula but, thanks to its elevated prolificity, this species has been able to colonize diverse ecosystems reaching continents far from their original distribution area (Alves et al., 2008). For example, in some areas of Australia, where the wild rabbit was introduced in 1859, this species has become a plague, with a large economic and environmental impact. Hares and jackrabbits, also with a global distribution, are larger than rabbits and are grouped in the genus *Lepus*, the most diverse lagomorph genus, with a total of 32 species (Duarte et al., 2020). Several of them face conservation challenges and, according to the International Union for Conservation of Nature (IUCN), some species are categorized as vulnerable, threatened or critically endangered (IUCN, 2024).



**Figure 1.** The order Lagomorpha with genera in families *Ochotonidae* (pikas) and *Leporidae* (leporids). Adapted from Asis et al. (2024).

## 2. Lagomorph species in Spain

Four lagomorph species inhabit Spain: the European wild rabbit, the Iberian hare (*Lepus granatensis*), the European brown hare (*Lepus europaeus*) and the Broom hare (*Lepus castroviejoii*) (Smith et al., 2018). The Broom hare is limited to a restricted area in the Cantabrian Mountains, and the European brown hare, the only non-exclusively endemic hare in the Iberian Peninsula, inhabits northern and northeastern Spain (Figure 2). In contrast, the European wild rabbit and the Iberian hare cover extensive areas of this country (Figure 2), standing out as the most relevant lagomorphs in terms of distribution, abundance and hunting interest (Delibes-Mateos et al., 2008). The high densities of these species, that have favored the maintenance of their genetic diversity, are due to biological characteristics that make them very adaptable to different ecological conditions. Remarkably, the European wild rabbit is the most widespread mammal across Spain, where two subspecies are identified: *O. c. algirus* in the southwestern half of the country and *O. c. cuniculus* in the northeast half (Díaz-Ruiz et al., 2023), being the Iberian Peninsula the only region in the world where these two rabbit subspecies currently co-exist in natural conditions (Ferreira, 2012).



**Figure 2.** Distribution of wild lagomorphs in Spain. A) Iberian hare; B) European wild rabbit; C) European brown hare; D) Broom hare. Adapted from MITECO (2024).

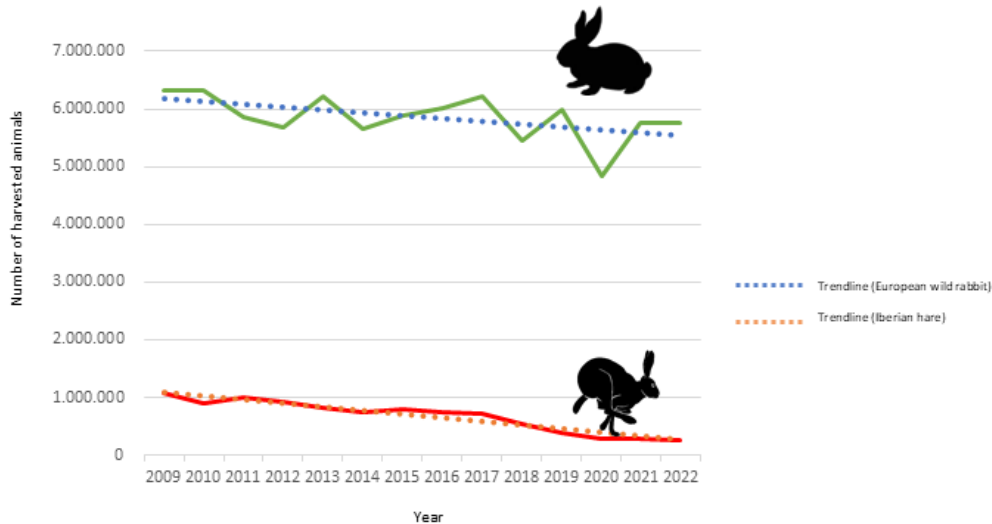
Despite being in the same family and sharing strong morphological similarities, the Iberian hare and the European wild rabbit have distinctive characteristics. Thus, wild rabbits use burrows and holes for hiding and breeding, while Iberian hares do not use burrows but only small depressions in the ground (Delibes-Mateos et al., 2023). Within hares, the Iberian hare exhibits specific morphological characteristics that clearly differentiate it from the other two hare species found in Spain, being the smallest hare in the region (Palacios, 1989). Likewise, genetic differences among these species have also been confirmed through molecular analyses (Melo-Ferreira et al., 2012; Ferreira et al., 2021).

### 2.1. Lagomorphs' population trends in Spain

European wild rabbit and Iberian hare populations of Spain have significantly declined since the mid-20<sup>th</sup> century, currently showing a heterogeneous distribution in this region. Thus, in some areas of the country, the European wild rabbit remains overly abundant and is considered a pest due to the damage it causes to crops. However, its

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populations have nearly disappeared in other parts, including in regions where they were once abundant. To illustrate this drop, data collected from hunting bags (number of animals harvested during a hunting season), show a declining trend in the number of European wild rabbits and Iberian hares hunted in recent years in Spain (Figure 3) (MITECO, 2024).



**Figure 3.** Wild rabbit and Iberian hare population trends in Spain based on hunting bags (Adapted from: MITECO, 2024).

The decline of wild lagomorph populations is likely attributed to a combination of factors, including predation, hunting pressure, climate change, degradation of habitat, the use of phytosanitary products and diseases (Alves et al., 2023; Delibes-Mateo et al., 2023). Lagomorph reproduction depends on the quality of food, which is linked with habitat and climatic conditions. In this context, the reduction of adequate habitat has been suggested as a factor implicated in the decline of their populations (Villafuerte et al., 1997). In addition, the wild rabbit and the Iberian hare face significant pressure from both hunting and predators. Thus, illegal game hunting added to inadequate management causes important populations declines (Ballesteros et al., 1996) and, although predation is not typically the primary cause of populations trends, it can limit population growth or recovery, particularly when numbers are already low (Trout and Tittensor, 1989). Nevertheless, the impact of epizootic diseases stands out as a key factor in the decline of populations, with human activities significantly contributing to their spread (Rouco et al., 2020). Of the diseases that affect the European wild rabbit, myxomatosis (MYXV) and rabbit haemorrhagic disease virus (RHDV) are undoubtedly the principal causes of mortality associated with declining populations (Villafuerte et al., 1995). It is estimated

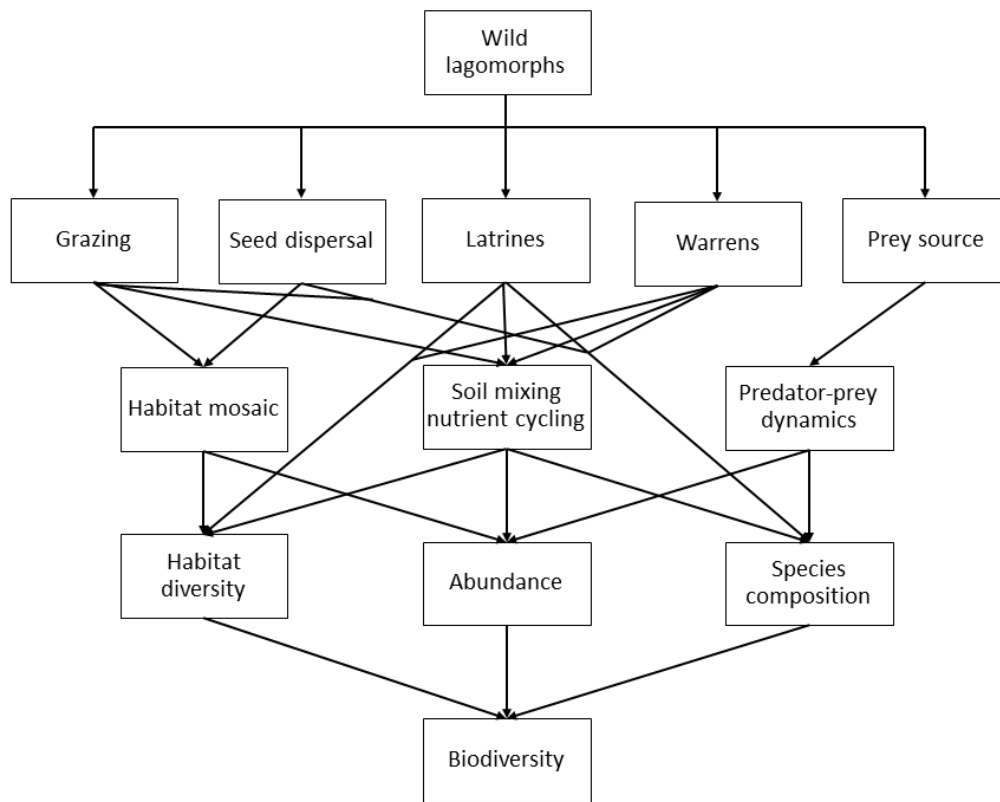
that these two diseases have contributed to a decline in wild rabbit populations of up to 73% in some regions of Spain, affecting the ecosystem equilibrium (Monterroso et al., 2016). Consequently, the wild rabbit is now considered as endangered within its native range (IUCN, 2024). Although hares are susceptible to diverse infectious diseases (Wibbelt and Frölich, 2005), MYXV has been particularly prominent in the Iberian hare since 2018, when significant mortalities were detected in this species in Andalusia (García-Bocanegra et al., 2019). Following these initial cases, the number of affected areas increased sharply impacting most regions of the Iberian Peninsula where this lagomorph species is present (García-Bocanegra et al., 2021; Cardoso et al., 2024).

### **3. Importance of wild lagomorphs in Spain**

In Spain, the population density and wide distribution of European wild rabbits and Iberian hares have converted them in key ecological species. A keystone species is one that is critical to the organization and diversity of its ecological communities, such that its loss would lead to further species extinctions (Mills et al., 1993). Likely, the most recognized attribute of wild lagomorphs is their role as prey, since they are herbivores that play key roles in ecosystems due to their medium size and their position in the food chain. They are prey for more than 30 predators, including emblematic species such as the Iberian lynx (*Lynx pardinus*) and the Spanish imperial eagle (*Aquila adalberti*), significantly contributing to the biodiversity and ecosystem balance (Delibes-Mateos et al., 2008). Thereby, a decline in wild lagomorph populations can reduce overall species diversity within the predator community, leading to various functional and numerical responses from predators, some of which may be unpredictable (Ferrerías et al., 2011). Such is the importance of the European wild rabbit that a higher conservation value has been given to areas where this species reaches high density (Delibes-Mateos et al., 2007).

It is also highlighted the value of wild lagomorphs as modulators of the landscape (Figure 4). Wild lagomorphs act as ecosystem engineers because they modify vegetation through grazing, while also contributing to seed dispersion. Wild rabbit warrens also serve as refuge for numerous vertebrate and invertebrate species and have a notable influence on the flora of herbaceous vegetation (Delibes-Mateos et al., 2009). Additionally, rabbit latrines have a clear impact on soil chemical fertility and plant growth (Willot et al., 2000), while also serving as feeding sites for various invertebrates (Galante and Cartagena, 1999). Thereby, the presence and abundance of wild lagomorphs serve as

indicators of ecosystem health, as changes in their populations may reflect shifts in vegetation, climate, or the overall well-being of the ecosystem (Anderson et al., 2009).



**Figure 4.** Dominant pathways by which wild lagomorphs affect ecosystem structure and function (Adapted from: Delibes-Mateos et al., 2008).

Additionally, from a socio-economical point of view, the European wild rabbit and the Iberian hare have great importance as game species. Hunting of these species date back thousands of years in Europe and still today they remain among the most relevant small game species in terms of population and hunting interest in Spain. More than 30,000 private hunting areas cover over 70% of the Spanish territory (Villafuerte et al., 1998), with many of these areas dedicated to wild lagomorph hunting, particularly in the south. As a result, wild rabbits and Iberian hares are highly managed and intensively harvested, even in regions where their populations are not particularly abundant (Piorno, 2006). Wild lagomorphs maintain a large part of the agricultural income in disadvantaged rural areas where hunting may be an important resource. Thus, about 5.8 million European wild rabbits and 248 thousand Iberian hares are harvested annually in Spain, with an estimated economic value of almost 10 million € per year (MITECO, 202

From a public health perspective, lagomorphs can play a relevant role in the epidemiology of several viruses, bacteria and parasites, with the potential for transmission to other sympatric species, including humans. Although many of those pathogens may have a limited implication on the lagomorph population, these acquire greater relevance from a public health point of view. In Spain, the circulation of different zoonotic pathogens has been evidenced in wild rabbit and Iberian hare populations (Millán et al., 2012; Fernández-Aguilar et al., 2013; García et al., 2014; González-Barrio et al., 2015; Espinosa et al., 2020; Remesar et al., 2021), thus suggesting that the human population may be at risk of exposure of different zoonotic pathogens direct or indirectly transmitted from these species.

### 3.1. Importance of wild lagomorphs in Andalusia

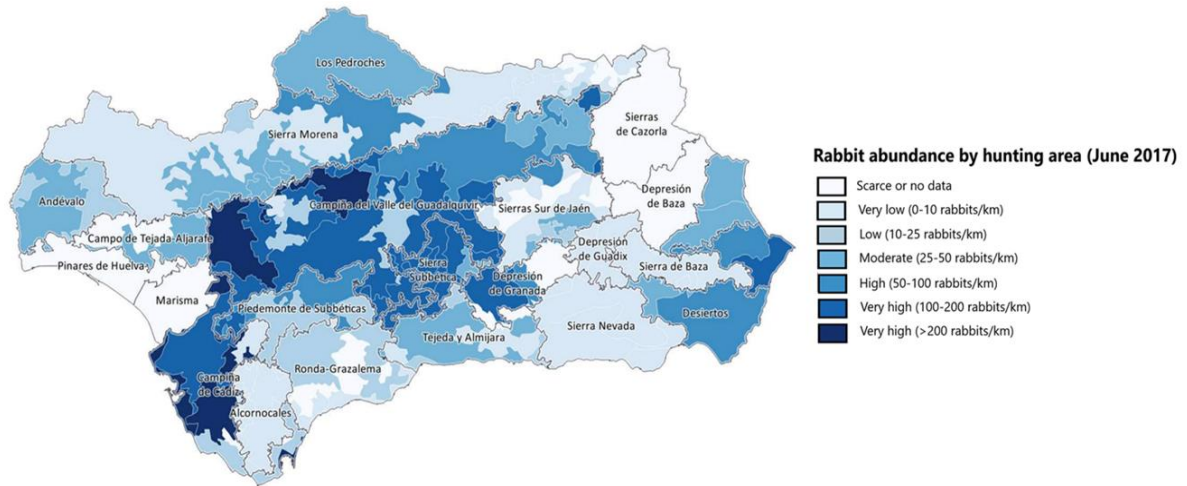
Andalusia (36°N-38°60'N, 1°75'W-7°25'W), with an area of 87,268km<sup>2</sup>, is the second largest autonomous community in Spain. It covers 17.3% of the country's territory and is comparable in size and complexity to many European countries, such as Austria, or Czech Republic. One of the most distinctive features of the region is its wide climatic contrasts, shaped by its geographic location. For instance, the arid Tabernas Desert stands in stark contrast to the Sierra de Grazalema Natural Park, which is the rainiest area in Spain (AEMET, 2024). In general, Andalusia has a Mediterranean climate, characterized by hot, dry summers and mild, humid winters. The region is dominated by a Mediterranean ecosystem, featuring the agroforestry system of the “dehesa”, interspersed with Mediterranean forests. In these areas, multiple land uses such as agriculture, livestock grazing, and hunting are often practiced simultaneously. Andalusia boasts also a rich biodiversity, with a wide variety of both flora and fauna, being one of the most well-preserved European regions and a key area for conservation (Martin and Ferrer, 2013). In addition to hosting a large number of species, Andalusia has a remarkable degree of endemism (approximately 15%) (Tortonese, 1985). Among small herbivores, the European wild rabbit and the Iberian hare stand out, forming the basis of the diet for most carnivorous species in this Mediterranean ecosystem. For instance, the drastic decline of the Iberian lynx, one of the most endangered wild felid species in the world, during the last decades of the 20<sup>th</sup> century was associated with the reduction in the number of its staple prey, the European wild rabbit (López et al., 2014). It was estimated that in 2002 there were only about 100 Iberian lynxes, all of them located in Andalusia, in two isolated areas (Simón et al., 2012). Since 2000, several projects have been launched to



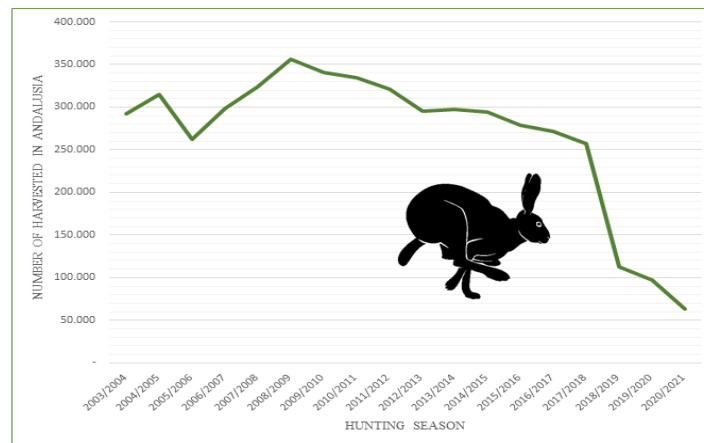
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save the Iberian lynx from extinction and most conservation efforts have focused on fostering and monitoring rabbit populations. In fact, the condition of rabbit populations is the key indicator for selecting reintroduction areas and triggering the emergency plan in the event of an Iberian lynx's population collapse (Vargas, 2009).

From a socio-economic perspective, hunting is highly important in Andalusia, ranking first among autonomous communities in the number of hunting licenses issued and second in the number of harvested big game animals (MITECO, 2024). There are seven big game species in Andalusia: Iberian wild goat (*Capra pyrenaica*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), mouflon (*Ovis musimon*), barbary sheep (*Ammotragus lervia*), and wild boar (*Sus scrofa*). Approximately 123,000 animals of these species are harvested annually, with red deer and wild boar being the most abundant. Among small game species, the European wild rabbit and the Iberian hare hold significant importance in Andalusia, which also ranks as the second-highest autonomous community in terms of the number of small game mammals hunted annually, with more than 1.4 million and 200 thousand of European wild rabbit and Iberian hares, respectively, harvested each year (MITECO, 2024). Nevertheless, wild lagomorphs' populations have shown a decreasing tendency in recent years, with drastically reduced or nearly nonexistent populations in vast areas, some of those considered as important biodiversity hotspots, such as the Cazorla, Segura y las Villas, Sierra Nevada, and Doñana National Parks (Figure 5). This drop was particularly remarkable for Iberian hares following the emergence of the myxoma virus in this species in 2018. Thus, the number of hares hunted between the 2008/2009 and 2020/2021 hunting seasons has been reduced by 82.0% (Figure 6).



**Figure 5.** Wild rabbit abundance map in Andalusia (Adapted from: CSMA, 2024).



**Figure 6.** Iberian hare population trends in Andalusia based on hunting bags (adapted from: CSMA, 2024).

#### 4. Major epizootic diseases affecting the European wild rabbit and the Iberian hare

The population decline of wild lagomorphs, not only in Andalusia but throughout the Iberian Peninsula, is attributed to various anthropogenic and environmental factors, of which transmissible diseases are notable as an important limiting factor in the demography of these species. In the last decades, mortality caused by transmissible diseases, among which MYXV and RHDV stand out, has been detected in the European wild rabbit and Iberian hare populations in the Iberian Peninsula.

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### 4.1. Myxomatosis

MYXV, belonging to the genus *Leporipoxvirus*, was first described in Uruguay in European wild rabbits in 1898, a highly susceptible species with high mortality rates to the virus (Fenner and Fantini, 1999). In contrast, the same virus does not seem to significantly affect *Sylvilagus* rabbits, a species native to South America, in which infection is usually asymptomatic or results in mild clinical symptoms (Bertagnoli and Marchandeu, 2015). During the 1950s, this virus was intentionally introduced into several countries, including Australia and France, to reduce wild rabbit populations. As a result, not only was the target population in France reduced, but the virus also spread uncontrollably across this and into neighboring countries.

In Spain, the first sporadic cases of myxomatosis were detected in 1953 in the northeast, though the first major epizootic outbreaks occurred one year later (Sánchez et al., 1954). This caused a significant reduction in European wild rabbit populations, leading to local extinctions in some Spanish regions. However, over the following decades mortality rates gradually decreased, due to increased genetic resistance, the development of acquired immunity, and the presence of more attenuated viral strains. Currently, myxomatosis is endemic in Spain, with annual outbreaks that can cause high mortality rates in vulnerable populations (Kerr, 2012). Remarkably, MYXV host range is not only restricted to rabbits. The virus had previously been reported in hare species, such as European brown hares and Italian hare (*Lepus corsicanus*) (Lucas et al., 1953; Collins et al., 1955; Barlow et al., 2014; Rossini et al., 2024), often epidemiologically linked to outbreaks in wild rabbits. While sporadic cases of MYXV was reported in this hare species, in July 2018, a novel recombinant hare MYXV provoked several outbreaks in Iberian hares in southern Spain with high mortality and lethality rates (Dalton et al., 2019; García-Bocanegra et al., 2019). During the subsequent months, this emerging virus rapidly spread throughout the Iberian Peninsula (Carvalho et al., 2020; García-Bocanegra et al., 2021; Cardoso et al., 2024), contributing to a reduction of 77.2% in Iberian hare numbers from 2018 to 2021 (Cardoso et al., 2024).

### 4.2. Rabbit haemorrhagic disease

Rabbit haemorrhagic disease (RHD) is an important and highly contagious viral disease of both wild and domestic European rabbits, caused by RHDV belonging to the family *Caliciviridae*. The International Committee on Taxonomy of Viruses (ICTV)

merged the European brown hare syndrome virus (EBHSV) and RHDV into *Lagovirus europaeus* (ICTV, 2024). This genus is divided into two genogroups: GI and GII. These genogroups are further divided into different genotypes. The GI. 1 genotype includes the first identified or “classic” strains of RHDV, while the GI. 2 genotype represents more recent strains classified as RHDV2 (ICTV, 2024).

RHDV was first described in China in 1984 in Angora rabbits imported from Germany (Liu et al., 1984). Two years later, it appeared in Europe, starting in southern Italy (Cancellotti and Renzi, 1991), and reached Spain in 1988 (Argüello et al., 1988), where it caused high mortality in both domestic rabbit farms and wild populations (Villafuerte et al., 1995). RHDV spread rapidly and became endemic, mortality rates dropping from the initial 55-75% to about 30% (Villafuerte et al., 1995). This decline in mortality was likely due to the increasing number of immune rabbits owing to the constant circulation of the virus in previous years (Calvete et al., 2002). However, RHDV2 emerged in France in 2010 and spread rapidly worldwide, including Spain (Rouco et al., 2019), where it was detected in 2011, affecting both domestic and wild rabbits (Le Gall-Reculé et al., 2011). Since its introduction, RHDV2 has caused all subsequent RHD outbreaks in Spain, indicating that this new strain has likely replaced the “classic” RHDV. In southern Spain, following the arrival of RHDV2, there was a 57% reduction in the wild rabbit populations (Guerrero-Casado et al., 2016). Unlike the original strain, RHDV2 is lethal to rabbits of all ages, including subadults and kittens as young as 11 days (Dalton et al., 2012), and have a broader host range within the *Leporidae* family (Asin et al., 2024). While hares are naturally resistant to RHDV, RHDV2 outbreaks or sporadic cases have been detected in European brown hares (Velarde et al., 2021) and Iberian hares (MITECO, 2024). However, hares do not seem to play a significant role in the virus's spread, though a future species jump is possible due to caliciviruses' high mutation rate (Velarde et al., 2021). Regarding GII, between 2020 and 2021, severe mortality was reported in European hares in Spain during an outbreak of EBHS (Almeida et al., 2024), though Iberian hares appear unaffected by this virus.

#### 4.3. Other transmissible diseases affecting wild lagomorph species in Spain

Although with less impact on populations, bacterial and parasitic pathogens, have also been linked to mortality and disease in wild lagomorphs inhabiting Spain. Of these, *Francisella tularensis* and *Yersinia enterocolitica* have been associated with systemic disease causing mortality, especially for Iberian hares in the case of *F. tularensis*. In a

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long-term study carried out in northern Spain, it was evidenced that 31.0% and 12.7% of the hares and lagomorphs found dead were associated with *F. tularensis* and *Y. enterocolitica* (Espinosa et al., 2020). Mortality has also been attributed to gastrointestinal pathogens, including bacteria such as *Escherichia coli* and *Clostridium* spp., as well as parasites, such as *Eimeria* spp. (Espinosa et al., 2020). Regarding other parasitic pathogens, lagomorphs serve as intermediate hosts for *Taenia pisiformis*, whose infection in these species can cause granulomas in the liver, induce weight loss (Betancourt-Alonso et al., 2011) and can be sometimes fatal (Espinosa et al., 2020). In southern Spain, 2.8% of the wild rabbits sampled in a long-term study were infected with this parasite (Remesar et al., 2021). Furthermore, not only endoparasites but also ectoparasites are important for wild lagomorphs. In fact, mortality associated with the zoonotic *Sarcoptes scabiei* have been reported in both European wild rabbit (Millán, 2010) and Iberian hare populations (Cardells et al., 2021).

The pathogens mentioned above are significant contributors to immunosuppression and local mortality of wild rabbit and Iberian hare populations, with potential implications for regional biodiversity and public health. However, information on these pathogens remains scarce and generally limited to specific geographic areas.

## 5. European wild rabbit and Iberian hare as a source of zoonotic pathogens

Lagomorphs have been shown to be natural reservoirs of a wide range of pathogens with zoonotic potential or may act as asymptomatic or spillover hosts of new, emerging and/or potential zoonotic pathogens worldwide (Fontanesi et al., 2016) (Tables 1-3). Even though it is not uncommon to find outbreaks of zoonotic diseases in humans that are related to lagomorphs, the European Food Safety Authority (EFSA) has recently recognized the importance of surveillance in wild lagomorphs for the early detection of zoonotic diseases (ENETWILD-consortium et al., 2023). Transmission of zoonotic pathogens from wild lagomorphs to humans can occur through the consumption or handling of hunted animals, or indirectly through vectors or environmental contamination.

**Table 1.** Zoonotic viral pathogens detected since 2010 in European wild rabbits.

Pathogen	Species	Country	Reference
Hepatitis E virus	European wild rabbit	Australia	Jenckel et al., 2021
		France	Izopet et al., 2012
			Lhomme et al., 2015
			Eiden et al., 2016
		Germany	Hammerschmidt et al., 2017
		Italy	Parisi et al., 2019
		Portugal	Lopes and Abrantes, 2020
			Santos-Silva, 2024
The Netherlands	Burt et al., 2016		
United Kingdom	Parisi et al., 2019		

**Table 2.** Zoonotic bacterial pathogens detected since 2010 in European wild rabbits and Iberian hares.

Pathogen	Species	Country	Reference
<i>Coxiella burnetii</i>	European wild rabbit	Spain	González et al., 2019
			González-Barrio et al., 2015
			Bolaños-Rivero et al., 2017
			Sánchez et al., 2022
<i>Francisella tularensis</i>	European wild rabbit	Algeria	Ammam et al., 2022
		France	Moinet et al., 2016
		Germany	Runge et al., 2011
		Portugal	Lopes et al., 2016
		Spain	Mínguez-González et al., 2021
	Iberian hare	Spain	Isidro et al., 2024

		Mínguez-González et al., 2021	
		Espinosa et al., 2020	
<i>Staphylococcus</i> spp	European wild rabbit	Portugal	Sousa et al., 2020
		Slovakia	Pipová et al., 2012
		Spain	Moreno-Grúa et al., 2020
<i>Escherichia coli</i>	European wild rabbit	Italy	Dotto et al., 2015
		Portugal	Marinho et al., 2014
		Spain	Silva et al., 2010
		Spain	Martínez et al., 2011
<i>Yersinia pseudotuberculosis</i>	European wild rabbit	Tunisia	Ben-Said et al., 2020
		Spain	Martínez et al., 2011
		Iberian hare	
<i>Bartonella alsatica</i>	European wild rabbit	Spain	Márquez 2015
		The Netherlands	Márquez 2010 Kik eet al., 2021
<i>Anaplasma</i> spp	European wild rabbit	Greece	Athanasidou et al., 2021

**Table 3.** Zoonotic parasitic pathogens detected since 2010 in European wild rabbits and Iberian hares.

Pathogen	Species	Country	Reference
<i>Sarcoptes scabiei</i>	European wild rabbit	Spain	Millán, 2010 Millán et al., 2012
	Iberian hare	Spain	Cardells et al., 2021
<i>Toxoplasma gondii</i>	European wild rabbit	Australia	McKenny et al., 2020
		Greece	Athanasidou et al., 2021
		Portugal	Coelho et al., 2020 Waap et al., 2016
		Scotland	Mason et al., 2015

*European wild rabbit and Iberian hare as a source of zoonotic pathogens*

	Iberian hare	Spain	Fernández-Aguilar et al., 2013		
<i>Leishmania infantum</i>	European wild rabbit	Greece	Tsakmakidis et al., 2019 Athanasiou et al., 2021		
		Italy	Abbate et al., 2019 Barbero-Moyano et al., 2024 Chitimia et al., 2011 García et al., 2014 Moreno et al., 2014 Ortega et al., 2017		
		Spain	Ortega-García et al., 2019 Díaz-Sáez et al., 2014 Martín-Sánchez et al., 2024 Martín-Sánchez et al., 2021 Cabezón et al., 2024 Risueño et al., 2018 Jiménez et al., 2014 Ayuso-Sáinz et al., 2022 de la Cruz et al., 2016		
			Iberian hare	Spain	Barbero-Moyano et al., 2024 Ortega et al., 2017 Ortega-García et al., 2019 Ruiz-Fons et al., 2013 de la Cruz et al., 2016
			European wild rabbit	Spain	Díaz-Sáez et al., 2014 Merino-Espinosa et al., 2016
			<i>Babesia caballi</i>	European wild rabbit	Greece
	<i>Thelazia callipaeda</i>		European wild rabbit	Portugal	Gama et al., 2016
	<i>Encephalitozoon spp</i>		European wild rabbit	Germany	Breuninger et al., 2024
		Spain		Martínez-Padilla et al., 2020 Baz-González et al., 2022 Rego et al., 2023	
		Iberian hare	Spain	Martínez-Padilla et al., 2020	



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<i>Enterocytozoon bieneusi</i>	European wild rabbit	Spain	Martínez-Padilla et al., 2020
<i>Cryptosporidium</i> spp	European wild rabbit	Spain The Netherlands	Rego et al., 2023 Robinson et al., 2010
	Iberian hare	Spain	Rego et al., 2023
<i>Giardia duodenalis</i>	European wild rabbit	Spain	Rego et al., 2023
	Iberian hare	Spain	Rego et al., 2023
<i>Blastocystis</i>	European wild rabbit	Spain	Rego et al., 2023
<i>Eimeria stiedae</i>	European wild rabbit	Australia	Peacock et al., 2022
		Greece	Athanasidou et al., 2023
		Iran	Razavi et al., 2010
		Portugal	Silva et al., 2015
		United Kingdom	Bochyńska et al., 2022
<i>Cysticercus pisiformis</i>	European wild rabbit	Poland	Samorek-Pieróg et al., 2021
		Spain	Remesar et al., 2021 Fernández-Álvarez et al., 2013
	Iberian hare	Spain	Espinosa et al., 2020 de Vega et al., 2018

### 5.1. Foodborne pathogens

Zoonotic water and foodborne cases epidemiologically linked to wild rabbits due to **hepatitis E virus** (HEV) (Burt et al., 2016), of which rabbits represent the major reservoir of the emergent HEV-3ra subtype (Cossaboom et al., 2011), *Cryptosporidium cuniculus* (Puleston et al., 2014), or to *F. tularensis* have already been reported in different European countries, including France (Abravanel et al., 2012), Germany (Jacob et al., 2020), Cyprus (Uncu et al., 2017), England or Serbia (Djordjevic-Spasic et al., 2011). In Spain, the first outbreak of tularemia was linked to hunting and handling hares (García-Peña, 1998). Since then, *F. tularensis* has caused recurrent epidemic outbreaks in northwestern Spain (Castilla y León), with outbreaks reported in 2007, 2014, and 2019,

primarily caused by human contact with infected hosts, including Iberian hares (Minguez-González et al., 2021). Also, *Coxiella burnetii*, which causes Q fever, is a zoonotic multi-host bacterium transmitted through different pathways, including the handling of infected animals. In this regard, human cases have already been related with exposure to infected wild rabbits (Marrie et al., 1986). In Spain, the country that recorded the highest number of Q fever cases in recent years (EFSA, 2022), the European wild rabbit is considered a natural reservoir of *C. burnetii* (González-Barrio et al., 2015).

## 5.2. Vector-borne pathogens

Lagomorphs are important key hosts in granting survival and abundance of vector populations. Thus, ticks, fleas, mosquitoes and sandflies are particularly notable for their frequency and abundance in burrows and on the animals themselves. Numerous flea and tick species have been found on wild lagomorphs in the Iberian Peninsula (Table 4), some of those having an important role in the transmission of zoonotic pathogens.

**Table 4.** Species of ticks and fleas detected on European wild rabbits and Iberian hares inhabiting Spain

<b>Genus</b>	<b>Species found on European wild rabbits</b>	<b>Species found on Iberian hares</b>
<b>Ticks</b>		
<i>Rhipicephalus</i>	<i>Rhipicephalus pusillus</i> (Ciceroni et al., 1988)	<i>Rhipicephalus pusillus</i> (Remesar et al., 2022)
	<i>Rhipicephalus sanguineus s.l.</i> (Remesar et al., 2022)	<i>Rhipicephalus sanguineus s.l.</i> (Remesar et al., 2022)
	<i>Rhipicephalus bursa</i> (González et al., 2016)	
<i>Hyalomma</i>	<i>Hyalomma lusitanicum</i> (González et al., 2016)	<i>Hyalomma lusitanicum</i> (Remesar et al., 2022)
<i>Haemaphysalis</i>	<i>Haemaphysalis hispanica</i> (Lopes de Carvalho et al., 2016)	--
<i>Ixodes</i>	<i>Ixodes ventralloi</i> (Gilot and Aubert, 1985)	--

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<i>Dermacentor</i>	<i>Dermacentor marginatus</i> (González et al., 2016)	--
<b>Fleas</b>		
<i>Odontopsyllus</i>	<i>Odontopsyllus quirosi</i> (Márquez, 2015)	--
<i>Spylopsyllus</i>	<i>Spylopsyllus cuniculi</i> (Márquez, 2015)	--
<i>Xenopsylla</i>	<i>Xenopsylla cunicularis</i> (Márquez, 2015)	--
<i>Nosopsyllus</i>	<i>Nosopsyllus fasciatus</i> (González et al., 2021)	--
<i>Echidnophaga</i>	<i>Echidnophaga iberica</i> (Osácar et al., 2001)	--
<i>Caenopsylla</i>	<i>Caenopsylla laptevi</i> (Osácar et al., 2001)	--
<i>Pulex</i>	<i>Pulex irritans</i> (Osácar et al., 2001)	--

Moreover, zoonotic vector-borne pathogens such as such as *Anaplasma* spp. (Remesar et al., 2023), Crimean Congo haemorrhagic fever virus (CCHFV), *Rickettsia* spp. (Estrada-Peña et al., 2012; Márquez, 2008), *Ehrlichia* spp. (Remesar et al., 2022) or *C. burnetii* (Bolaños-Rivero et al., 2017), have been detected in ticks collected from wild lagomorphs inhabiting Spain, indicating the potential role of these mammals in the epidemiology of these pathogens. It should be noted the outbreak of human leishmaniosis that occurred in metropolitan Madrid (Ruiz-Fons et al., 2013), which was the largest outbreak of human leishmaniosis ever reported in Europe, with more than 800 cases (Arce et al., 2013), was linked to the large population of vectors, as well as the presence of wild rabbits and Iberian hares in the city's urban and peri-urban parks. In this context, it was noted that lagomorphs acted as natural reservoirs of these parasites, increasing the risk of transmission to people who shared the same habitat (Arce et al., 2013).

Despite the significant and potential role that European wild rabbits and Iberian hares play in the transmission and maintenance of many zoonotic pathogens, there is still

very limited scientific information about the role of wild lagomorphs in the epidemiology of these and other zoonotic diseases.

## **6. Epidemiological surveillance in wild lagomorphs**

Surveillance, defined as the monitoring of pathogens within a population, is one of the most effective tools for gaining knowledge in the epidemiology of zoonotic pathogens and for the early detection of emerging diseases that threaten public health. This approach forms the foundation of the One Health strategy, which emphasizes the interconnected health of humans, animals, and their shared environment (WOAH, 2024a).

There are two primary types of epidemiological surveillance: active surveillance and passive surveillance. While both surveillances are important for assessing population health, statistical epidemiological estimates and analyses are more effectively applied to data from active surveillance, as it involves structured, purposeful data collection, in contrast to the often irregular and incomplete nature of passive surveillance.

Diseases at the wildlife-livestock-human interface are an increasing concern for public health, animal health and animal conservation authorities worldwide (Kuiken et al., 2011). Moreover, transmissible diseases affecting wildlife are at the top of human emerging diseases (Jones et al., 2008). Basic epidemiologic knowledge is pivotal for targeted prevention and control measures of wildlife-associated diseases. Thereby, the World Organization for Animal Health (WOAH) recommends the incorporation of wildlife in epidemiological surveillance programs, since they can play a role as reservoirs of pathogens or serve as health risk indicators for humans and domestic animals (WOAH, 2024a).

Epidemiological surveillance programs in domestic animals have been common practice for decades, while their application in wildlife is much more recent. The oldest global surveillance network for wildlife diseases was created by WOAH in 1993. In the last decade, improvements have also been introduced in the notification system and collection of data, with the development of the World Animal Health Information System (WAHIS) (WOAH, 2024b). Regarding Europe, the first wildlife surveillance programs were established in the 1930s in Scandinavian countries (Morner et al., 2002). Nevertheless, there are still some countries in Europe without established national wildlife disease surveillance programs, and in most cases, are focused on passive surveillance (Cardoso et al., 2022).

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In Spain, wildlife health surveillance began in the 1980s in response to the health emergency caused by the outbreak of RHD in European wild rabbits (Boadella et al., 2011). However, it was not until 2013 that the Ministry of Agriculture, Fisheries and Food (MAPA) implemented the National Wildlife Health Surveillance Plan (PNVFS) (MAPA, 2024). In that plan, active surveillance focuses on species considered most significant from a public or animal health perspective, in which the European wild rabbit and the Iberian hare were listed. Specifically, three diseases are monitored in the PNVFS in lagomorphs: two of them (myxomatosis and RHD) are of animal health importance, and the third (tularemia) is of both animal and public health concern (RASVE, 2024).

The PNVFS should be coordinated with the surveillance programs implemented by the different Autonomous Communities. In Andalusia, the Epidemiological Surveillance Program in Wildlife (PVE) began in 2009, making it a pioneering initiative, as it was launched before the PNVFS. Currently, it includes an active surveillance program for nine game species, thus aiming to determine disease prevalence and identify the risk factors associated with their occurrence. For lagomorphs, the number of diseases actively monitored are higher than those listed in the PNVFS, and it includes myxomatosis, RHD, EBHS, tularemia, cysticercosis, and the presence of digestive parasites.

In 2010, the PVE also launched a passive surveillance program through the Wildlife Health Alert Network (RASFAS), which activates the PVE's Health Emergency Protocol in cases of abnormal mortality among wild species. This network was crucial in detecting the new variant of RHD, as well as identifying the interspecies transmission of the MYXV to the Iberian hare in 2018 (CSMA, 2024).

However, most of the pathogens actively monitored in lagomorphs both at regional and national level are selected by their importance in animal health. Given that, as mentioned above, lagomorphs can serve as reservoirs for relevant zoonotic pathogens, their role in disease surveillance should be emphasized, as highlighted by the EFSA (ENETWILD-consortium et al., 2023). Despite this, epidemiological information on the circulation of pathogens of public health importance in wild lagomorphs in Spain is still very scarce. Given their key ecological role in different Spanish ecosystems, the circulation of these zoonotic pathogens that are not monitored may have direct consequences for other domestic and wild sympatric species, including human being, and

hampers the development, if required, of adequate control measures to limit the risk of transmission. Therefore, public health concerns clearly indicate the need for epidemiological research of zoonotic pathogens in wild lagomorphs in Spain.

This PhD thesis focuses on determining the role of the European wild rabbit and the Iberian hare in the epidemiology of zoonotic pathogens in southern Spain. We hypothesise that wild lagomorphs could act as reservoir, and therefore be involved in maintenance and transmission, of zoonotic pathogens in Andalusia.

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## OBJECTIVES

The general goal of the present PhD is **to assess the potential epidemiological role of the European wild rabbit and the Iberian hare in the transmission and maintenance of zoonotic pathogens in Mediterranean ecosystems of southern Spain**. Thus, to achieve this general objective different specific goals are considered:

### 1. Viral pathogens:

This objective has been addressed in **Chapter 1**, which includes:

**Chapter 1.1.**, to determine the seroprevalence and prevalence of HEV in wild lagomorphs in Andalusia and to determine potential risk factors associated with HEV exposure in this species.

**Chapter 1.2.**, to assess exposure to CCHFV in wild lagomorphs in southern Spain, a CCHFV endemic area, and to determine the presence of the virus in ticks feeding on these species.

**Chapter 1.3.**, to evaluate the circulation and spatial distribution of WNV in European wild rabbit and Iberian hare populations inhabiting Spanish Mediterranean ecosystems.

### 2. Bacterial pathogens:

This objective has been addressed in **Chapter 2**, which includes:

**Chapter 2.1.**, to determine the prevalence and risk factors associated with *C. burnetii* circulation in European wild rabbits and Iberian hares and to assess the presence of this pathogen in ticks that feed on them in Mediterranean ecosystems in Spain, the country with the highest number of reported cases of Q fever in Europe.

**Chapter 2.2.**, to assess the seroprevalence and risk factors associated with *C. burnetii* exposure in wild lagomorphs in Andalusia.

**Chapter 2.3.**, to determine the occurrence of *F. tularensis* using molecular techniques in wild lagomorphs in Mediterranean ecosystems of southern Spain.

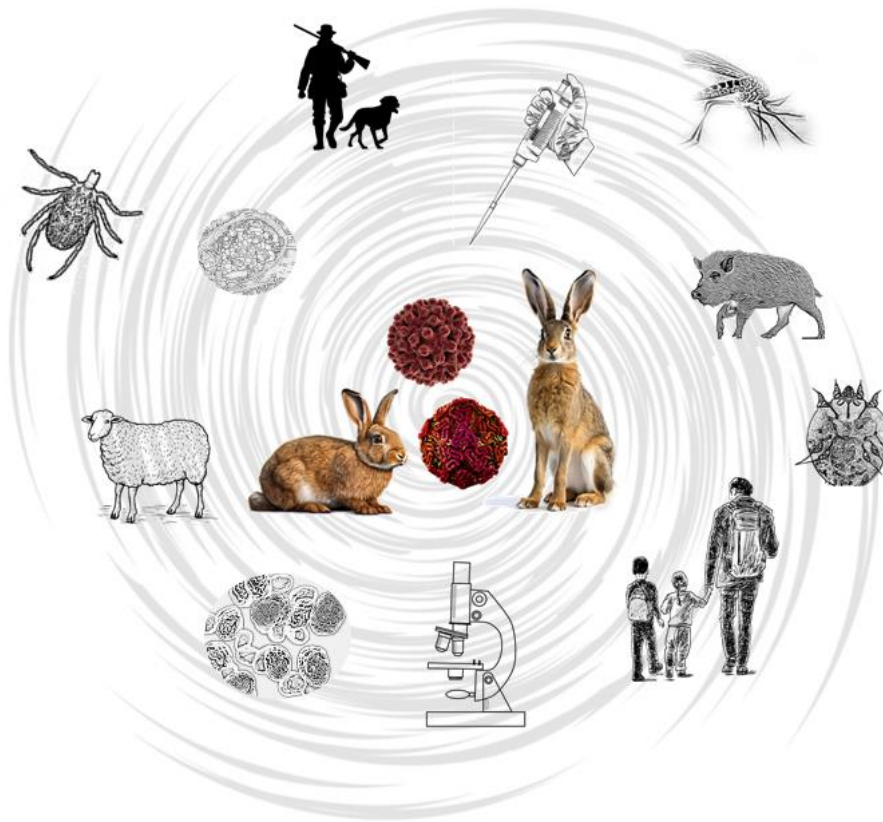
### 3. Parasitic pathogens:

**Chapter 3.1.**, to evaluate the seroprevalence, potential risk factors and spatial distribution of *S. scabiei* in wild lagomorphs in Mediterranean ecosystems of southern Spain.



# CHAPTER 1

## Viral pathogens







## Chapter 1.1.

### Monitoring of hepatitis E virus in wild lagomorphs in Spanish Mediterranean ecosystems



Castro-Scholten, S., Caballero-Gómez, J., Rivero-Juarez, A., Cano-Terriza, D., Gómez-Guillamón, F., Jiménez-Martín, D., Rivero, A., & García-Bocanegra, I. (2023). **Monitoring of Hepatitis E Virus in Wild Lagomorphs in Spanish Mediterranean Ecosystems.** *Transboundary and Emerging Diseases*, 2023(1), 7947220.

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**Abstract**

Hepatitis E virus (HEV) is an emerging zoonotic pathogen in Europe. Even though swine species are considered the main host of the zoonotic HEV-3 genotype, rabbits are recognized as the main reservoir of the divergent HEV-3ra subtype. However, the role of wild lagomorphs in the epidemiology of this virus in Mediterranean ecosystems is under debate. The aims of this study were to assess exposure of HEV in wild rabbit (*Oryctolagus cuniculus*) and Iberian hare (*Lepus granatensis*) populations in southern Spain and to determine potential risk factors associated with HEV exposure in these species. Between 2018 and 2021, blood and fecal samples from 370 wild rabbits and 60 Iberian hares were collected. A total of 29 (6.7%; 95%CI: 4.4–9.1) out of 430 sampled animals showed anti-HEV antibodies. By species, the seroprevalences in wild rabbit and Iberian hare were 6.8% (29/370; 95%CI: 4.2–9.3) and 6.7% (4/60; 95%CI: 0.4–13.0), respectively. Seropositive animals were detected on 17 (26.2%; 95%CI: 15.4–36.8) of the 65 sampled hunting estates. The generalized estimating equations model showed that geographical area was a risk factor potentially associated with HEV exposure in wild lagomorphs in the study region. HEV RNA was not detected in any of the 242 (0.0%; 95%CI: 0.0-1.5) fecal samples tested. This is the first large-scale serosurvey performed in wild rabbits in the Iberian Peninsula and in Iberian hares worldwide. Our results provide evidence of low, widespread and heterogeneous distribution of HEV among wild rabbit and Iberian hare populations in Spanish Mediterranean ecosystems, which indicates a limited role of wild lagomorphs in the maintenance of the virus and a low risk of transmission of HEV to other species, including humans.

**Keywords:** hepatitis E, emerging HEV-3ra, risk factors, survey, wild rabbit, Iberian hare

## Introduction

*Paslahepevirus balayani* (also known as hepatitis E virus [HEV]; family *Hepeviridae*) is the main cause of human acute viral hepatitis worldwide (Lhomme et al., 2019). Eight different genotypes of HEV have been recognized so far (HEV-1 to 8), of which HEV-3, HEV-4 and HEV-7 are zoonotic. Among them, HEV-3 is the genotype with the greatest geographical distribution, being the most prevalent in many high-income regions, including Europe (Treagus et al., 2021). In this continent, HEV-3 human cases have considerably increased during the last few years and contact with infected animals and the consumption of raw or undercooked animal products have been shown to be the major transmission routes of HEV infection (EFSA et al., 2017). Although wild boar (*Sus scrofa*) and domestic pig (*Sus scrofa domesticus*) are recognized as the main hosts of HEV-3 (Pavio et al., 2017), rabbits, which are also susceptible to swine HEV-3 related strains (Hammerschmidt et al., 2017; Parisi et al., 2019), represent the major reservoir of the divergent but also emergent and zoonotic HEV-3ra subtype (Cossaboom et al., 2011).

Since the first description of HEV-3ra in domestic and wild rabbits in France in 2012 (Izopet et al., 2012), HEV RNA and anti-HEV antibodies have been detected in lagomorphs in several European countries, including France (Lhomme et al., 2015), United Kingdom, Italy (Di Bartolo et al., 2016; Parisi et al., 2019), The Netherlands (Burt et al., 2016), Poland (Bigoraj et al., 2020) and Germany (Eiden et al., 2016; Hammerschmidt et al., 2017; Ryll et al., 2018; Corman et al., 2019) with prevalence and seroprevalence values that ranged from 5.0% to 60.0% and from 2.6% to 37.3%, respectively. In the Iberian Peninsula, the European wild rabbit (*Oryctolagus cuniculus*) and the Iberian hare (*Lepus granatensis*) are two endemic species, being the most important small game lagomorph species in terms of abundance and hunting interest. These species are considered an important source of food for humans, especially in rural areas and usually for self-consumption, as rabbit meat is one of the most nutritional white meats (Dalle Zotte & Szendrő, 2011). Over the last decade, circulation of HEV has been confirmed in sympatric wildlife from this European region, such as wild boar (Rivero-Juarez et al., 2018), red deer (*Cervus elaphus*) (Kukielka et al., 2015) and also the Iberian lynx (*Lynx pardinus*) (Caballero-Gómez et al., 2022), whose staple prey is the European wild rabbit. Even though active HEV infection was not found in wild lagomorphs in southern Spain (Caballero-Gómez et al., 2020), only a single local study has assessed HEV exposure in a limited number of wild lagomorphs in the Iberian Peninsula (southern

Portugal), detecting seropositivity (Lopes & Abrantes, 2020). Therefore, the involvement of these species in the epidemiology of HEV in the Iberian Peninsula is under debate. The aims of the present study were to assess the role of wild lagomorphs in the epidemiology of HEV in Mediterranean ecosystems of southern Spain and to determine potential risk factors associated with HEV exposure in these species.

## **Material and Methods**

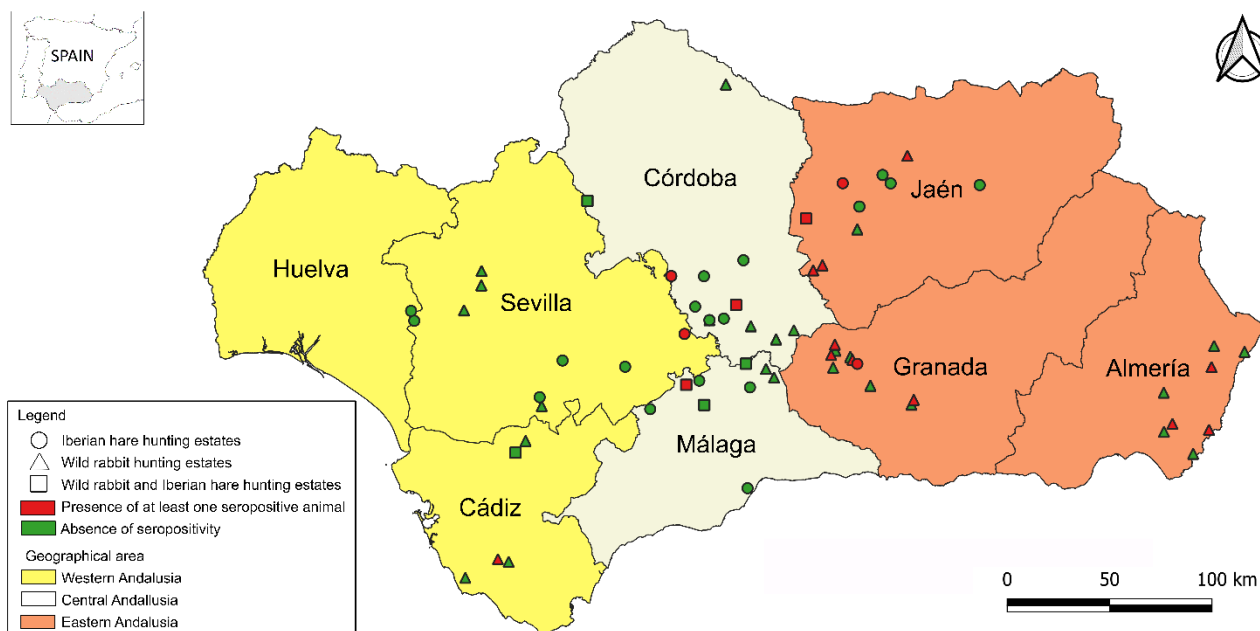
### *Study design and sampling*

A cross-sectional study was carried out in Andalusia (southern Spain; 36°N-38°60'N, 1°75'W-7°25'W), one of the most important regions in Spain in terms of small game population (Garrido et al., 2019). About 1.4 million wild rabbits and 251 thousand hares are harvested annually in Andalusia, being the second Spanish community with the highest number of lagomorphs hunted annually (Garrido et al., 2019; CAGPD, 2022). The Andalusian Mediterranean ecosystems are characterized by the “dehesa” agroforestry system interspersed with Mediterranean forest, where different land uses such as agriculture, farming and/or hunting are simultaneously exploited. The climate is a Continental thermos Mediterranean climate with hot, dry summers and mild winters. The western region presents higher mean humidity and less extreme mean temperatures than the central and eastern regions.

Whenever possible, 60 wild rabbits were sampled per province in order to ensure a 95% probability of detecting at least one positive animal, assuming a minimum seroprevalence of 5% in each province (Thrusfield & Christley, 2018). In addition, for each province, sampling sites (hunting states) were randomly selected and between 1 and 15 (mean: 8.6) wild rabbits were kindly provided by hunters in each of the hunting estates to collect samples (Figure 1). Between 2020 and 2021, serum and fecal samples were collected from a total of 370 wild rabbits sampled in 43 hunting estates. In addition, serum and feces from 60 Iberian hares were taken between 2018 and 2021 in 28 hunting estates in the same study region using a convenience sampling.

Blood samples from all animals were taken from the heart or thoracic cavity and sera were then obtained after blood centrifugation at 400 g for 10 min. Feces were collected directly from the rectum. Samples were stored at -80°C until laboratorial analysis.

Information about each animal, including location, sampling date, age and sex, were recorded whenever possible (Table 1). Bodyweight and length were used as indicators of age (Morris, 1972). Three groups of age were considered: young (< 40 days old), subadult (from 40 days to 8 months) or adult (over 8 months). Epidemiological data related to hunting estates were also gathered through personal interviews with the gamekeepers using a standardized questionnaire (Table 1).



**Figure 1.** Map of the study area (Andalusia, southern Spain) showing the location of the sampled wild lagomorphs hunting estates.

**Table 1.** Distribution of the seroprevalence against HEV in wild lagomorphs in Andalusia (southern Spain) by animal and hunting estate categories and results of the bivariate analysis.

Variable	Categories	No. positives/	Seroprevalence	<i>P</i>
		Overall*	(%)	
Species	Wild rabbit	25/370	6.8	0.620
	Iberian hare	4/60	6.7	
Age	Adult	15/265	5.7	0.228
	Subadult	7/117	6.0	
	Young	5/39	12.8	

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Sex	Male	14/213	6.6	0.536
	Female	13/206	6.3	
Kidney fat index	0	2/56	3.6	0.371
	1	9/122	7.4	
	2	7/98	7.1	
	3	8/67	11.9	
Bodyweight (kg)	0.1-0.9	12/121	9.9	0.274
	1.0-1.1	7/141	5.0	
	1.2-3.1	6/97	6.2	
Body length (cm)	28-37	12/142	8.5	0.836
	38-40	8/121	6.6	
	41-56	6/86	7.0	
Geographical area	Western	3/89	3.4	0.002
	Central	3/136	2.2	
	Eastern	23/205	11.2	
Hunting season	2017/2018	0/1	0.0	0.925
	2018/2019	1/10	10.0	
	2019/2020	2/24	8.3	
	2020/2021	25/365	6.8	
	2021/2022	1/30	3.3	
Burrow density	High	22/348	6.3	0.615
	Medium	0/12	0.0	
	Low	3/38	7.9	
Presence of ticks	Yes	20/293	6.8	0.313
	No	5/105	4.8	
Presence of fleas	Yes	21/274	7.7	0.066
	No	4/124	3.2	
Last restocking (months)	No	12/244	4.9	0.277
	≤6	2/39	5.1	
	6-12	5/65	7.7	
	≥12	6/50	12.0	
Origin of the restocking	Same game estate	11/114	9.6	0.425
	Another game estate	2/32	6.3	



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Cases of myxomatosis in the last year	Yes	24/356	6.7	0.233
	No	1/42	2.4	
Cases of RHD in the last year	Yes	19/304	6.3	0.563
	No	6/94	6.4	
Fenced hunting estate	Yes	0/26	0.0	0.175
	No	25/372	6.7	
Distance to urban areas (Km)	< 10	25/352	7.1	0.105
	10-20	0/32	0.0	
Presence of wild boar ( <i>Sus scrofa</i> )	Yes	17/143	10.6	0.261
	No	6/78	7.1	
Presence of red deer ( <i>Cervus elaphus</i> )	Yes	0/35	0.0	0.071
	No	29/351	7.6	
Presence of wildcat ( <i>Felis silvestris</i> )	Yes	14/135	10.4	0.016
	No	11/263	4.2	
Presence of Iberian lynx ( <i>Lynx pardinus</i> )	Yes	3/24	12.5	0.184
	No	22/374	5.9	
Presence of badger ( <i>Meles meles</i> )	Yes	22/348	6.3	0.614
	No	3/50	6.0	
Presence of marten ( <i>Martes foina</i> )	Yes	19/256	7.4	0.148
	No	6/142	4.2	
Presence of weasel ( <i>Mustela nivalis</i> )	Yes	19/301	6.3	0.591
	No	6/97	6.2	
Presence of polecat ( <i>Mustela putorius</i> )	Yes	11/198	5.6	0.350
	No	14/200	7.0	
Presence of domestic cat ( <i>Felis silvestris</i> <i>catus</i> )	Yes	24/368	6.5	0.420
	No	1/30	3.3	
Presence of dog ( <i>Canis</i> <i>familiaris</i> )	Yes	22/308	7.1	0.142
	No	3/90	3.3	
Presence of cattle ( <i>Bos</i> <i>taurus</i> )	Yes	0/33	0.0	0.097
	No	24/335	7.2	
	Yes	15/152	9.9	0.025

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Presence of goat ( <i>Capra aegagrus hircus</i> )	No	9/216	4.2	
Presence of sheep ( <i>Ovis aries</i> )	Yes	13/199	6.5	0.582
	No	11/169	6.5	
Presence of farmed rabbit ( <i>Oryctolagus cuniculus</i> )	Yes	2/14	14.3	0.217
	No	23/384	6.0	
Presence of domestic pig ( <i>Sus scrofa domestica</i> )	Yes	0/26	0.0	0.162
	No	24/343	7.0	
Presence of rabbit feeders	Yes	14/214	6.5	0.492
	No	11/184	6.0	
Feed supplementation in rabbits	Yes	5/144	3.5	0.060
	No	20/254	7.9	
Presence of swamps	Yes	7/49	14.3	0.023
	No	18/349	5.2	
Presence of waterholes	Yes	14/174	8.0	0.142
	No	11/224	4.9	
Presence of sources	Yes	10/340	7.5	0.313
	No	15/264	5.7	
Presence of troughs	Yes	18/323	5.6	0.093
	No	7/63	11.1	
Presence of streams	Yes	13/187	7.0	0.377
	No	12/211	5.7	
The hunting estate is weeded	Yes	8/71	11.3	0.057
	No	17/327	5.2	
Cleaning watering places	Yes	9/126	7.1	0.389
	No	16/272	5.9	
Presence of artificial burrows	Yes	3/33	9.1	0.344
	No	22/365	6.0	
Crops intended for hunting	Yes	11/161	6.8	0.431
	No	14/237	5.9	

	High (51-100)	5/25	20.0	
Lagomorph density**	Medium (26-50)	5/36	13.9	0.037
(animal hunted/km <sup>2</sup> )	Low (11-25)	2/38	5.3	
	Very low (0-10)	4/94	4.3	

\*Missing values omitted.

\*\*Calculated from the average number of animals hunted in the last 10 years in the sampled hunting estate.

#### *Laboratory analysis*

The presence of anti-HEV antibodies was assessed using a commercial double-antigen multi-species ELISA (HEV ELISA 4.0v; MP Diagnostics, Illkirch, France), in accordance with the manufacturer's instructions. Samples were considered positive when the optical density (OD) at 450 nm of the sample was superior to the cut-off value (cut-off value = mean OD of the negative control + 0.2). This assay is based on the highly conserved recombinant protein ET2.1 (Hu et al., 2008) and detects the presence of total antibodies (IgM, IgG and IgA) against the virus in sera or plasma in a wide range of animal species.

RNA from feces of wild lagomorphs from seropositive hunting states was extracted using the IndiSpin Pathogen Kit (Indical Biosciences, Germany) following the manufacturer's instructions. For HEV RNA detection, two RT-PCR assays were carried out in parallel. A broad-spectrum real-time RT-PCR (CFX Connect Real-Time PCR System) capable of detecting all *P. balayani*, genotypes, including HEV-3ra subtype, with the QIAGEN One-Step RT-PCR kit (QIAGEN, Hilden, Germany) was used as previously described (Frias et al., 2021). In addition, a nested broad-spectrum RT-PCR that is capable to amplify the four genera of hepevirus was also carried out according with Johne et al. (2010). For the first round the QIAGEN One-Step RT-PCR kit was used whereas the nested PCR was carried out with the premixed 2X solution Taq DNA polymerase, dNTPs and reaction buffer kit (Promega). The second PCR products were examined on 1.5% agarose gel stained with RedSafe™ Nucleic Acid Staining solution.

#### *Statistical analyses*

The prevalence and seroprevalence against HEV was determined from the proportion of positive and seropositive animals, respectively, to the total number of

examined, using the two-sided exact binomial test, 95%CI. Firstly, associations between results and independent variables were screened using the Pearson's Chi-square or Fisher's exact test, as appropriate. All variables with a  $p < 0.05$  in the bivariate analysis were selected for further analyses. Collinearity between pairs of variables was tested by Cramer's V coefficient. Finally, a generalized estimating equation (GEE) analysis was carried out to study the effect of the variables selected based on the bivariate analysis. The number of positive animals was assumed to follow a binomial distribution, and "hunting estate" was included as the subject variable. The model was re-run until all remaining variables showed statistically significant values ( $p < 0.05$ ). SPSS 25.0 software (Statistical Package for Social Sciences, Inc., Chicago, IL, USA) was used for all statistical analyses.

## **Results and discussion**

In recent decade, new viral strains of HEV have emerged in different European countries, which may have important clinical and epidemiological implications (Abravanel et al., 2017; Bouamra et al., 2014; Caballero-Gómez et al., 2019; Oeser et al., 2019). Since 2012, an increasing number of HEV-3ra human cases has been confirmed in France, Belgium, Switzerland and, recently, also in Spain (Izopet et al., 2012; Abravanel et al., 2017; Sahli et al., 2019; Suin et al., 2019; Rivero-Juarez et al., 2020) and the high homology between HEV-3ra isolates from humans and rabbits supports the hypothesis of zoonotic transmission linked to consumption of these animals (EFSA, 2017). In addition, HEV-3ra seems to produce chronic hepatitis E cases in immunosuppressed patients more often than other subtypes (Sahli et al., 2019). In this context, understanding the role of lagomorphs in the epidemiology of HEV-3ra is an important key issue to control this emerging genotype.

The present study provides new epidemiological data on HEV in wild lagomorph populations in Mediterranean ecosystems of southern Spain. The major strengths of the survey include a large sample size, which is representative of the whole study area. A total of 29 (6.7%; 95%CI: 4.4-9.1) out of 430 sampled animals showed anti-HEV antibodies, which confirm that wild lagomorphs are naturally exposed to HEV in Iberian Mediterranean ecosystems. The distribution of seropositivity according to the explanatory variables is shown in Table 1. The seroprevalence in wild rabbit was 6.8% (29/370; 95%CI: 4.2-9.3). Our result is consistent with that previously found in this species in Australia (9.1%; 33/362) (Jenckel et al., 2021), and slightly higher to those reported in

the United Kingdom (3.3%; 1/30) (Parisi et al., 2019) and also in a local study conducted in southern Portugal, where 3 out of 74 showed anti-HEV antibodies (4.1%) (Lopes & Abrantes, 2020). By contrast, higher seropositivity values were obtained in wild rabbits in Japan (33.3%; 20/60) (Mendoza et al., 2021), Germany (30.8%; 4/13 - 37.3%; 47/126) (Eiden et al., 2016; Hammerschmidt et al., 2017), Italy (42.9%; 15/35) (Parisi et al., 2019) and Burkina Faso (60.0%; 60/100) (Ouoba et al., 2019).

In the present study, seropositivity was detected in four (6.7%; 95%CI: 0.4-13.0) of the 60 Iberian hares sampled. It should be noted that only a very few numbers of serosurveys have been conducted to date in hares worldwide. Two of them were carried out in Germany in European brown hare (*Lepus europaeus*) and found lower seroprevalences (2.2%; 14/624 - 2.6%; 25/944) (Hammerschmidt et al., 2017; Corman et al., 2019), whereas higher seropositivity values were found in *Lepus africanus* in Burkina Faso (52.6%; 10/19) (Ouoba et al., 2019). Comparisons between studies should be made with caution, given the differences in the serological methods used, study design and species and number of animals sampled. Nevertheless, we would like to state that the seroprevalence of HEV in wild rabbits and Iberian hares in the study area should be considered low.

Statistical differences among ages were not found ( $p = 0.228$ ) (Table 1). In line, seropositivity was detected in 6.0% (7/117) of subadults and 12.8% (5/39) of young lagomorphs in all the years of the study period. Although the presence of maternal antibodies in yearling individuals cannot be ruled out, our results suggest endemic HEV circulation in wild lagomorph populations in Iberian Mediterranean ecosystems during the study period. Seropositive animals were found in all the provinces of the study area and in 17 (26.2%; 95%CI: 15.4-36.8) of the 65 sampled hunting estates (Figure 1).

Although variables related with the hunting estate (“geographical area”, “presence of wildcat”, “presence of goat”, “presence of swamps” and “lagomorph density”) showed association ( $p < 0.05$ ) with the dependent variable in the bivariate analyses (Table 1), only the variable “geographical area” was retained in the GEE model. Significantly higher seropositivity was detected in eastern (11.2%; 95%CI: 6.9-15.5;  $p = 0.009$ ; OR = 6.5 [1.6-26.4]) compared to central Andalusia (2.2%; 95%CI: 0.0-4.7) but not with western region (3.4%; 95%CI: 0.0-7.1;  $p = 0.456$ ; OR = 1.9 [0.3-10.1]). These findings denote a widespread but not homogeneous distribution of HEV in wild rabbit and Iberian hare populations from southern Spain.

Experimental studies reported that HEV can be detected in lagomorphs' feces between two- and ten-weeks post infection (Cheng et al., 2012). Previous studies have confirmed natural circulation of HEV in wild lagomorphs with prevalence values that ranged between 1.7% and 16.0% (Izopet et al., 2012; Burt et al., 2016; Mendoza et al., 2021). In the present study, none of the 242 (0.0%; 95%CI: 0.0-1.5) feces analyzed were positive to HEV RNA, which indicate a limited active HEV circulation (Cheng et al., 2012) in wild lagomorphs in southern Spain. Our results are also consistent with the absence of infection reported previously in liver from these species in the same study region (Caballero-Gómez et al., 2020) and confirm a limited risk of transmission of HEV from wild lagomorphs to other sympatric species, including humans.

In conclusion, the serological results obtained in the present study indicate natural exposure to HEV but low, widespread and heterogeneous viral circulation in wild rabbits and Iberian hare populations in southern Spain. The low seroprevalence as well as the absence of active HEV infection point that these wild lagomorphs play a limited role in the epidemiology of this virus in Mediterranean ecosystems of southern Spain. Further studies in different regions of the Iberian Peninsula are warranted to get a deeper and broader understanding of the epidemiological situation of HEV in these lagomorph species.

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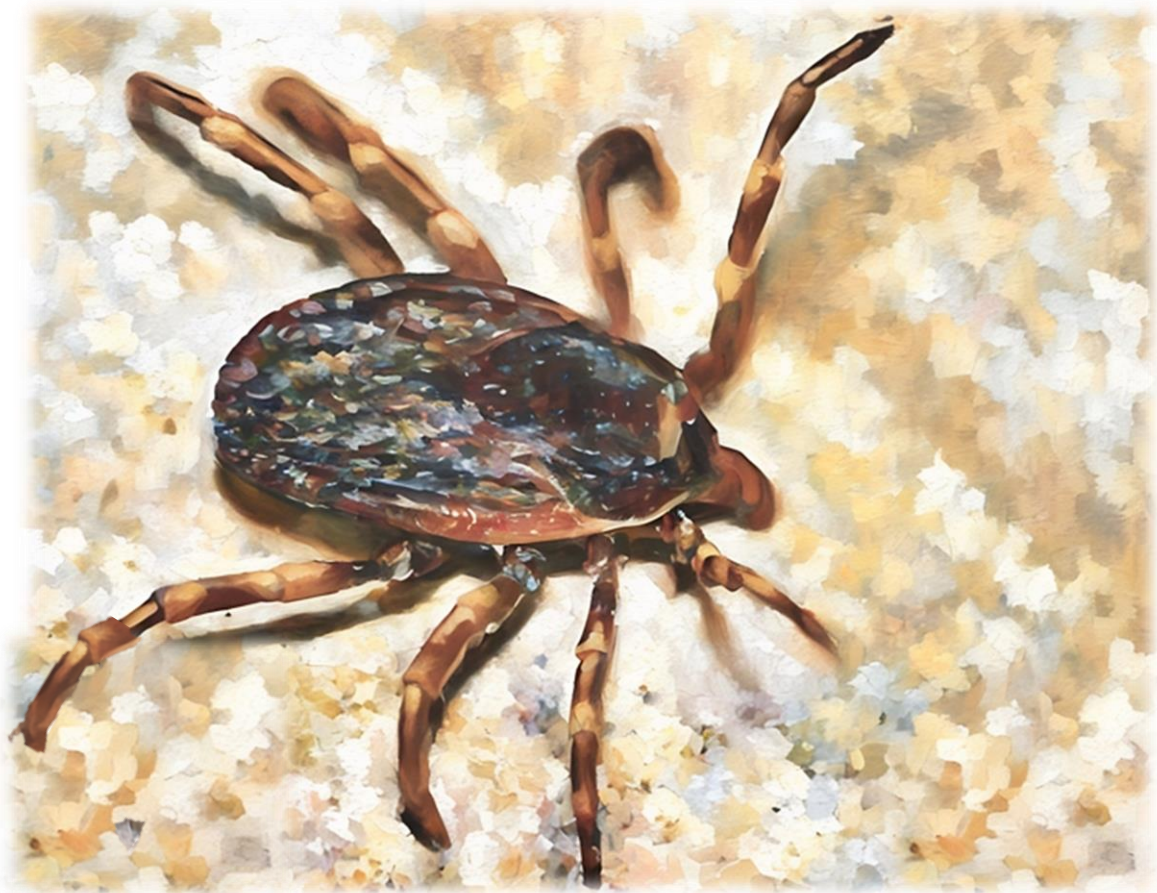
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## Chapter 1.2.

# Absence of Crimean-Congo hemorrhagic fever virus in wild lagomorphs and their ticks in Spanish Mediterranean ecosystems



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**Abstract**

Crimean-Congo hemorrhagic fever virus (CCHFV) is an emerging tick-borne pathogen in different European countries. Since 2013, clinical and fatality cases associated with CCHFV infection have been reported in humans in Spain. During the last few years, endemic circulation of this virus has been detected in ticks and wild ungulates in this country, but the role of other sympatric wild species in the sylvatic cycle of this multi-host virus remains poorly understood. The aims of the present study were to assess exposure to CCHFV in wild lagomorphs in southern Spain, a CCHFV endemic area, and to determine the presence of the virus in ticks feeding on these species. Serum samples from 473 European wild rabbits (*Oryctolagus cuniculus*) and 162 Iberian hares (*Lepus granatensis*), and 120 ticks feeding on 85 of these wild lagomorphs were collected on 133 hunting grounds between 2018/2019 and 2021/2022 hunting seasons. The presence of antibodies against CCHFV was assessed in all serum samples using a commercial ELISA, whereas ticks were tested for the presence of CCHFV-RNA by a multiplex RT-qPCR that detects all known genotypes of this virus. None of the 635 (0.0%; 95%CI: 0.0-0.6) lagomorphs tested had anti-CCHFV antibodies and CCHFV-RNA was not found in any of the 120 (0.0%; 95%CI: 0.0-3.0) ticks analyzed. To the best of the authors knowledge, this is the first epidemiological study conducted on CCHFV in Iberian hare worldwide. Our findings indicate absence of exposure to CCHFV in European wild rabbit and Iberian hare populations, as well as in their ticks, which suggests that they do not seem to play a relevant role in the epidemiology of CCHFV in Mediterranean ecosystems of southern Spain.

**Keywords:** CCHFV, emerging diseases, wild lagomorphs, tick, One Health

## Introduction

Crimean-Congo hemorrhagic fever virus (CCHFV) (*Orthonairovirus haemorrhagiae*, family *Nairoviridae*) is a tick-borne multi-host zoonotic virus of great public health concern (Hawman and Feldmann, 2018), inducing severe and frequently lethal hemorrhagic disease in humans (Ergonul, 2006). This virus, recognized as the most widespread tick-borne hemorrhagic pathogen, currently lacks a specific vaccine or treatment (Gavier-Widen et al., 2023). Given its significant pathogenicity and potential to cause public health emergency, the World Health Organization (WHO) has listed CCHFV as a priority pathogen for research (WHO, 2018). In Europe, the disease has traditionally been reported in south-eastern countries (Mesina et al., 2015; Papa et al., 2015), but Spain has experienced a growing number of human CCHF cases since 2013 (Portillo et al., 2021), with 13 clinical and four fatality cases reported so far (RENAVE, 2024), being currently considered an emerging and underdiagnosed disease in this country (Monsalve Arteaga et al., 2021).

CCHFV is mainly acquired through tick bites, although exposure to the blood or tissues of infected animals or humans can also lead to transmission (Ergonul, 2012). Different tick species have been suggested to contribute to the circulation of the virus (Gargili et al., 2017; Cuadrado-Matías et al., 2024). However, the distribution area of CCHFV is primarily linked with environmental conditions that are most suitable for the presence of ticks from the *Hyalomma* genus, its only proven competent vectors so far (Mesina et al., 2015).

The reported host range of CCHFV, particularly in wildlife species, has considerably increased during the last years through numerous serological and molecular studies, highlighting the need of identifying all potential reservoirs to control this zoonotic pathogen. The virus has been detected in different ungulate, lagomorph, artiodactyl, perissodactyl and bird species, and do not show apparent signs of disease (Cooper et al., 2004; Espunyes et al., 2021; Nurettin et al., 2022; Shahhosseini et al., 2021; Baz-Flores et al., 2024b). In this context, the European Food Safety Authority (EFSA) recently listed CCHFV as a priority pathogen for surveillance in European wildlife (Gavier-Widen et al., 2023).

In Iberian Mediterranean ecosystems, the European wild rabbit (*Oryctolagus cuniculus*) and the Iberian hare (*Lepus granatensis*) are keystone species and constitute

an important source of human food, generally destined for home consumption and without veterinary controls. Although outbreaks of CCHF have been suggested to be epidemiological linked to wild lagomorphs (Bente et al., 2013) and these species are the main hosts of immature stages of *Hyalomma lusitanicum* (Valcárcel et al., 2020), there is not information on the role of wild lagomorphs in the epidemiology of this virus in Spain (Espunyes et al., 2023). Thus, the aims of the present study were to investigate the exposure to CCHFV in European wild rabbit and Iberian hare populations from Mediterranean ecosystems in southern Spain, a CCHFV endemic area, and to assess the presence of the virus in ticks feeding on these lagomorph species.

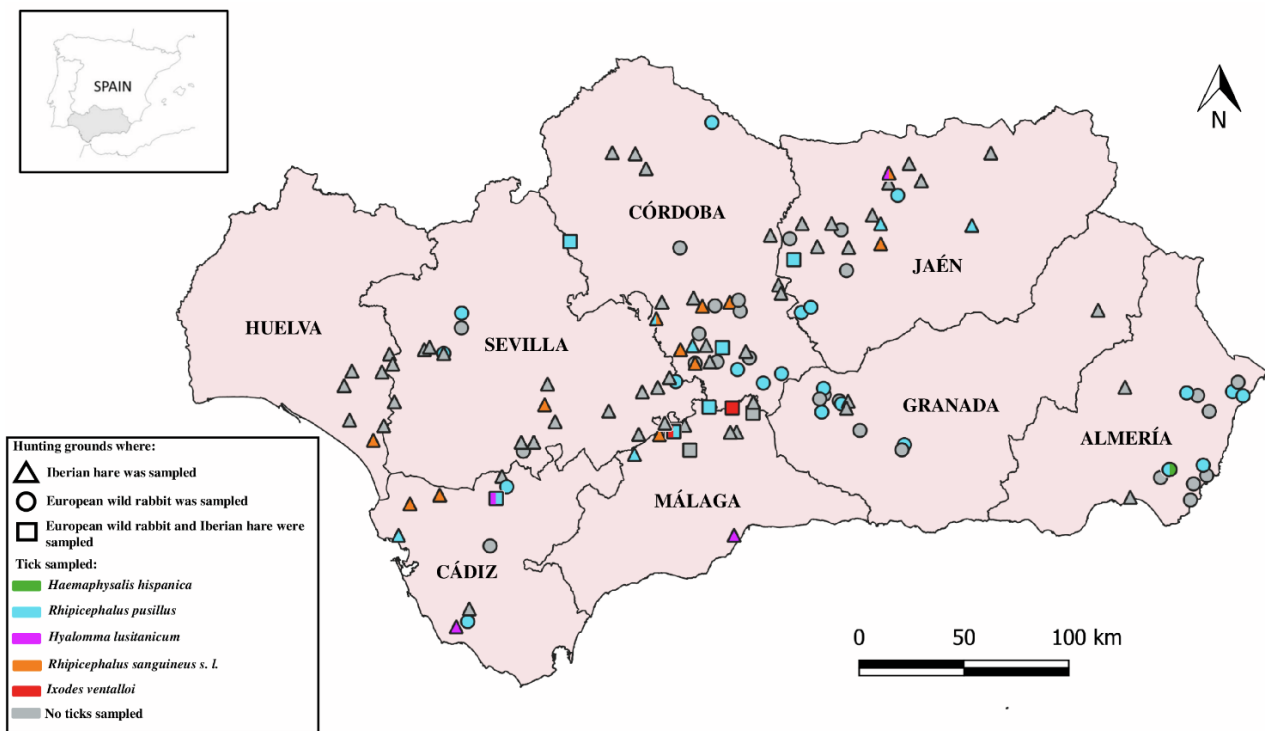
## **Material and Methods**

### *Study area and sampling collection*

The study was carried out in Andalusia, a region of 87,268 Km<sup>2</sup> located in southern Spain (36°N-38°60'N, 1°75'W-7°25'W) (Figure 1), identified recently as part of a high-risk area of exposure to CCHFV (Baz-Flores et al., 2024a). The region is characterized by the Mediterranean ecosystem, with the “dehesa” agroforestry system interspersed with Mediterranean forest, where different land uses such as agriculture, farming, and/or hunting are simultaneously exploited. The climate is Mediterranean with dry summers and mild, humid winters (AEMET, 2023).

Sampling size was calculated as a minimum of 600 animals, to ensure a 95% probability of detecting at least one positive animal for an assuming prevalence of 0.5%. Between hunting seasons 2018-2019 and 2021-2022, a total of 635 lagomorphs, including 473 European wild rabbits and 162 Iberian hares, legally hunted in 59 and 83 hunting grounds, respectively, were sampled across all eight provinces of Andalusia (Figure 1). Blood samples from all animals were obtained by post-mortem heart puncture. Serum samples were obtained after blood centrifugation at 400g for 10 min. Both sera and ticks were stored at -80°C until serological and molecular analysis were undertaken.





**Figure 1.** Spatial distribution of sampled hunting grounds in the study region (Andalusia, southern Spain).

After visual inspection, a total of 120 ticks feeding on 54 and 31 of the rabbits and hares sampled, respectively, were also collected and stored in individually labeled plastic bags. Ticks were already identified in a previous survey conducted by Remesar et al. (2021). Morphological keys were used for the identification to species level (Pérez-Eid, 2007), and a subset of each tick species was further molecularly analyzed (Norris et al., 1996) to confirm microscopic identification.

During sampling, an epidemiological questionnaire was filled out by directly interviewing the gamekeepers of each hunting ground. In addition, individual information, including species, location, year of sampling, age and sex, was recorded for each lagomorph sampled (Table 1). Body weight and body length were used as indicators of age (Morris, 1972).

**Table 1.** Distribution of the wild lagomorphs sampled by categories.

Variable	Categories	No. of wild lagomorphs* (relative frequency (%))	No. of European wild rabbit* (relative frequency (%))	No. of Iberian hare * (relative frequency (%))
Species	European wild rabbit	473 (74.5)	-	-
	Iberian hare	162 (25.5)	-	-
Age	Adult	418 (67.0)	292 (63.0)	126 (78.8)
	Subadult	153 (24.5)	131 (28.2)	22 (13.8)
	Young	53 (8.5)	41 (8.8)	12 (7.5)
Sex	Male	321 (51.4)	240 (51.3)	81 (51.6)
	Female	304 (48.6)	228 (48.8)	76 (48.4)
Province	Almería	55 (8.7)	52 (11.0)	3 (1.9)
	Cádiz	77 (12.1)	66 (14.0)	11 (6.8)
	Córdoba	164 (25.8)	121 (25.6)	43 (26.5)
	Granada	69 (10.9)	63 (13.3)	6 (3.7)
	Huelva	9 (1.4)	0 (0.0)	9 (5.5)
	Jaén	77 (12.2)	45 (9.5)	32 (19.8)
	Málaga	76 (12.0)	45 (9.5)	31 (19.1)
	Sevilla	108 (17.0)	81 (17.1)	27 (16.7)
Hunting season	2018/2019	66 (10.4)	1 (0.2)	65 (40.1)
	2019/2020	62 (9.8)	11 (2.3)	51 (31.5)
	2020/2021	472 (74.3)	428 (90.5)	44 (27.2)
	2021/2022	35 (5.5)	33 (7.0)	2 (1.2)

\*Missing values omitted

### *Serological analysis*

The presence of antibodies against CCHFV was tested using a commercial multi-species double antigen ELISA kit (IDvet Screen<sup>®</sup>, Montpellier, France), in accordance with the manufacturer's instructions. Interpretation of tested samples is based on the ratio of the sample optical density measured at 450 nm (OD<sub>S</sub>) to the optical density of the positive control (OD<sub>PC</sub>) expressed as percentage: (OD<sub>S</sub> / OD<sub>PC</sub>) x 100 (hereafter S/P (%)). A sample was classified as positive if S/P (%) was over 30%. Serum samples with S/P (%) below or equal to 30% were considered as negative.

*Molecular detection*

The 120 ticks were analyzed for molecular detection. RNA was extracted using the NucleoMag<sup>®</sup> Vet Kit (Macherey-Nagel, Düren, Germany). The presence of CCHFV RNA was assessed by means of QuantiTect<sup>®</sup> Probe RT-PCR Kit (QIAGEN, Hilden, Germany) using the multiplex RT-qPCR protocol described elsewhere (Sas et al., 2018), which has been shown to detect all currently known CCHFV genotypes. The RT-qPCR was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA).

**Results and discussion**

In Spain, the first report of CCHFV dates back to 2010, when the virus was detected in *H. lusitanicum* ticks collected from red deer (*Cervus elaphus*) (Estrada-Peña et al., 2012). Since then, endemic circulation and high genetic diversity of CCHFV have been evidenced in several regions of this country. To date, CCHFV exposure has been detected in different tick species (Moraga-Fernández et al., 2021; Sánchez-Seco et al., 2022; Cuadrado-Matías et al., 2024), in wildlife, with seroprevalences ranging between 19.4% and 78.6% in red deer (Cuadrado-Matías et al., 2022 a, b), Iberian ibex (*Capra pyrenaica*) (Espunyes et al., 2021) and wild boar (*Sus scrofa*) (Baz-Flores et al., 2024a; Cuadrado-Matías et al., 2022a), as well as in domestic mammal species, with seroprevalence values of 2.8% and 3.4% detected in Iberian pigs (Frías et al., 2024) and small ruminants (Baz-Flores et al., 2024b), respectively.

In the present study, which was conducted in the CCHFV endemic region in southern Spain, none of the 473 (0.0%; 95%CI: 0.0-0.8) wild rabbits and 162 (0.0%; 95%CI: 0.0-2.3) Iberian hares sampled showed anti-CCHFV antibodies. These results indicate absence of exposure to CCHFV in wild lagomorph populations in southern Spain, which suggests that they do not seem to play a relevant role in the epidemiology of CCHFV in Mediterranean ecosystems of southern Spain. These findings are consistent with the only previous serosurvey conducted in wild rabbits worldwide, using the same ELISA than in the present study, in which antibodies against CCHFV were not found in any of the 35 individuals sampled in the northeastern region of Spain (Espunyes et al., 2021).

To the best of the author's knowledge, this is the first study to assess CCHFV exposure in Iberian hares. Previous studies conducted in other hare species such as the

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European brown hare (*Lepus europaeus*) evidenced exposure to this virus, with seroprevalence values ranging between 6.0% (12/198) in Hungary (Németh et al., 2013), 20.0% (4/20) in Russia (Zarubinsky et al., 1975) and 23.8% (5/21) in Turkey (Nurettin et al., 2022). In contrast, no anti-CCHFV antibodies were detected in the four European brown hares analyzed in Albania (Papa et al., 2009). The Iberian hare is distributed throughout most of the Iberian Peninsula and although the European brown hare is not distributed in the study area, it inhabits north and northeastern regions of Spain. Therefore, further studies on these lagomorph species covering different areas of the Iberian Peninsula are warranted to determine their role in the epidemiology of CCHFV in this country.

Fourteen different tick species belonging to the genera *Dermacentor*, *Ixodes*, *Haemaphysalis*, *Hyalomma* and *Rhipicephalus* (Family Ixodidae) have been detected in southern Spain so far (Ruiz-Fons et al., 2006; Millán et al., 2007; San José et al., 2022). Wild lagomorphs have been shown to be important hosts for immature stages of ticks of the *Hyalomma* genus (Gargily et al., 2017). However, no studies have been carried out on CCHFV in ticks collected from European wild rabbits or Iberian hares so far. In the present study, none of the 120 (0.0%; 95%CI: 0.0-3.0) ticks were positive for CCHFV RNA. A previous study conducted in Turkey analyzed 216 *Hyalomma* spp., *Haemaphysalis* spp. and *Rhipicephalus* spp. ticks collected from European brown hares, detecting one CCHFV positive pool of *Haemaphysalis parva* (Orkun et al., 2017). By contrast, individual or pool prevalences between 3.2% to 21.0% were found in ticks collected from wild ungulates in Spain (Moraga-Fernández et al., 2021; Sánchez-Seco et al., 2022), being CCHFV mainly detected in *H. lusitanicum*, but also in *Ixodes ricinus*, *Dermacentor marginatus*, *Hyalomma marginatum* and *Rhipicephalus annulatus* (Estrada-Peña et al., 2012; Moraga-Fernández et al., 2021; Negrodo et al., 2019; Sánchez-Seco et al., 2022). In the present study, five different tick species were analyzed, being *Rhipicephalus pusillus*, in which CCHFV has never been detected, and *Rhipicephalus sanguineus* s.l., in which the virus has recently been detected in Spain (Cuadrado-Matías et al., 2024), the most abundant collected species followed by *Hyalomma lusitanicum*, *Haemaphysalis hispanica*, and *Ixodes ventalloi* (Table 2). Thus, our results suggest that the particular ticks parasitizing wild lagomorphs in the study area are not involved in the maintenance and transmission of CCHFV. Nevertheless, further studies including a larger

number of ticks from different species and at different stages, specifically nymphs, sampled throughout the year are warranted to support this hypothesis.

**Table 2.** Distribution of the tick species analyzed for the detection of Crimean-Congo hemorrhagic fever virus RNA collected from European wild rabbits and Iberian hares.

Host species	N° ticks			Total
	Nymph	Adult female	Adult male	
<b>Wild rabbit</b>				
<i>Rhipicephalus pusillus</i>	0	24	31	55
<i>Haemaphysalis hispanica</i>	1	3	3	7
<i>Hyalomma lusitanicum</i>	1	0	0	1
<i>Ixodes ventralloi</i>	0	3	0	3
TOTAL	2	30	34	66
<b>Iberian hare</b>				
<i>Rhipicephalus pusillus</i>	0	7	9	16
<i>Rhipicephalus sanguineus</i> s.l.	0	4	27	31
<i>Hyalomma lusitanicum</i>	2	4	1	7
TOTAL	2	15	37	54
<b>TOTAL</b>	<b>4</b>	<b>45</b>	<b>71</b>	<b>120</b>

In conclusion, our findings indicate absence of exposure to CCHFV in European wild rabbit and Iberian hare populations, as well as in their ticks, during the study period. These findings suggest a limited risk of transmission of CCHFV from these species to other mammals, including humans, in Spanish Mediterranean ecosystems. Further studies are required to assess CCHFV circulation in wild lagomorphs from other areas of the Iberian Peninsula, a European region where this emerging virus has emerged during the last decade.

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## Chapter 1.3.

### **Exposure to West Nile virus in wild lagomorphs in Spanish Mediterranean ecosystems**



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## **Abstract**

**Background:** West Nile virus (WNV) is the most widely distributed mosquito-borne flavivirus. Over the past decade, its spread across Europe has raised significant concerns for both public and animal health. Although WNV exposure has been evidenced in various wild mammal species in Spain, no seroepidemiological studies have been conducted on this flavivirus in wild lagomorphs so far.

**Aim:** This study aimed to assess WNV exposure in European wild rabbit (*Oryctolagus cuniculus*) and Iberian hare (*Lepus granatensis*) populations inhabiting Spanish Mediterranean ecosystems.

**Methods:** Sera from 540 wild lagomorphs (399 European wild rabbit and 141 Iberian hares), from 106 hunting grounds distributed throughout Andalusia (southern Spain), were collected between the 2018/2019 and 2022/2023 hunting seasons.

**Results:** Antibodies against flavivirus were detected by blocking enzyme-linked immunosorbent assay (bELISA) in 5.0% (27/540; 95%CI: 3.2-6.8) of the wild lagomorphs. Exposure to WNV was confirmed in 4.8% (19/394; 95%CI: 2.7-6.9) of wild rabbits and 0.7% (1/141; 95%CI: 0.0-2.1) of Iberian hares by virus microneutralization test. Anti-WNV antibodies were found in wild lagomorphs sampled from three (2.8%) hunting grounds located in western Andalusia during the seasons 2020-2021 and 2021-2022. Remarkably, this spatiotemporal distribution overlaps with the largest outbreak of WNV in Spain. Antibodies against Usutu virus and Bagaza virus were not detected in the wild lagomorph populations analyzed.

**Conclusions:** This study constitutes the first report of WNV exposure in wild rabbit in Spain and in Iberian hare worldwide. While these species seem not play a primary role in the epidemiology of the virus, they could serve as sentinel for monitoring WNV in Iberian Mediterranean ecosystems.

**Keywords:** arbovirus, *Orthoflavivirus*, serosurveillance, zoonosis, One Health, vector borne diseases

## **Impacts**

- First report of WNV exposure in Iberian hare worldwide.
- Seropositivity found in wild lagomorphs overlaps with the largest WNV outbreak in Spain.

### *Chapter 1.3*

- Wild lagomorphs could serve as sentinels for monitoring WNV in Iberian Mediterranean ecosystems.

## **Introduction**

Flaviviruses (genus *Orthoflavivirus*, family *Flaviviridae*) are endemo-epidemic *vector-borne pathogens* in Europe, representing a significant threat for public health. Among them, West Nile virus (WNV) has the broadest geographical spread (Cuervo et al., 2022), being considered as the leading cause of arboviral encephalitis in humans worldwide (Ciota, 2017). During the past two decades the WNV has spread to various regions of eastern and central Europe, while circulating endemically in the Mediterranean basin (ECDC, 2024).

In Spain, WNV circulation has been confirmed in bird, mammal and mosquito's species since the early 2000s (Figuerola et al., 2007; Jiménez-Clavero et al., 2008). However, cases in humans were sporadic until 2020, when an unprecedented WNV epidemic occurred in southwestern Spain, with 77 clinical cases and 7 deceased (Rodríguez-Alarcón et al., 2021). Since then, human cases have been reported annually in this area (ECDC, 2024), establishing the virus as endemic in this region.

The natural enzootic cycle of WNV primarily involves birds as primary hosts and mosquitoes, especially those of the *Culex* genus, serving as competent vectors (Vogels et al., 2017), while mammals, mainly horses and humans, are generally considered dead-end hosts due to their low levels of viremia (Blitvich, 2008). Due to the significance of these viruses, a growing number of studies focused on wildlife have identified an increasing number of species susceptible to WNV, thereby expanding the range of potential hosts beyond the primary ones (Jeffrey, 2013). In Iberian Mediterranean ecosystems, WNV exposure has been evidenced in different wild mammal species, including wild ruminants (García-Bocanegra et al., 2016), red foxes (*Vulpes vulpes*) and wild boar (*Sus scrofa*) (Gutiérrez-Guzmán et al., 2012).

The European wild rabbit (*Oryctolagus cuniculus*) and the Iberian hare (*Lepus granatensis*) are two endemic and keystone species in the Iberian Peninsula (Delibes-Mateos et al., 2007) and are among the most abundant and significant species in terms of hunting interest. These species are widespread across the Iberian Peninsula, inhabiting diverse environments, including urban and peri-urban areas. They can reach high densities in certain regions, thereby increasing the risk of zoonotic pathogens transmission (Jiménez et al., 2014). In this regard, these wild lagomorph species have been shown to be natural reservoirs for several zoonotic vector-borne pathogens such as

*Coxiella burnetii*, *Francisella tularensis* or *Leishmania infantum* (Jiménez et al., 2014; Gonzalez-Barrio et al., 2015; Castro-Scholten et al., 2024) and they have been considered to be useful sentinel species for some vector-borne diseases (Carvalho et al., 2014). Furthermore, natural exposure to WNV has been evidenced in European wild rabbits (Arthur et al., 1990) and European brown hares (*Lepus europaeus*) (Juřicová & Hubálek, 1999) although upon infection, these species remain asymptomatic (McLean et al., 2002). However, there are no seroepidemiological studies on WNV in European wild rabbit in Spain and Iberian hare worldwide. Thus, the aim of the present study was to assess exposure to WNV in wild lagomorph populations of Spanish Mediterranean ecosystems.

## **Material and Methods**

### *Study area and sampling collection*

A cross-sectional epidemiological study was conducted in Andalusia, southern Spain (36°N-38°60'N, 1°75'W-7°25'W), across the hunting seasons 2018/2019 to 2022/2023. This region accounted for nearly 83% of the total WNV cases detected in human and horses in Spain since the first detection in 2010 (RNVE, 2023; RASVE, 2024). For the European wild rabbits, sample size was calculated as a minimum of 385 animals, assuming a prevalence of 50%, with a 95% confidence interval (95%CI) and a desired precision of  $\pm 5\%$ . Whenever possible, 42 wild rabbits were sampled in each of the eight provinces within the study area to ensure a 95% probability of detecting at least one positive animal, assuming a minimum within-province prevalence of 7% (Thrusfield & Christley, 2018). Hunting grounds were randomly selected for sampling in each province. Ultimately, a total of 399 wild rabbits from 53 hunting grounds distributed across all eight provinces were sampled during the study period. A convenience sampling was used for the Iberian hare, as its geographic distribution is restricted to certain areas due to recent population declines, primarily resulting from the impact of myxomatosis on this species (García-Bocanegra et al., 2019, 2021). A total of 141 Iberian hares from 62 hunting grounds were sampled in the same study area and period. Blood samples were collected from the hearth or thoracic cavity of all animals and centrifuged at 400x g for 10 min. The resulting serum was stored at -80°C until serological analysis were performed.

During sampling, an epidemiological questionnaire was completed through direct interviews with gamekeepers at each hunting ground. The information collected included the characteristics of the hunting ground, the presence of diseases (myxomatosis and

rabbit hemorrhagic disease) and control measures, management practices, and the presence of other sympatric species. Additionally, data on each individual animal were recorded, including species, location, year of sampling, age (determined by bodyweight and body length; Morris, 1972), kidney fat index and sex.

#### *Serological analysis*

Sera from wild lagomorphs were analyzed using a commercial blocking enzyme-linked immunosorbent assay (bELISA) (10.WNV.K3 IngezimWest Nile Compac<sup>®</sup>, Gold Standard Diagnostics, Madrid, Spain) to detect antibodies that block the reaction of a horseshoe peroxidase-conjugated monoclonal antibody specifically recognizing an epitope within the envelope protein domain III of WNV. The assay was performed according to the manufacturer's instructions. Results were expressed as a percentage of inhibition (PI) and it was calculated as follows:  $PI = 100 - [(OD_{\text{sample}}/OD_{\text{NC}}) \times 100]$ , in which  $OD_{\text{sample}}$  is the optical density of a sample and  $OD_{\text{NC}}$  is the mean optical density of the negative control (NC). Samples with PI values >40% were considered positive, <30% negative, and between 30% and 40% doubtful.

Positive and doubtful sera were further tested by micro virus neutralization test (micro-VNT) to confirm the presence of specific neutralizing antibodies against WNV as well as against Usutu virus (USUV) and Bagaza virus (BAGV), two other flaviviruses which could cross-react in the ELISA tests (Llorente et al., 2019), and whose circulation has been previously evidenced in the study area (García-Bocanegra et al., 2013; Jurado-Tarifa et al., 2016). Micro-VNTs were performed according to the previously described protocol (Llorente et al., 2019). The immune response was considered specific for a WNV when neutralizing antibodies showed titres equal or higher than 1:10 by micro-VNT and at least fourfold higher than titres obtained for any of the other viruses analyzed (Calisher et al., 1989). When titres differences did not reach this threshold, the result was considered inconclusive and the specific virus to which the animal was exposed could not be determined.

#### *Statistical analysis*

Animals were classified as seropositive for WNV if the sera tested positive/doubtful by ELISA and positive for specific WNV antibodies by micro-VNT. Seroprevalence was calculated by dividing the number of seropositive animals by the total number of animals tested, using two-side exact binomial 95% confidence intervals



(95%CI). Continuous variables were categorised taking the percentiles 33 and 66 as cut points to homogenise the scales of the explanatory variables. Associations between WNV seroprevalence and epidemiological variables were assessed through a Pearson's chi-square or Fisher's exact tests, as appropriate. Variables with a  $p < 0.05$  in the bivariate analysis were selected for further examination. Collinearity between pairs of variables was assessed using Cramer's V test. In cases in which a correlation was detected (Cramer's V coefficient  $\geq 0.6$ ), the variable with the highest a priori biological association with flavivirus was retained. Finally, the selected variables were included in a multiple logistic regression model to assess the risk factors potentially associated with WNV exposure in wild lagomorphs. The model was re-run until all remaining variables presented statistically significant values ( $p < 0.05$ ). Statistical analyses were conducted using SPSS v25.0 software (Statistical Package for Social Sciences, Inc., Chicago, IL, USA).

## Results and discussion

During the last decade, the number of outbreaks and the spatial distribution of WNV have drastically increased, providing evidence of the emergence and/or re-emergence of the virus across Europe (ECDC, 2024). In particular, the Mediterranean Basin has witnessed a significant increase in the number of notified WNV outbreaks caused by both lineages 1 and 2, raising considerable concerns for public and animal health. In Spain, Andalusia stands out as a hotspot for WNV circulation, attributed to favorable climatic conditions, the presence of competent vector species and the influx of susceptible migratory wild birds (Jourdain et al., 2007; Cuervo et al., 2022). In these epidemiological scenarios, different studies have suggested that surveillance in large game species could be a useful complementary tool for national surveillance programs to monitor WNV (Gutiérrez-Guzmán et al., 2012; García-Bocanegra et al., 2016).

In the present study, flavivirus antibodies were detected in 27 (4.8%; 95%CI: 3.0-6.6) out of 540 wild lagomorphs tested by bELISA and only one sample was doubtful, specifically one European wild rabbit. Five serum samples positive by ELISA could not be analyzed by micro-VNT due to low sample volume. Specific neutralizing antibodies against WNV were detected in 20 (90.9% of ELISA positive/doubtful) animals, supposing an overall individual seroprevalence to WNV of 3.7% (95%CI: 2.1-5.3). Also, one positive and the doubtful European wild rabbit by bELISA had micro-VNT titres but they did not allow to differentiate the presence of WNV, USUV or BAGV specific

antibodies (Table 1). The bELISA PI and titres of neutralizing antibodies to WNV, USUV and BAGV are shown in table 1. WNV seroprevalence was determined only from samples that tested positive by both ELISA and micro-VNT, which may have led to a slight underestimation.

Table 1. Values obtained in the serological analysis.

Animal identification	Species	Province	bELISA PI (%)	WNV Micro-VNT titre	USUV Micro-VNT titre	BAGV Micro-VNT titre	Result
1	Wild rabbit	Almeria	42.67	1:20	1:20	<1:10	Undetermined <i>Orthoflavivirus</i>
3	Wild rabbit	Cádiz	94.04	1:320	1:10	<1:10	WNV
4	Wild rabbit	Cádiz	92.26	>1:1280	1:20	<1:10	WNV
5	Wild rabbit	Cádiz	73.22	>1:1280	1:160	<1:10	WNV
6	Wild rabbit	Cádiz	94.93	>1:1280	1:20	<1:10	WNV
7	Wild rabbit	Cádiz	91.46	>1:1280	1:40	1:10	WNV
8	Wild rabbit	Cádiz	94.40	1:640	1:10	<1:10	WNV
9	Wild rabbit	Cádiz	94.13	1:640	1:20	<1:10	WNV
10	Wild rabbit	Cádiz	94.13	>1:1280	1:20	1:10	WNV
11	Wild rabbit	Cádiz	94.13	1:640	1:80	<1:10	WNV
12	Wild rabbit	Cádiz	92.79	1:640	1:20	1:10	WNV
14	Wild rabbit	Cádiz	94.31	>1:1280	1:20	<1:10	WNV
15	Wild rabbit	Cádiz	92.88	1:320	1:10	<1:10	WNV
16	Wild rabbit	Cádiz	93.42	1:640	1:20	<1:10	WNV
17	Wild rabbit	Cádiz	90.92	1:640	1:10	<1:10	WNV
18	Wild rabbit	Cádiz	92.35	1:640	1:20	<1:10	WNV
19	Wild rabbit	Cádiz	88.97	>1:640	1:10	<1:5	WNV
20	Wild rabbit	Cádiz	92.08	>1:1280	1:20	1:10	WNV
21	Wild rabbit	Cádiz	94.31	1:320	1:40	--	WNV
25	Wild rabbit	Granada	30.56	1:10	1:10	<1:10	Undetermined <i>Orthoflavivirus</i>
27	Wild rabbit	Huelva	92.44	1:320	1:40	1:10	WNV
28	Iberian hare	Sevilla	78.65	1:320	1:80	<1:10	WNV

**bELISA:** blocking enzyme-linked immunosorbent assay; **PI:** percentage of inhibition; **WNV:** West Nile virus; **USUV:** Usutu virus; **BAGV:** Bagaza virus; **Micro-VNT:** micro virus neutralization test.

Nineteen (4.8%; 95%CI: 2.7-6.9) out of 394 European wild rabbits were positive for anti-WNV antibodies. Although our results were higher, they align with the only previous serosurvey conducted on this species globally. In that study, one (0.4%) of the 269 animals sampled in southeast France reacted positively to WNV antigen in a hemagglutination inhibition (HI) test (Arthur et al., 1990). These findings confirm that European wild rabbits are naturally exposed to this virus. On the other hand, only one (0.7%; 95%CI: 0.0-2.1) Iberian hare showed specific antibodies against WNV. To our knowledge this is the first report of WNV exposure in this hare species, thus increasing the range of susceptible mammals to this zoonotic pathogen. Previous surveys detected positive reaction to WNV antigen in 18 (9.3%) out of 193 European brown hares in Czech Republic by HI test (Juřicová & Hubálek, 1999) and in the five (100%) hares of this species sampled in Israel using the same diagnostic method (Akov & Goldwasser, 1966). Although accurate comparisons are difficult due to variations in the number of animals tested, epidemiological contexts, and serological methods used, our results suggest a limited but relevant exposure of the European wild rabbit and, to a lesser extent, Iberian hare populations, to WNV in the Mediterranean ecosystems of southern Spain. However, additional studies on these lagomorph species, including a larger number of animals and broader geographic coverage, are warranted to determine their role in the epidemiology of WNV in the Iberian Peninsula.

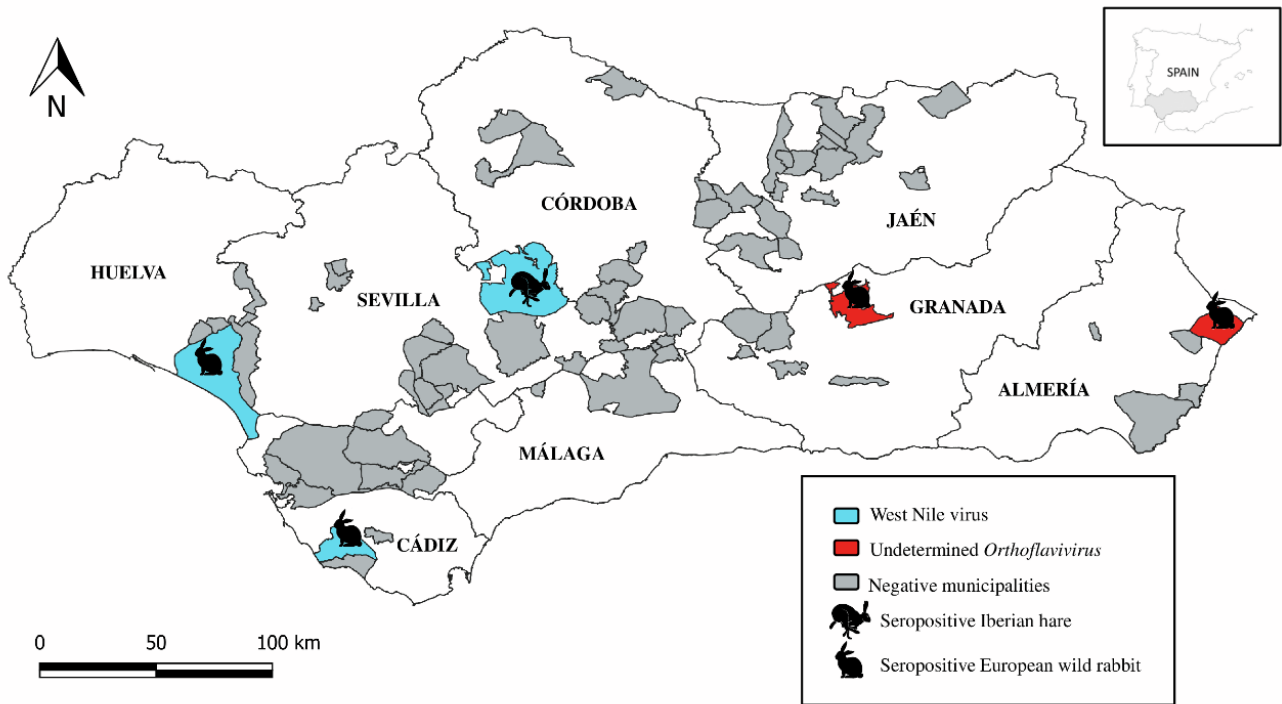
The distribution of WNV seroprevalence according to species, age, sex, hunting season and province is shown in Table 2. Although species, hunting season and province showed  $p < 0.05$  in bivariate analysis, neither of these variables were retained in the final multiple logistic regression model. Seropositive animals were detected in hunting seasons 2020-2021 and 2021-2022 and anti-WNV antibodies were found in a seropositive yearling Iberian hare sampled in 2020. In addition, seropositivity to WNV was confirmed in three (2.8%) of the 106 sampled hunting grounds, these belonging to three different provinces located in southwestern Spain (Huelva, Cádiz and Sevilla) (Figure 1). All these findings denote WNV circulation in wild lagomorphs during the last years as well as heterogeneous spatiotemporal distribution of the virus in these mammal species in the study region. This spatial pattern is in line with the outbreaks of WNV reported in human and horses in Spain. Since the first case notified in 2010, a total of 131 human cases and 364 outbreaks in horse herds have been reported in Andalusia, with 98.4% detected in western region and 43.6% between 2020 and 2022 (RNVE, 2023; SAS, 2024; RASVE,

2024). Interestingly, 18 of the 20 WNV-seropositive wild lagomorphs were sampled in September 2020 in a single hunting ground located in the municipality of Vejer de la Frontera (province of Cádiz; southwestern Spain). It should be noted that August and September were the highest-risk period for WNV outbreaks during the epidemic of 2020 and, notably, both outbreaks in horses and human cases were reported in this municipality (González et al., 2023).

**Table 2.** Distribution of seroprevalence against West Nile virus in wild lagomorphs in Andalusia (southern Spain) by animal categories and results of bivariate analysis.

Variable	Categories	No. positives/ Overall <sup>†</sup>	Seroprevalence (%)	<i>P</i>
Species	Wild rabbit	19/394	4.8	<b>0.016</b>
	Iberian hare	1/141	0.7	
Age	Adult	19/384	4.9	0.051
	Subadult	0/112	0.0	
	Young	1/35	2.9	
Sex	Male	9/263	3.4	0.427
	Female	11/268	4.1	
Hunting season	2018/2019	0/42	0.0	<b>0.010</b>
	2019/2020	0/43	0.0	
	2020/2021	19/296	6.4	
	2021/2022	1/102	1.0	
	2022/2023	0/51	0.0	
Province	Almería	0/45	0.0	<b>&lt; 0.001</b>
	Cádiz	18/54	33.3	
	Córdoba	0/92	0.0	
	Granada	0/51	0.0	
	Huelva	1/53	1.9	
	Jaén	0/81	0.0	
	Málaga	0/93	0.0	
	Sevilla	1/66	1.5	

<sup>†</sup>Missing values omitted



**Figure 1.** Map of the Autonomous Region of Andalusia (southern Spain) showing the sampling areas and the geographical distribution of the samples from wild lagomorphs that tested positive for WNV and undetermined *Orthoflavivirus*.

Specific antibodies against USUV and/or BAG were not detected in the bELISA-positive lagomorphs. Nevertheless, exposure to more than one of these viruses cannot be ruled out in these animals, particularly in those two individuals which showed similar titres against both WNV and USUV. Our results denote that positivity found by the bELISA is essentially due to WNV exposure and not to these other flaviviruses in the wild lagomorphs analyzed. This finding is in line with the absence of BAGV RNA or antibodies in any of the mammal species studied so far (Llorente et al., 2015; Magallanes et al., 2023). In contrast, anti-USUV antibodies have been detected in equids (Guerrero-Carvajal et al., 2021), red deer (*Cervus elaphus*) (García-Bocanegra et al., 2016) and zoo animals (Caballero-Gómez et al., 2020) from southwestern Spain. Although active circulation of USUV has been evidenced in the study area (Bravo-Barriga et al., 2021, 2023; Figuerola et al., 2022), our results suggest a limited exposure to USUV of wild lagomorphs in southern Spain. Nevertheless, future studies are needed to assess the circulation of other flaviviruses in wild lagomorph populations from this region.

In conclusion, our findings confirm that the European wild rabbit and the Iberian hare populations are naturally exposed to WNV, increasing the range of susceptible species to this zoonotic virus. Even though these species do not appear to play a relevant role in the epidemiology of WNV, they could serve as sentinel species for monitoring this mosquito-borne virus. This study underscores the importance of continued surveillance efforts in wildlife populations to better understand the dynamics of flaviviruses transmission and inform public health interventions.

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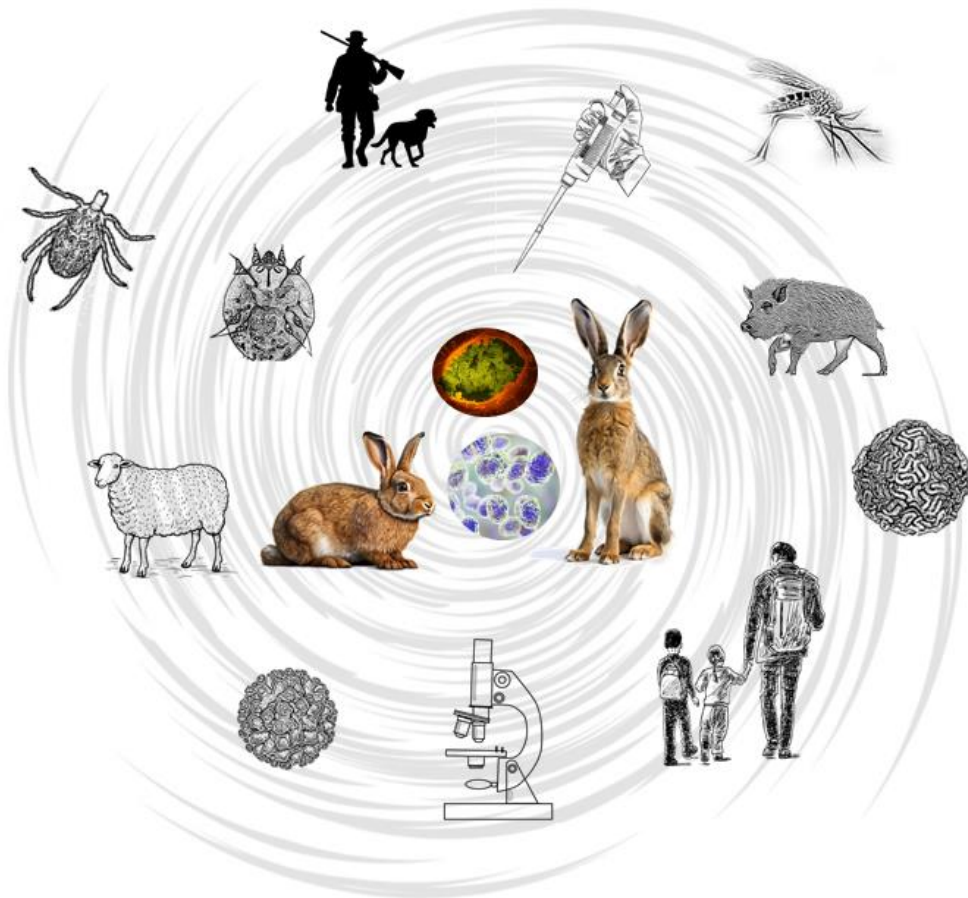
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## CHAPTER 2

### Bacterial pathogens





## Chapter 2.1.

### Occurrence of *Coxiella burnetii* in wild lagomorphs and their ticks in Spanish Mediterranean ecosystems



Castro-Scholten, S., Caballero-Gómez, J., Martínez, R., Nadales-Martín, B.J., Cano-Terriza, D., Jiménez-Martín, D., Remesar, S., Jiménez-Ruiz, S., Gómez-Guillamón, F., & García-Bocanegra, I. (2024). **Occurrence of *Coxiella burnetii* in wild lagomorphs and their ticks in Spanish Mediterranean ecosystems.** *Zoonoses and Public Health*, 71, 549–559.

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## **Abstract**

**Background:** *Coxiella burnetii*, the causative agent of Q fever, is a zoonotic multi-host vector-borne pathogen of major public health importance. Although the European Food Safety Authority has recently made the monitoring of this bacterium in wildlife a priority, the role of wild lagomorphs in the transmission and maintenance of *C. burnetii* is poorly understood.

**Aims:** The aims of this study were to determine the prevalence and risk factors associated with *C. burnetii* circulation in European wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*) and to assess the presence of this pathogen in ticks that feed on them in Mediterranean ecosystems in Spain, the country with the highest number of reported cases of Q fever in Europe.

**Methods:** A total of 574 spleen samples were collected from 453 wild rabbits and 121 Iberian hares, and 513 ticks (processed in 120 pools) between the 2017/2018 and 2021/2022 hunting seasons.

**Results:** *Coxiella burnetii* DNA was detected in 103 (17.9%; 95%CI: 14.8-21.1) of the 574 wild lagomorphs tested. By species, prevalence was 16.3% (74/453; 95%CI: 12.9-19.7) in the European wild rabbit and 24.0% (29/121; 95%CI: 16.4-31.6) in the Iberian hare. At least one positive lagomorph was found on 47.9% of the 96 hunting estates sampled and in every hunting season since 2018/2019. Two risk factors associated with *C. burnetii* infection were: outbreak of myxomatosis on the hunting estate in the month prior to sampling and high tick abundance observed by gamekeepers on the hunting estate. *Coxiella burnetii* DNA was also found in 33 out of the 120 (27.5%; 95%CI: 19.5-35.5) tick pools tested. The pathogen was detected in 66.7% (4/6), 29.2% (26/89) and 21.4% (3/14) of *Haemaphysalis hispanica*, *Rhipicephalus pusillus* and *Hyalomma lusitanicum* pools, respectively.

**Conclusions:** This study provides new epidemiological data on *C. burnetii* in European wild rabbits and is the first survey on this zoonotic pathogen performed in Iberian hares. Our results indicate widespread endemic circulation of *C. burnetii* and highlight the importance of both wild lagomorph species as natural reservoirs of this zoonotic bacterium in Mediterranean ecosystems in southern Spain, which may be of public and animal health concern. The high prevalence and wide diversity of positive tick species



## Chapter 2.1

suggests the possible role of ticks in the epidemiological cycle of *C. burnetii*, with the potential risk of transmission to sympatric species, including humans.

**Keywords:** Q fever, European wild rabbit, Iberian hare, tick, risk factors.

### Impacts

- Widespread circulation of *C. burnetii* in wild rabbits and Iberian hares and wide variety of tick species positive for *C. burnetii* DNA.
- First report of *C. burnetii* infection in the Iberian hare.
- The prevalence detected in both wild lagomorphs and their ticks may be of public and animal health concern and underlines the need to establish a coordinated surveillance system for Q fever in wild lagomorph species.

## **Introduction**

*Coxiella burnetii* (family *Coxiellaceae*) is a globally distributed obligate intracellular zoonotic bacterium that causes Q fever, a disease with a major impact on public and animal health (EFSA & ECDC, 2022). The European Food Safety Authority (EFSA) recently included this disease as a priority for the establishment of a coordinated surveillance system (EFSA et al., 2023).

Q fever in humans is mainly acquired zoonotically through environmental contamination, the result of infected animals excreting bacteria in their feces, but also through foodborne and tickborne transmission (EFSA, 2014). More rarely, *C. burnetii* can be transmitted through blood transfusions and congenital or sexual transmission (CDC, 2019). In Europe, more than 700 human cases per year and a fatality rate of around 1.7% have been reported in recent years; Spain has the highest number of cases (EFSA & ECDC, 2022).

Domestic ruminants are considered to be the main reservoirs of *C. burnetii* (Maurín & Raoult, 1999). In these species, the disease is frequently associated with reproductive disorders, which has a considerable economic impact due to production losses and the cost of implementing control programs (van Asseldonk et al., 2013). However, this bacterium has a complex eco-epidemiology and its host range has expanded in recent decades, so that it is currently considered a multi-host pathogen (Celina & Cerný, 2022). Different studies suggest that wildlife plays a major epidemiological role in the maintenance and transmission of *C. burnetii*, not only to other wildlife species, but also to domestic animals and humans (González-Barrio et al., 2015a, 2021). It should also be noted that, in recent years, an increased number of human cases of Q fever have been associated with exposure to wildlife (González-Barrio & Ruiz-Fons, 2019). Ticks are known to be important vectors of transmission of *C. burnetii* among wild animals (Moraga-Fernández et al., 2023), either via tick saliva at the site of the bite, or by direct contact or inhalation of tick feces (Philip, 1948). Different studies point out that these parasites may act as reservoirs of the bacterium in nature since ticks can spread the pathogen to their progeny by transovarial transmission (Walker & Fishbein, 1991). To date, the bacterium has been detected in more than 30 tick species collected from wildlife, some of which have already been shown to be competent vectors (Duron et al., 2015; Eldin et al., 2017).

It has been suggested that the European wild rabbit (*Oryctolagus cuniculus*) is a natural reservoir of *C. burnetii* (González-Barrio et al., 2015b) and human infections have already been linked to this species (Marrie et al., 1986). Transmission can be direct, while handling hunted animals being prepared for consumption for example, or indirect, either by tick bite or by coming into contact with contaminated environment, given the high resistance of the bacterium. In Spanish Mediterranean ecosystems, both the European wild rabbit and the Iberian hare (*Lepus granatensis*) are the most important small game species in terms of hunting interest, distribution and abundance, and constitute an important source of human food, generally destined for home consumption and without veterinary controls. Although the EFSA has highlighted the need for the surveillance of *C. burnetii* in wild lagomorphs (ENETWILD-consortium et al., 2023), to date, there is very little information on these species and their associated ticks. The aims of this study therefore were to molecularly investigate the presence and associated risk factors of *C. burnetii* in spleen samples from European wild rabbits and Iberian hares and ticks feeding on these lagomorph species in Mediterranean ecosystems of southern Spain.

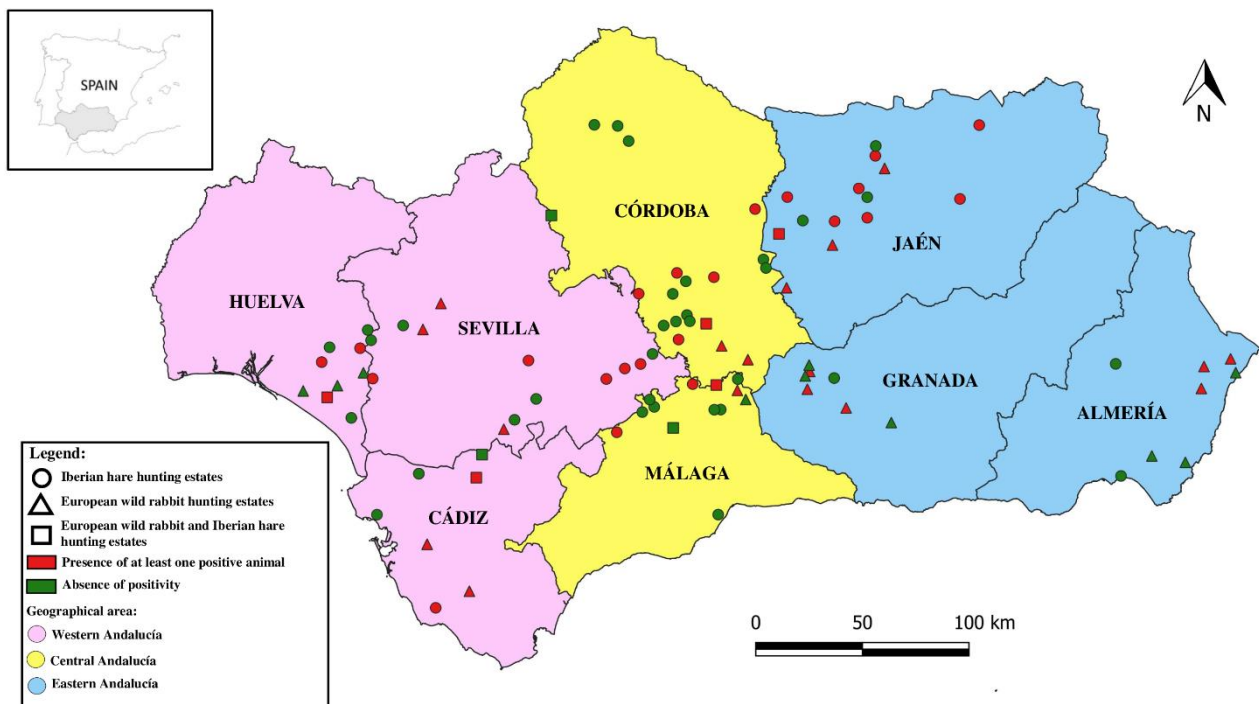
## **Material and methods**

### *Study design and sampling*

Between the 2017/2018 and 2021/2022 hunting seasons, a cross-sectional epidemiological study was carried out in the region of Andalusia (southern Spain; 36°N-38°60'N, 1°75'W-7°25'W), the second largest in Spain with an area of 87,300km<sup>2</sup>, occupying more than 17% of the national territory. For the European wild rabbits, sample size was calculated as a minimum of 385 animals, assuming a prevalence of 50%, with a 95% confidence interval (95%CI) and a desired precision of ±5%. Whenever possible, 60 wild rabbits were sampled in each of the eight provinces comprising the study area, in order to ensure a 95% probability of detecting at least one positive animal, assuming a minimum within-province prevalence of 5% (Thrusfield & Christley, 2018). Sampling sites (hunting estates) were randomly selected in each province. On each of these hunting estates (Figure 1), hunters kindly provided between 3 and 25 (mean: 11.9) wild rabbits for sampling. A total of 453 wild rabbits from 38 hunting estates distributed across all eight provinces were ultimately sampled during the study period. Between hunting seasons 2017/2018 and 2021/2022, samples were also collected from 121 Iberian hares from 66 hunting estates in the same study area using convenience sampling. The spleens were removed from these animals aseptically and stored in individually labeled plastic

tubes. After visual inspection, a total of 513 ticks feeding on 127 and 30 of the sampled rabbits and hares, respectively, were also collected using tweezers. Spleen and tick samples were kept refrigerated until arrival at the laboratory and immediately frozen at -20 °C.

Information on each individual was recorded, including species, location, year of sampling, age (according to bodyweight and body length; Morris, 1972), kidney fat index and sex. During each sampling, epidemiological data related to the hunting estates were also gathered through personal interviews with gamekeepers using a standardized questionnaire (Table S1). The information obtained included the characteristics of the hunting estate, the presence of disease and control measures, management practices, and presence of other sympatric species.



**Figure 1.** Spatial distribution of the prevalence of *Coxiella burnetii* infection among wild lagomorphs in the study region (Andalusia, southern Spain).

#### *Tick identification and pooling*

Ticks collected in the present study were already identified in a previous survey conducted by Remesar et al. (2021). Identification to species level was performed using morphological keys (Pérez-Eid, 2007), and a subset of each tick species was further molecularly analyzed (Norris et al., 1996) to confirm microscopic identification. Tick

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specimens collected from the same hunting estate were pooled according to species, development stage and host species.

### *Molecular analysis*

Total DNA from spleen samples was extracted with the commercial NucleoSpin Tissue<sup>®</sup> kit (Macherey-Nagel, Germany), while DNA from tick pools was obtained using the commercial High Pure PCR Template Preparation Kit<sup>®</sup> (Roche Diagnostics, Mannheim, Germany), both following the manufacturer's instructions. Extracted DNA was stored at -80 °C until molecular analyses. To test for the presence of *C. burnetii* DNA, real time PCR (RT-PCR; Bio-Rad CFX Connect Real-Time PCR System, Feldkirchen, Germany) was performed, targeting the IS1111a insertion element of this pathogen, as previously described (Tilburg et al., 2010), using GoTaq<sup>®</sup>qPCR Master Mix technology (Promega). One positive control in duplicate, obtained after DNA extraction of *C. burnetii* Phase I antigens derived from the Serion<sup>®</sup> ELISA classic *Coxiella burnetii* (Serion GmbH, Würzburg), and two no-template controls were used in each run of PCR.

### *Statistical analyses*

The prevalence of *C. burnetii* in the wild lagomorphs was determined from the proportion of positive animals to the total number of individuals analyzed by PCR, whereas the percentage of *C. burnetii*-positive ticks was calculated by taking into account the number of pools analyzed, using the two-sided exact binomial test, with 95% CI in both. Cut-off points for continuous variables were determined at the 33rd and 66th percentiles. Pearson's Chi-square or Fisher's exact test was first used, as appropriate, to screen for associations between the prevalence of *C. burnetii* in wild lagomorphs and the percentage of positive tick pools with explanatory variables. All variables with a  $p < 0.05$  in the bivariate analysis were selected for further analysis. Collinearity between pairs of variables was then tested by Cramer's V coefficient, and finally a generalized estimating equations (GEE) analysis was carried out to study the effect of the variables selected from the bivariate analysis. The number of positive animals and tick pools was assumed to follow a binomial distribution, and "hunting estate" was included as the subject variable. Forward selection was used for introduction of variables, starting with the variable with the lowest  $p$ -value in the bivariate analysis, until all remaining variables showed statistically significant values ( $p < 0.05$ ). SPSS 25.0 software (Statistical Package for Social Sciences, Inc., Chicago, IL, USA) was used for all statistical analyses.

## Results

*Coxiella burnetii* DNA was detected in 103 (17.9%; 95%CI: 14.8-21.1) of the 574 wild lagomorphs analyzed. Prevalence by species was 16.3% (74/453; 95%CI: 12.9-19.7) in the European wild rabbit and 24.0% (29/121; 95%CI: 16.4-31.6) in the Iberian hare. The pathogen was detected in animals sampled during each hunting season since 2018/2019, with prevalence values ranging from 12.7% (48/379; 95%CI: 9.3-16.0) in 2020/2021 to 33.3% (12/36; 95%CI: 17.9-48.8) in 2019/2020 (Table 1). Positive animals were detected on 46 out of 96 (47.9%; 95%CI: 37.9-57.9) hunting estates and in every province in the study region (Figure 1).

**Table 1.** Distribution of the prevalence of *Coxiella burnetii* in wild lagomorphs in Andalusia (southern Spain) according to animal and hunting estate variables and results of bivariate analysis.

Variable	Categories	No. positives/ Overall <sup>†</sup>	Seroprevalence (%)	P
<b>Data recorded from the sampled animals</b>				
Species	Wild rabbit	74/453	16.3	<b>0.038</b>
	Iberian hare	29/121	24.0	
Age	Adult	73/405	18.0	0.846
	Subadult	25/129	19.4	
	Young	5/33	15.2	
Sex	Male	44/279	15.8	0.099
	Female	59/291	20.3	
Kidney fat index	0	33/135	24.4	0.059
	1	24/139	17.3	
	2	17/108	15.7	
	3	10/92	10.9	
Bodyweight (kg)	0.4-0.9	32/165	19.4	0.347
	1.0-1.2	22/143	15.4	
	1.3-3.1	26/116	22.4	
Body length (cm)	19-37	31/159	19.5	0.917
	38-40	27/142	19.0	
	41-59	20/114	17.5	

	2017/2018	0/1	0.0	
	2018/2019	9/44	20.5	
Hunting season	2019/2020	12/36	33.3	<b>&lt; 0.001</b>
	2020/2021	48/379	12.7	
	2021/2022	34/114	29.8	
<b>Hunting estate's characteristics</b>				
	Western	39/186	21.0	
Geographical area	Central	19/181	10.5	<b>0.007</b>
	Eastern	45/207	21.7	
	High	69/405	17.0	
Burrow density	Medium	11/30	36.7	<b>0.002</b>
	Low	5/65	7.7	
High abundance of ticks in the hunting estate	Yes	63/286	22.0	
	No	11/140	7.9	<b>&lt; 0.001</b>
High abundance of fleas in the hunting estate	Yes	52/264	19.7	
	No	22/162	13.6	0.067
Presence of rabbit feeders	Yes	42/297	14.1	
	No	43/203	21.2	<b>0.027</b>
Feed supplementation in rabbits	Yes	34/216	15.7	
	No	51/284	18.0	0.298
Presence of swamps	Yes	20/82	24.4	
	No	65/418	15.6	<b>0.040</b>
Presence of troughs	Yes	69/419	16.5	
	No	16/81	19.8	0.283
Presence of streams	Yes	52/262	19.8	
	No	33/238	13.9	<b>0.048</b>
The hunting estate is weeded	Yes	12/109	11.0	
	No	73/391	18.7	<b>0.037</b>
Presence of artificial burrows	Yes	3/35	8.6	
	No	82/465	17.6	0.122
Fenced hunting estate	Yes	5/37	13.5	0.375

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	No	80/463	17.3	
	High (51-100)	3/27	11.1	
Lagomorph density (animal/km <sup>2</sup> )	Medium (26-50)	5/48	10.4	0.057
	Low (11-25)	6/38	15.8	
	Very low (0-10)	31/118	26.3	
<b>Detection of outbreaks of other infectious diseases</b>				
Outbreaks of myxomatosis in the last year	Yes	84/477	17.6	<b>0.074</b>
	No	1/23	4.3	
Outbreaks of RHD <sup>‡</sup> in the last year	Yes	68/405	16.8	<b>0.450</b>
	No	17/95	17.0	
Outbreaks of myxomatosis in the last month	Yes	81/408	19.9	<b>&lt; 0.001</b>
	No	4/92	4.3	
Outbreaks of RHD <sup>‡</sup> in the last month	Yes	24/115	10.9	0.133
	No	61/385	15.8	
<b>Presence of other sympatric species in the hunting estate</b>				
Wild boar ( <i>Sus scrofa</i> )	Yes	45/297	15.2	<b>0.048</b>
	No	54/258	20.9	
Red deer ( <i>Cervus elaphus</i> )	Yes	4/57	7.0	<b>0.013</b>
	No	95/498	19.1	
Wildcat ( <i>Felis silvestris</i> )	Yes	27/153	17.6	0.445
	No	58/347	16.7	
Iberian lynx ( <i>Lynx pardinus</i> )	Yes	8/68	11.8	0.143
	No	77/432	17.8	
Domestic cat ( <i>Felis silvestris catus</i> )	Yes	74/449	16.5	0.231
	No	11/51	21.6	
Dog ( <i>Canis familiaris</i> )	Yes	65/371	17.5	0.353
	No	20/129	15.5	
Cattle ( <i>Bos taurus</i> )	Yes	5/25	20.0	0.223
	No	46/361	12.7	
Goat ( <i>Capra aegagrus hircus</i> )	Yes	21/144	14.6	0.321
	No	30/242	12.4	



Sheep ( <i>Ovis aries</i> )	Yes	26/203	12.8	0.461
	No	25/183	13.7	
Farmed rabbit ( <i>Oryctolagus cuniculus</i> )	Yes	8/49	17.1	0.541
	No	77/451	16.3	
Domestic pig ( <i>Sus scrofa domesticus</i> )	Yes	6/40	15.0	0.439
	No	45/346	13.0	
<b>Climate characteristics of the hunting estate</b>				
Mean temperature (°C)	12.3-16.8	23/209	11.0	<b>0.004</b>
	16.9-17.4	16/93	17.2	
	17.5-18.5	37/152	24.3	
Max temperature (°C)	18.9-23.0	17/171	9.9	<b>&lt; 0.001</b>
	23.1-24.2	29/194	14.9	
	24.3-27.4	20/65	30.8	
Rainfall (mm)	273.3-563.8	21/157	13.4	0.333
	563.9-597.9	34/175	19.4	
	598.0-1134.6	21/122	17.2	
Humidity (g/m <sup>3</sup> )	33-56	10/26	38.5	0.107
	57-65	23/119	19.3	
	66-100	15/67	22.4	

†Missing values omitted

‡RHD: rabbit hemorrhagic disease

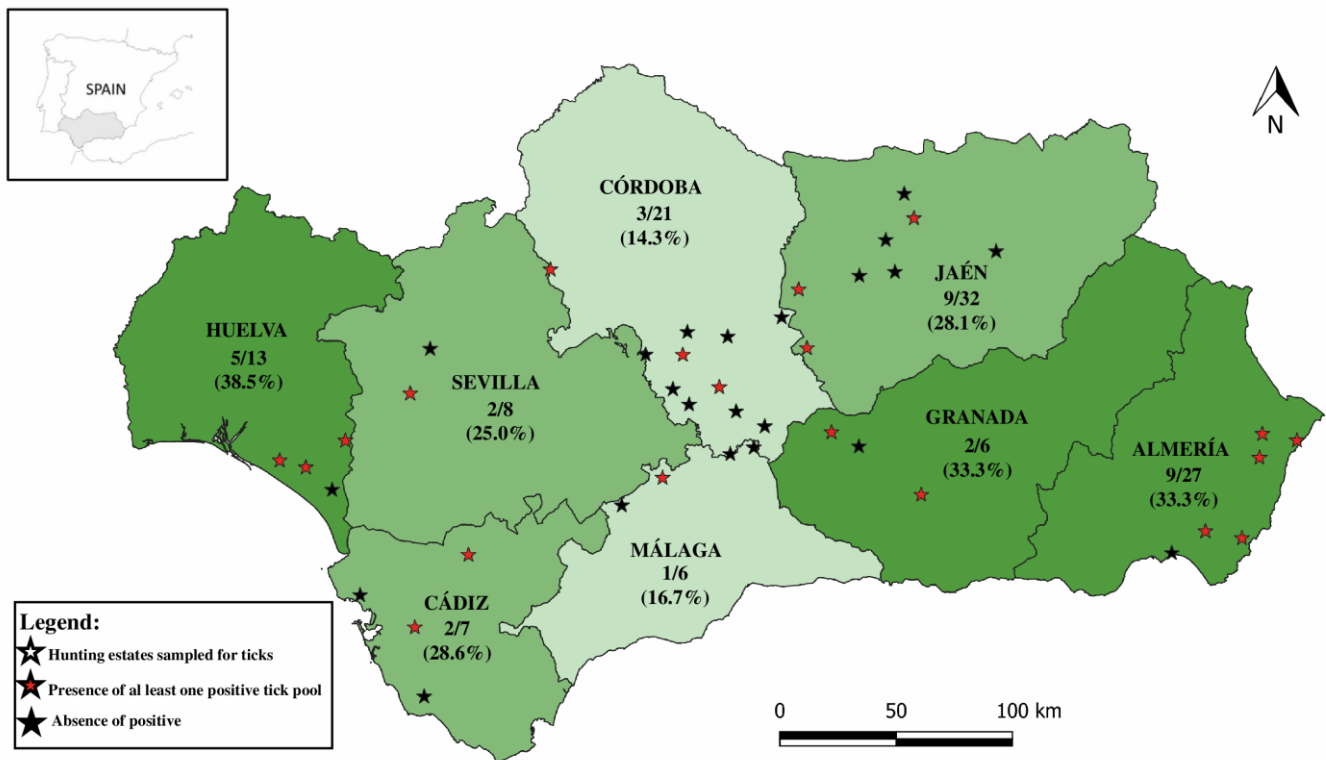
The final GEE model showed that the risk of being infected by *C. burnetii* was significantly higher in lagomorphs from hunting estates that had reported outbreaks of myxomatosis during the month prior to sampling (19.9%) than in those with no outbreaks (4.3%) ( $p = 0.028$ ). In addition, significantly higher positivity was found in animals from hunting estates where gamekeepers observed a high tick abundance (22.0%) than in those where a high tick abundance was not detected (7.9%) ( $p = 0.022$ ) (Table 2).

**Table 2.** Generalized estimating equations (GEE) analysis of risk factors associated with *Coxiella burnetii* infection in wild lagomorphs in Andalusia (southern Spain).

Variable	Categories	<i>p</i> -value	OR 95%CI
High abundance of ticks in the hunting estate	Yes	0.022	2.3 (1.1-4.9)
	No	a	a
Outbreaks of myxomatosis in the last month	Yes	0.028	3.8 (1.2-12.7)
	No	a	a

<sup>a</sup>Reference Category

Five tick species were identified: *Rhipicephalus pusillus*, *Rhipicephalus sanguineus sensu lato*, *Haemaphysalis hispanica*, *Hyalomma lusitanicum* and *Ixodes ventralloi* (Table 3). *Coxiella burnetii* DNA was detected in 33 (27.5%; 95%CI: 19.5-35.5) of the 120 tick pools tested (Table 3). According to tick species, *C. burnetii* DNA was found in 29.2% (26/89; 95%CI: 19.8-38.7) of *R. pusillus* pools, 21.4% (3/14; 95%CI: 0.0-42.9) of *H. lusitanicum* pools and 66.7% (4/6; 95%CI: 29.0-100) of *H. hispanica* pools. None of the *R. sanguineus s.l.* (0/9; 95%CI: 0.0-33.6) or *I. ventralloi* (0/2; 95%CI: 0.0-84.2) pools tested positive (Table 3). Positive tick pools were observed during all hunting seasons sampled, and at least one positive pool was found on 20 out of 42 (47.6%; 95%CI: 32.5-62.7) hunting estates (Figure 2). *C. burnetii*-positive lagomorphs were detected on ten (50%) of these 20 hunting estates, and on 14 (63.6%) of the 22 hunting estates where *C. burnetii* DNA was not present in ticks.



**Figure 2.** Distribution of hunting estates where tick samples were collected from wild lagomorphs and frequency of positive pools by province in the study region.

The final GEE model for tick pools identified the variable “lagomorph species” as potentially associated with the positivity to *C. burnetii* found in these parasites. In connection with this, the frequency of positive tick pools collected from the European wild rabbits (33.0%; 30/91; 95%CI: 23.3-42.6;  $p = 0.031$ ; OR = 4.3 (95%CI: 1.1-16.3)) was significantly higher than the 10.3% (3/29; 95%CI: 0.0-21.4) detected in those collected from Iberian hares.

**Table 3.** Total number of ticks captured from European wild rabbits and Iberian hares in Andalusia (southern Spain) and pools processed, with percentage of pools positive for *Coxiella burnetii*, according to tick developmental stage.

Host species	Nymph		Adult female		Adult male		Total	
	N° ticks	% positives/ n° pools	N° ticks	% positives/ n° pools	N° ticks	% positives/ n° pools	N° ticks	% positives/ n° pools
<b>European wild rabbit</b>								
<i>Rhipicephalus pusillus</i>	161	12/28	127	6/24	111	5/20	399	23/72
<i>Rhipicephalus sanguineus s.l.</i>	0	-	0	-	0	-	0	-
<i>Haemaphysalis hispanica</i>	13	4/6	0	-	0	-	13	4/6
<i>Hyalomma lusitanicum</i>	35	3/11	0	-	0	-	35	3/11
<i>Ixodes ventraloi</i>	0	-	3	0/2	0	-	3	0/2
<b>TOTAL</b>	<b>209</b>	<b>19/45</b>	<b>130</b>	<b>6/26</b>	<b>111</b>	<b>5/20</b>	<b>450</b>	<b>30/91</b>
<b>Iberian hare</b>								
<i>Rhipicephalus pusillus</i>	1	0/1	14	2/8	20	1/8	35	3/17
<i>Rhipicephalus sanguineus s.l.</i>	0	-	0	-	23	0/9	23	0/9
<i>Haemaphysalis hispanica</i>	0	-	0	-	0	-	0	-
<i>Hyalomma lusitanicum</i>	1	0/1	4	0/2	0	-	5	0/3
<i>Ixodes ventraloi</i>	0	-	0	-	0	-	0	-
<b>TOTAL</b>	<b>2</b>	<b>0/2</b>	<b>18</b>	<b>2/10</b>	<b>43</b>	<b>1/17</b>	<b>63</b>	<b>3/29</b>
<b>TOTAL</b>	<b>211</b>	<b>19/47</b>	<b>148</b>	<b>8/36</b>	<b>154</b>	<b>6/37</b>	<b>513</b>	<b>33/120</b>

s.l.: sensu lato.

## Discussion

In view of the current epidemiological situation of Q fever in Europe, *C. burnetii* has been recognized as an important zoonotic pathogen of public health concern, with Q fever being listed by the EFSA as a priority disease for coordinated surveillance under a One Health approach. During the study period, a total of 1402 human cases were reported in Spain, with Andalusia being the region with the second highest number of reported cases after the Canary Islands (EFSA et al., 2023).

The present study provides new epidemiological data on *C. burnetii* in wild lagomorphs and adds information relevant to the role of ticks in the epidemiology of this zoonotic bacterium in wild animals. To date, only four studies have assessed the presence of *C. burnetii* DNA in European wild rabbits, all of them carried out in Spain. The prevalence found in wild rabbit populations (16.3%) in Andalusia in the present study was lower than in those detected in central regions of this country, where 43.4-47.2% of European wild rabbits were positive (González et al., 2019; Sánchez et al., 2022). By contrast, lower prevalence values ranging between 1.5% and 4.4%, were observed in the Canary Islands and the south of Spain, respectively (González-Barrio et al., 2015b; Bolaños-Rivero et al., 2017).

To the best of the author's knowledge, this is the first study on *C. burnetii* in Iberian hares. Our results confirm the susceptibility of this hare species to *C. burnetii*, thereby enlarging its host range. Previous studies have evidenced circulation of this bacterium in other hare species, such as the European brown hare (*Lepus europaeus*). In Cyprus, 15 out of 31 (48.4%) pools of blood samples from 247 animals of this species were positive for *C. burnetii* (Psaroulakia et al., 2014), while no infection was found in any of the 51 and 105 animals sampled in Italy (Rochigiani et al., 2018) and Greece (Tsokana et al., 2020), respectively. A regional study conducted in northern Spain detected this pathogen in 9.1% of the 22 European brown hares sampled (Astobiza et al., 2011). The overall prevalence of *C. burnetii* detected in our study of European wild rabbits and Iberian hares (17.9%) indicates that it is actively circulating in these populations, which is of public health and animal health concern. These findings, together with the importance of wild lagomorphs as prey for a large number of species, point to the risk of interspecies transmission of *C. burnetii*, thus favoring the maintenance of this bacterium in Iberian Mediterranean ecosystems (Delibes & Hiraldo, 1981; González-Barrio et al., 2022).

The prevalence of infection was significantly higher in lagomorphs from hunting estates where there had been an outbreak of myxomatosis during the month prior to sampling (19.9%) than in those estates where there had been no cases (4.3%), suggesting a possible epidemiological link. Myxomatosis is a viral disease that causes significant immunosuppression in both rabbits and hares (Jeklova et al., 2008; García-Bocanegra et al., 2019), which may favor infection by other pathogens, including *C. burnetii*. Our result is consistent with previous studies that also detected a relationship between myxomatosis virus infection and other pathogens in European wild rabbits. García-Bocanegra et al. (2010) noted that myxomatosis virus infection may favor infection by rabbit hemorrhagic disease virus (RHDV), and Boag et al. (2013) found an increased number of *Eimeria stiedae* oocysts shed in feces by affected rabbits. Similarly, Mason et al. (2015) found that *Toxoplasma gondii* seropositivity could be associated with exposure to myxomatosis virus, although further studies are needed to confirm this hypothesis.

*Coxiella burnetii* infection in wild lagomorphs was also significantly higher in animals from hunting estates where gamekeepers had observed high tick abundance (Table 2). This, together with the detection of bacterial DNA in 27.5% of the tick pools analyzed (Table 3), highlights the importance of ticks in the epidemiological cycle of *C. burnetii* in Iberian Mediterranean ecosystems. Interestingly, although the prevalence of infection in wild rabbits was lower than in Iberian hares, the frequency of positive tick pools collected from the former was significantly higher than in those collected from hares. This finding could be explained by a differential exposure of the two wild lagomorph species to vector ticks (Table 3), and indicating that the European wild rabbit could play a more important role in disseminating *C. burnetii*-positive ticks. It should be noted that the European wild rabbit is widespread in Spain, and is frequently present in urban and peri-urban areas, which increases the risk of zoonotic transmission of *C. burnetii*, as has been shown for other vector-borne pathogens (Jiménez et al., 2014).

Three of the five tick species analyzed had *C. burnetii* DNA. In Spain, the bacterium has previously been detected in *H. lusitanicum*, *Rhipicephalus turanicus*, *R. sanguineus*, *Rhipicephalus bursa*, *R. pusillus*, *Haemaphysalis sulcata*, *Haemaphysalis punctata* and *Dermacentor marginatus* (Körner et al., 2021). However, to the best of our knowledge, this is the first report of *C. burnetii* infection in *H. hispanica*. Positive pools of *R. pusillus* and *H. hispanica* pools were detected on 10 hunting estates where no positive wild lagomorphs were observed. The presence of positive ticks feeding on

negative hosts could indicate that the pathogen was acquired at an earlier stage of tick development and from another host. This has already been evidenced in previous studies in *H. lusitanicum* and was associated with transovarial and transstadial transmission of *C. burnetii* (González et al., 2019). The same finding could also be related to recent infestation or to the vector competence of these tick species. It should be noted that while *C. burnetii* has previously been detected in all positive tick species found in the present study, research on vector competence has only been conducted in *R. sanguineus* (Smith, 1941) and *H. lusitanicum* (González et al., 2020) and to date has only been demonstrated in *H. lusitanicum* (González et al., 2020). Further studies are required to assess the vector competence of these tick species.

In conclusion, our results indicate the widespread endemic circulation of *C. burnetii* in European wild rabbits, Iberian hares and ticks feeding on these two lagomorph species. The prevalence values observed in these mammals and their ticks indicate that wild lagomorphs act as reservoirs of this bacterium in Mediterranean ecosystems of southern Spain, making them potential sources of *C. burnetii* for sympatric species, including humans. Monitoring programs for this zoonotic bacterium are justified to gain a deeper and broader understanding of the role of wildlife in the epidemiology of *C. burnetii* in different epidemiological scenarios.

### Supplementary material

**Table S1.** English version of the blank epidemiological questionnaire used in this study.

<b>Responsible for the questionnaire:</b>	<b>Date:</b>
<b>Game estate:</b>	<b>Licence number:</b>
<b>Person of contact:</b>	<b>Province:</b>

#### A) Species-related factors

##### 1. Burrow density

None  Low  High

##### 2. High abundance of ticks

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Yes  No

**3. High abundance of flies**

Yes  No

**B) Disease related factors**

**4. Disease outbreaks last year**

Myxomatosis in rabbits  Myxomatosis in hares  RHD in rabbits  Others:

**5. Disease outbreaks last month**

Myxomatosis in rabbits  Myxomatosis in hares  RHD in rabbits  Others:

**C) Environmental factors**

**6. Fencing**

No  Yes

**7. Presence of sympatric species**

Wild boar  Red deer  Wild cat  Iberian lynx  Domestic cat

Dog

**8. Presence of cattle/farm species**

No  Yes

Species	Presence (yes/no)	Extensively/intensively reared
Bovine cattle		
Goats		
Sheep		
Farmed rabbits		
Domestic pigs		
Others		



**9. Presence of rabbit feeders:**

Yes     No

**10. Feed supplementation in rabbits:**

Yes     No

**11. Water points**

Swamps     Drinking troughs     Streams     Others:

**12. Hunting improvements in the game estate**

Weeding     Artificial burrows     Others:

**Other observations:**

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## Chapter 2.2.

### **Exposure to *Coxiella burnetii* in Wild Lagomorphs in Spanish Mediterranean ecosystems**



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## Simple Summary

*Coxiella burnetii*, the causative agent of Q fever, is a multi-host zoonotic bacterium of public and animal health concern, with Spain being the European country with the highest number of Q fever cases in recent years. However, and despite that the European wild rabbit and the Iberian hare are two keystone species in the Iberian Peninsula and are considered important sources of food for humans, very little is known about the epidemiological role of these two species. To tackle this question, a cross-sectional study was carried out to determine the seroprevalence and risk factors associated with *C. burnetii* exposure in wild lagomorph populations of Southern Spain. Antibodies against this bacterium were found in 11.3% of 471 wild rabbits, and, for the first time, in 2.4% of 167 Iberian hares, which provides evidence of the moderate circulation of *C. burnetii* in wild lagomorph populations in Spanish Mediterranean ecosystems. Our results also demonstrated that wild lagomorphs from western Andalusia and those from hunting grounds in which sheep were present were at higher risk of exposure to *C. burnetii*. In this context, these risk factors should be prioritized in future risk-based surveillance programs for this zoonotic multi-host bacterium.

## Abstract

*Coxiella burnetii* is an important zoonotic pathogen of worldwide distribution that can infect a wide range of wild and domestic species. The European wild rabbit (*Oryctolagus cuniculus*) can play a role as a reservoir for this bacterium in certain epidemiological scenarios, but, to date, a very limited numbers of large-scale serosurveys have been conducted for this species worldwide. Although exposure in hare species has also been described, *C. burnetii* in Iberian hare (*Lepus granatensis*) has never been assessed. Here, we aimed to determine the seroprevalence and risk factors associated with *C. burnetii* exposure in wild lagomorphs in the Mediterranean ecosystems of southern Spain. Between the 2018/2019 and 2021/2022 hunting seasons, blood samples from 638 wild lagomorphs, including 471 wild rabbits and 167 Iberian hares, were collected from 112 hunting grounds distributed across all eight provinces of Andalusia (southern Spain). The overall apparent individual seroprevalence was 8.9% (57/638; 95%CI: 6.8–11.4). Antibodies against *C. burnetii* were found in 11.3% (53/471; 95%CI: 8.4–14.1) of the wild rabbits and 2.4% (4/167; 95%CI: 0.1–4.7) of the Iberian hares. Seropositive animals were detected for 16 (14.3%; 95%CI: 7.8–20.8) of the 112 hunting grounds tested and in all the hunting seasons sampled. A generalized estimating equations model showed that



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the geographical area (western Andalusia) and presence of sheep were risk factors potentially associated with *C. burnetii* exposure in wild lagomorphs. A statistically significant spatial cluster ( $p < 0.001$ ) was identified in the south-west of Andalusia. Our results provide evidence of moderate, endemic and heterogeneous circulation of *C. burnetii* in wild lagomorph populations in Spanish Mediterranean ecosystems. Risk-based strategies for integrative surveillance programs should be implemented in these species to reduce the risk of transmission of the bacterium to sympatric species, including humans.

**Keywords:** Q fever; wild rabbit; Iberian hare; One Health; risk factors

## **Introduction**

The agent that causes Q fever disease, *Coxiella burnetii* (family *Coxiellaceae*), is an important and highly environmentally resistant zoonotic bacterium with a worldwide distribution [1]. In recent years, Europe has reported over 700 annual human cases of Q fever. Among European countries, Spain has recorded the highest number of Q fever cases in recent years [1], making it the most frequent reportable zoonosis in the country [2]. Different modes of transmission have been reported and although inhalation is considered the main mode, oral and tick-borne transmissions have also been evidenced [3]. In humans, Q fever causes fever, malaise, headache, muscle pain and endocarditis [4], whereas in animals, and particularly ruminants, this disease is characterized by abortions during late pregnancy or weak offspring, causing significant economic losses in the live-stock industry [5].

Although domestic ruminants are the main reservoirs of the bacterium, a broad range of wild species can be infected with *C. burnetii*. The European wild rabbit (*Oryctolagus cuniculus*) has been denoted as a natural reservoir of *C. burnetii* in certain epidemiological contexts [6], and Q fever cases in humans have already been associated with indirect contact with wild rabbits [7]. Moreover, some studies have pointed out that hares may play a role in the maintenance and transmission of this zoonotic bacterium [8,9]. Nevertheless, information on the role of these lagomorph species in the epidemiology of Q fever is still very limited.

The European wild rabbit and the Iberian hare (*Lepus granatensis*) are two endemic and keystone species of the Iberian Peninsula [10,11], being a source of food for a large number of predators [12]. In Mediterranean ecosystems, both species have been shown to be natural reservoirs for a wide range of pathogens that can affect other species, including humans [13–16]. This, together with their wide distribution, gregarious behavior and close direct and indirect contact with other sympatric species, evidenced the potential role of wild lagomorphs in the maintenance and transmission of multi-host pathogens such as *C. burnetii* [17]. However, even though the European Food Safety Authority (EFSA) has included Q fever as a priority for the establishment of a coordinated surveillance system [18] and has highlighted the need for the epidemiological surveillance of *C. burnetii* in wild lagomorphs to assess the circulation of this zoonotic pathogen [17], no or only a very few serosurveys have been conducted to date for Iberian hares and European wild rabbits, respectively, worldwide. Here, we aimed to assess the

seroprevalence and risk factors associated with *C. burnetii* exposure in European wild rabbit and Iberian hare populations in Mediterranean ecosystems of southern Spain.

## Materials and Methods

### *Study Design and Sampling*

A cross-sectional study was carried out on wild lagomorph populations in the region of Andalusia (south-western Europe) (87,268 km<sup>2</sup>; 36° N–38°60' N, 1°75' W–7°25' W) between the 2018/2019 and 2021/2022 hunting seasons. The study area is characterized by a continental Mediterranean climate with mild wet winters and hot, dry summers. The western region presents higher mean humidity and less-extreme mean temperatures than the central and eastern regions [19].

The sample size was determined based on an assumed prevalence of 50%, which provides the maximum sample size in studies where the prevalence is unknown. The calculation was completed with a 95% confidence interval (95%CI) and a desired precision of ±5%. Whenever possible, 60 European wild rabbits were sampled in each of the eight provinces comprising the study area, in order to ensure a 95% probability of detecting at least one positive animal, assuming a minimum prevalence of 5% [20]. Sampling sites (hunting grounds) were randomly selected in each province. On each of these hunting grounds, hunters provided between 5 and 25 (mean: 12.1) European wild rabbits for sampling. A total of 471 wild rabbits from 38 hunting grounds distributed across all eight provinces were sampled during the study period. In addition, 167 Iberian hares from 82 hunting grounds were also sampled in the same study area and study period using a convenience sampling. In eight of these hunting grounds, both wild rabbit and Iberian hares were sampled.

Blood samples from all animals were obtained from the heart or thoracic cavity and centrifuged at 400×g for 10 min. The serum obtained was stored at –20 °C until serological analysis was performed. During sampling, an epidemiological questionnaire was also conducted through a direct interview with gamekeepers at each hunting grounds, wherever possible. The information obtained included the characteristics of the hunting ground, the presence of disease and control measures, management practices, and the presence of other sympatric species. Also, meteorological information for each sampling area [mean and maximum annual temperatures (°C), humidity (g/m<sup>3</sup>), and mean annual rainfall (mm)] was collected from the closest official meteorological station [19]. In

addition, individual information, including species, location, year of sampling, age, kidney fat index and sex, was recorded for each animal. Bodyweight and body length were used as indicators of age [21].

#### *Laboratory Analysis*

Serum samples were tested for antibodies against *C. burnetii* using the commercial in-direct and multispecies enzyme-linked immunosorbent assay (ELISA) ID Screen<sup>®</sup> Q Fever (IDvet, Grabels, France), according to the manufacturer's instructions. This assay has previously been used in wild lagomorphs, being a satisfactory alternative to detect *C. burnetii* specific antibodies, compared to other commercial ELISA kits [22]. Results were expressed as an ELISA percentage (E%), calculated using the following formula:  $[E\% = (\text{sample Op-tical Density (OD)}/\text{mean OD of positive controls}) \times 100]$ . The positive threshold values were set as suggested by the manufacturers: sera with  $E\% > 50$  were considered positive.

#### *Statistical Analysis*

The individual apparent prevalence of antibodies against *C. burnetii* was estimated from the proportion of seropositive animals to the total number of individuals analyzed, using the two-sided exact binomial test, with 95%CI. To homogenize the scales of the explanatory variables, cut-off points for continuous variables were determined at the 33<sup>rd</sup> and 66<sup>th</sup> percentiles. Coefficients and standard error values generated using an intercept only generalized estimating equation (GEE) binomial logistic regression model, with the hunting ground as the subject variable, were used to adjust the estimated seroprevalence and 95%CI for clustering at hunting ground level [23]. Pearson's Chi-square or Fisher's exact test was first used, as appropriate, to screen for associations between seroprevalence with explanatory variables. All variables with a  $p < 0.05$  in the bivariate analysis were selected for further analyses. Collinearity between pairs of variables was then tested using Cramer's V coefficient. When collinearity was detected (Cramer's V coefficient  $\geq 0.6$ ), the variable with the strongest a priori biological association with *C. burnetii* was retained. Finally, a GEE analysis was carried out to study the effect of the variables selected from the bivariate analysis. The number of seropositive animals was assumed to follow a binomial distribution, and "hunting ground" was included as the subject variable. Forward selection was used for introduction of variables, starting with the variable with the lowest  $p$ -value in the bivariate analysis. At each step,

the confounding effect of the included variable was assessed by calculating the change in odds ratio (OR). The model was re-run until all remaining variables showed statistically significant values ( $p < 0.05$ ). For the choice of the best model, the quasi-likelihood under the independence model criterion (QIC) was considered. SPSS 25.0 software (Statistical Package for Social Sciences, Inc., Chicago, IL, USA) was used for all statistical analyses.

#### *Spatial Cluster Analysis*

A spatial scan statistical analysis was applied using a Bernoulli model [24] to detect areas with significant aggregations of high seroprevalence at hunting ground level, using SaTScan™ v10.1.2 software. The number of Monte Carlo simulations was set to 1000 for the cluster scan statistic. SaTScan was used to estimate relative risk (RR), representing the relative frequency of seropositive individuals compared to baseline, for each cluster. Clusters were considered significant at  $p < 0.05$ .

### **Results and Discussion**

In the present study, we detected moderate and endemic circulation of *C. burnetii* in wild lagomorph populations of Spanish Mediterranean ecosystems. The overall apparent individual seroprevalence was 8.9% (57/638; 95%CI: 6.8–11.4) (Table 1). By species, antibodies against *C. burnetii* were found in 11.3% (53/471; 95%CI: 8.4–14.1) of European wild rabbits and 2.4% (4/167; 95%CI: 0.1–4.7) of Iberian hares. After adjustment for clustering, the estimated individual seroprevalences were 12.9% (95%CI: 7.2–22.3) in wild rabbit and 2.5% (95%CI: 0.9–6.5) in Iberian hare, which denote different exposure level to *C. burnetii* between these two lagomorph species. This finding could be related to differences in behavior. While Iberian hares are generally solitary [25], wild rabbits live in social groups in burrows [26], which might favor the transmission and maintenance of the bacterium.

**Table 1.** Distribution of the seroprevalence against *Coxiella burnetii* in wild lagomorphs in Andalusia (southern Spain) by animal and hunting ground categories and results of the bivariate analysis.

Variable	Categories	No. positives/ Overall <sup>†</sup>	Seroprevalence (%)	<i>P</i>
<b>Data recorded from the sampled animals</b>				
Species	Wild rabbit	53/471	11.3	<b>&lt; 0.001</b>
	Iberian hare	4/167	2.4	
Age	Adult	53/460	11.5	<b>0.001</b>
	Subadult	3/135	2.2	
	Young	0/36	0.0	
Sex	Male	28/307	9.1	0.516
	Female	29/326	8.9	
Kidney fat index	0	19/154	12.3	0.541
	1	16/148	10.8	
	2	10/114	8.8	
	3	7/99	7.1	
Bodyweight (kg)	0.4-0.9	13/166	7.8	0.420
	1.0-1.2	7/144	4.9	
	1.3-3.1	7/147	4.8	
Body length (cm)	19-37	6/161	3.7	0.075
	38-40	14/143	9.8	
	41-59	7/136	5.1	
Hunting season	2018/2019	2/60	3.3	<b>&lt; 0.001</b>
	2019/2020	2/48	4.2	
	2020/2021	24/391	6.1	
	2021/2022	29/139	20.9	
<b>Hunting ground's characteristics</b>				
Geographical area	Western	45/217	20.7	<b>&lt; 0.001</b>
	Central	3/196	1.5	
	Eastern	9/225	4.0	
Burrow density	High	43/418	10.3	<b>&lt; 0.001</b>

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	Medium	10/37	27.0	
	Low	0/76	0.0	
High abundance of ticks in the hunting ground	Yes	24/297	8.1	<b>0.001</b>
	No	1/142	0.7	
High abundance of fleas in the hunting ground	Yes	16/273	5.9	0.514
	No	9/166	5.4	
Fenced hunting ground	Yes	5/37	13.5	0.305
	No	48/494	9.7	
Presence of rabbit feeders	Yes	26/310	8.4	0.097
	No	27/221	12.2	
Feed supplementation in rabbits	Yes	32/217	14.7	<b>0.002</b>
	No	21/314	6.7	
Presence of swamps	Yes	18/85	21.2	<b>0.001</b>
	No	35/446	7.8	
Presence of troughs	Yes	50/442	11.3	<b>0.012</b>
	No	3/89	3.4	
Presence of streams	Yes	23/277	8.3	0.115
	No	30/254	11.8	
The hunting ground is weeded	Yes	17/118	14.4	0.054
	No	36/413	8.7	
Presence of artificial burrows	Yes	4/37	10.8	0.517
	No	49/494	9.9	
<b>Detection of clinical cases of other infectious diseases</b>				
Outbreaks of myxomatosis in the last year	Yes	53/508	10.4	0.084
	No	0/23	0.0	
Outbreaks of RHD <sup>†</sup> in the last year	Yes	47/435	10.8	0.120
	No	6/96	6.3	
Outbreaks of myxomatosis in the last month	Yes	51/434	11.8	<b>0.001</b>
	No	2/97	2.1	

*Exposure to Coxiella burnetii in Wild Lagomorphs in Spanish Mediterranean ecosystems*

Outbreaks of RHD <sup>‡</sup> in the last month	Yes	18/121	14.9	<b>0.034</b>
	No	35/410	8.5	
<b>Presence of other sympatric species in the hunting ground</b>				
Presence of wild boar ( <i>Sus scrofa</i> )	Yes	18/333	5.4	<b>0.003</b>
	No	34/286	11.9	
Presence of red deer ( <i>Cervus elaphus</i> )	Yes	3/63	4.8	0.199
	No	49/556	8.8	
Presence of wildcat ( <i>Felis silvestris</i> )	Yes	14/170	8.2	0.224
	No	39/361	10.8	
Presence of Iberian lynx ( <i>Lynx pardinus</i> )	Yes	14/86	16.3	<b>0.032</b>
	No	39/445	8.8	
Presence of domestic cat ( <i>Felis silvestris</i> <i>catus</i> )	Yes	45/476	9.5	0.168
	No	8/55	14.5	
Presence of dog ( <i>Canis</i> <i>familiaris</i> )	Yes	34/396	8.6	0.051
	No	19/135	14.1	
Presence of cattle ( <i>Bos</i> <i>taurus</i> )	Yes	5/25	20.0	<b>0.012</b>
	No	19/372	5.1	
Presence of goat ( <i>Capra aegagrus</i> <i>hircus</i> )	Yes	5/149	3.4	0.060
	No	19/248	7.7	
Presence of sheep ( <i>Ovis</i> <i>aries</i> )	Yes	21/192	10.9	<b>&lt; 0.001</b>
	No	3/205	1.5	
Presence of farmed rabbit ( <i>Oryctolagus</i> <i>cuniculus</i> )	Yes	7/60	11.7	0.390
	No	46/471	9.8	
Presence of domestic pig ( <i>Sus scrofa</i> <i>domesticus</i> )	Yes	5/40	12.5	0.081
	No	19/357	5.3	
<b>Climate characteristics of the hunting ground</b>				
	12.3-16.8	2/230	0.9	<b>&lt; 0.001</b>
Mean temperature (°C)	16.9-17.4	7/106	6.6	
	17.5-18.5	17/163	10.4	



	18.9-23.0	10/184	5.4	
Max temperature (°C)	23.1-24.2	8/218	3.7	0.060
	24.3-27.4	8/73	11.0	
Mean annual rainfall (mm)	273.3-563.8	3/169	1.8	<b>0.002</b>
	563.9-597.9	9/199	4.5	
	598.0-1134.6	14/131	10.7	
Humidity (g/m <sup>3</sup> )	33-56	0/26	0.0	0.346
	57-65	9/132	6.8	
	66-100	6/75	8.0	

†Missing values omitted

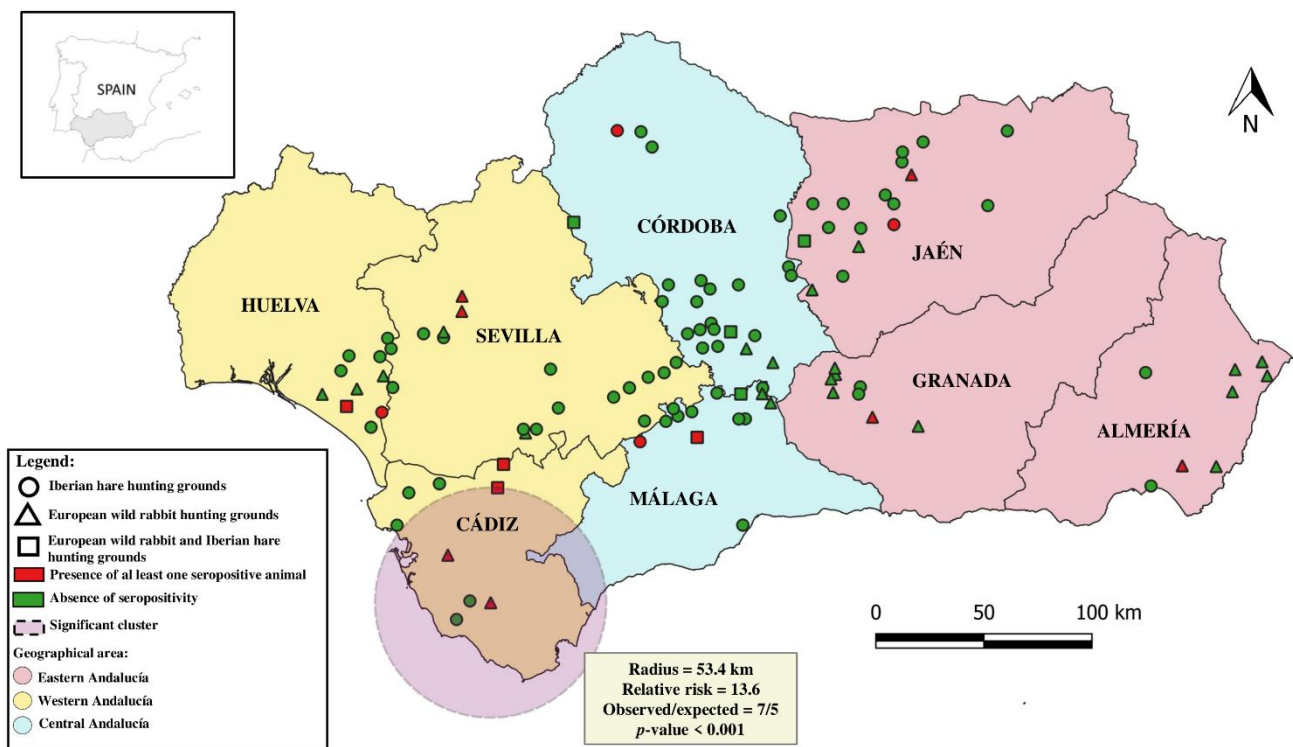
‡RHD: rabbit hemorrhagic disease

The prevalence of anti-*C. burnetii* antibodies detected in wild rabbits in the present study is lower than those reported in the only two previous serosurveys conducted on this species so far. A seroprevalence of 37.9% (176/464) was found in wild rabbit populations in a survey carried out in different regions of Spain, including Andalusia, where certain areas exhibited seroprevalence values ranging between 45.0% and 62.5% [27]. Higher seroprevalence (65.5%; 394/602) was also found in wild rabbits from central areas of this country [28]. The differences may be due to the presence of sympatric wild ungulates, particularly of red deer (*Cervus elaphus*) [28,29], which is considered an important node in the epidemiological cycle of this pathogen in Mediterranean ecosystems [27]. While both studies found higher seroprevalences in areas with a high density of this wild ruminant species, in our study, red deer were only present in 10 of the 112 (8.9%) hunting grounds analyzed. Nevertheless, a further large-scale serosurvey is warranted to assess differences among regions.

To the best of the authors' knowledge, this is the first report of *C. burnetii* exposure in Iberian hare, increasing the range of susceptible species to this zoonotic pathogen. The low seroprevalence detected in this species is consistent with the absence of antibodies against *C. burnetii* reported in European hare (*Lepus europaeus*) in Germany (0/78) [30], the Czech Republic (0/48) [31] and Greece (0/105) [32]. In contrast, higher seropositivity values were observed in other hare species, including the black-tailed jack rabbit (*Lepus californicus*) in the USA (39.4%; 99/251) [33] and in snowshoe

hares (*Lepus americanus*) in Canada (45.5%; 10/22) [7]. The seroprevalence detected in Iberian hare suggests that this species may be considered spillover hosts rather than true reservoirs for *C. burnetii* in the study area, although additional studies, including a high number of tested animals, are needed to support this hypothesis.

Temporally, anti-*C. burnetii* antibodies were detected in all the hunting season samples and an increasing trend in seroprevalence was found over the years. The lowest seroprevalence (3.3%) was detected during the 2018–2019 hunting season, rising to 20.9% during 2021/2022. Geographically, seropositive animals were detected in all provinces with frequencies of antibodies ranging between 0.9% (1/107; 95%CI: 0.0–2.8) in Córdoba and 46.5% (33/71; 95%CI: 34.9–58.1) in Cádiz, indicating a wide but heterogeneous spatial distribution of *C. burnetii* in wild lagomorphs in the Mediterranean ecosystems of southern Spain. Indeed, the GEE model revealed that the prevalence of anti-*C. burnetii* antibodies was significantly higher ( $p = 0.006$ ) in western (20.7%) compared to central (1.5%) Andalusia. Consistently, spatial analysis identified one statistically significant cluster of hunting grounds positive for anti-*C. burnetii* antibodies in southwestern Andalusia (RR: 13.6;  $p < 0.001$ ) (Figure 1). Previous studies have suggested that host density could be associated with a higher risk of *C. burnetii* exposure in mammal species, including wild lagomorphs [27,28,34]. In this respect, a higher mean of hunted wild lagomorphs, which is a reliable index of relative animal abundance [35], was reported in the western region of Andalusia compared with central and eastern regions [36]. In addition, the western region presents higher mean humidity and less-extreme mean temperatures than those observed in central and eastern regions [19]. In fact, the area identified in the spatial cluster includes the Spanish area with the highest average annual rainfall [19]. These climatic features may favor not only the persistence of the bacterium in pastures but also the presence and abundance of competent tick species in the environment. All these findings indicate that western Andalusia could be a hotspot area for *C. burnetii* circulation.



**Figure 1.** Spatial distribution of *C. burnetii* seroprevalence in wild lagomorphs in the study region (Andalusia, southern Spain). The discontinuous circle represents a significant cluster of seropositivity identified using spatial analysis.

The presence of sheep (*Ovis aries*) in the sampled hunting grounds was also identified as a risk factor potentially associated with *C. burnetii* exposure (Table 2), which is consistent with previous studies carried out on domestic ruminants [37]. Hunting grounds where sheep were present had 4.6 times more risk of *C. burnetii* exposure than those without sheep. Domestic ruminants are recognized as the main reservoirs of *C. burnetii* and sheep are frequently raised under extensive production systems in the study region [38]. In this respect, pastures contaminated with feces from *C. burnetii* positive livestock have been described as an important source for infection in wildlife [33], including wild rabbit and hare species [8,33,39]. This hypothesis is supported by the high seroprevalence of *C. burnetii* detected by our research group in sheep farms of Andalusia, where 100.0% of the farms had at least one positive sheep and 40.0% of the tested sheep were confirmed to be exposed to *C. burnetii* (unpublished data).

**Table 2.** Generalized estimating equations analysis of risk factors associated with exposure to *Coxiella burnetii* in wild lagomorphs in Andalusia (southern Spain).

Variable	Categories	<i>p</i> -value	OR 95%CI
Presence of sheep	Yes	0.023	4.6 (1.2-17.0)
	No	a	a
Geographical area	Western	0.006	19.9 (2.3-170.4)
	Eastern	0.133	5.2 (0.6-43.8)
	Central	a	a

<sup>a</sup>Reference Category

## Conclusions

In summary, the results obtained in the present study revealed widespread, but not homogeneous exposure to *C. burnetii* in wild lagomorph populations of Spanish Mediterranean ecosystems. The risk factors identified (western Andalusia and hunting grounds with presence of sheep) should be prioritized in future risk-based surveillance programs for *C. burnetii*. Additional molecular and serological studies are required to elucidate differences between wild rabbits and Iberian hares and to assess the risk of zoonotic transmission of *C. burnetii* from these wild lagomorph species in Iberian Mediterranean ecosystems.

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## Chapter 2.3.

### ***Francisella tularensis* in wild lagomorphs in southern Spanish Mediterranean ecosystems**



Castro-Scholten, S., García-Bocanegra, I., Rejón-Segura, S., Cano-Terriza, D., Jiménez-Martín, D., Rouco, C., Camacho-Sillero, L., Arenas, A., & Caballero-Gómez, J. (2024). ***Francisella tularensis* in wild lagomorphs in southern Spanish Mediterranean ecosystems.** *Animals. Under review*

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## Simple Summary

*Francisella tularensis*, the causative agent of tularemia, is a zoonotic bacterium of public and animal health concern. However, and despite wild lagomorphs are considered important reservoirs of this bacterium and are keystone species in the Iberian Peninsula, very little is known about the epidemiological role of these species. To tackle this question, a cross-sectional study was carried out, between 2017/2018 and 2022/2023 hunting seasons, to investigate the occurrence of *F. tularensis* in spleen samples from 774 European wild rabbits (*Oryctolagus cuniculus*) and 178 Iberian hares (*Lepus granatensis*) inhabiting Iberian Mediterranean ecosystems. None of the 952 wild lagomorphs sampled showed presence of *F. tularensis* DNA. Our results indicate low to no circulation of *F. tularensis* in European wild rabbit and Iberian hare populations, suggesting a limited risk of transmission to other sympatric species, including humans, in southern Spain.

## Abstract

*Francisella tularensis* is a vector-borne zoonotic bacterium that causes tularemia, a disease of great importance for animal and public health. Although wild lagomorphs are considered one of the major reservoirs of this bacterium, information about the circulation of *F. tularensis* in European wild rabbit (*Oryctolagus cuniculus*) and Iberian hare (*Lepus granatensis*) populations in Europe is still very limited. In Spain, *F. tularensis* is present in northern central regions, with recurrent outbreaks occurring annually. However, southern Spain has been neglected in terms of monitoring and research. Thus, the aim of this study was to investigate the occurrence of *F. tularensis* using molecular techniques in wild lagomorphs in Mediterranean ecosystems of southern Spain. Spleen samples from 774 European wild rabbits and 178 Iberian hares were collected on 135 hunting grounds between 2017/2018 and 2022/2023 hunting seasons. A primer set targeting the 16S rRNA gene was used for the detection of *F. tularensis* DNA. None of the 952 (0.0%; 95%CI: 0.0-0.4) wild lagomorphs sampled showed presence of *F. tularensis* DNA. Our finding indicates absence or very low circulation of the bacterium in wild rabbit and Iberian hare populations in Mediterranean ecosystems of southern Spain, denoting a limited risk of transmission of this pathogen from wild lagomorphs to other sympatric species, including human beings in this Iberian region.

**Keywords:** tularemia, vector-borne disease, wild lagomorphs, surveillance, zoonoses.

## Introduction

Tularemia, also known as rabbit or hare fever, is a multi-host vector-borne zoonoses caused by the highly virulent and infectious bacterium *Francisella tularensis*. The disease is often prolonged, debilitating and can be lethal to a wide variety of mammal species, including humans [1], who can acquire the infection through several routes: contact with infected animals, consumption of contaminated food or water, bites of infected vectors (such as ticks, flies, and mosquitoes) or inhalation of aerosols [2]. There are mainly two cycles of the disease that have been described: terrestrial and aquatic. In the terrestrial cycle, lagomorphs and rodent are the most important mammalian hosts and different species of ticks and insects act as competent vectors. In Europe, the most common tick species involved belong to the genus *Dermacentor*, *Ixodes* and *Haemaphysalis* [3]. Mosquitoes, especially those belonging to the genus *Aedes* [4], flies and to a lesser extent fleas, have also been described as vectors of *F. tularensis* [5]. In the aquatic cycle, beaver, muskrat and voles serve as host, contaminating the environment. Mosquitoes have been described as vectors connecting both cycles [6].

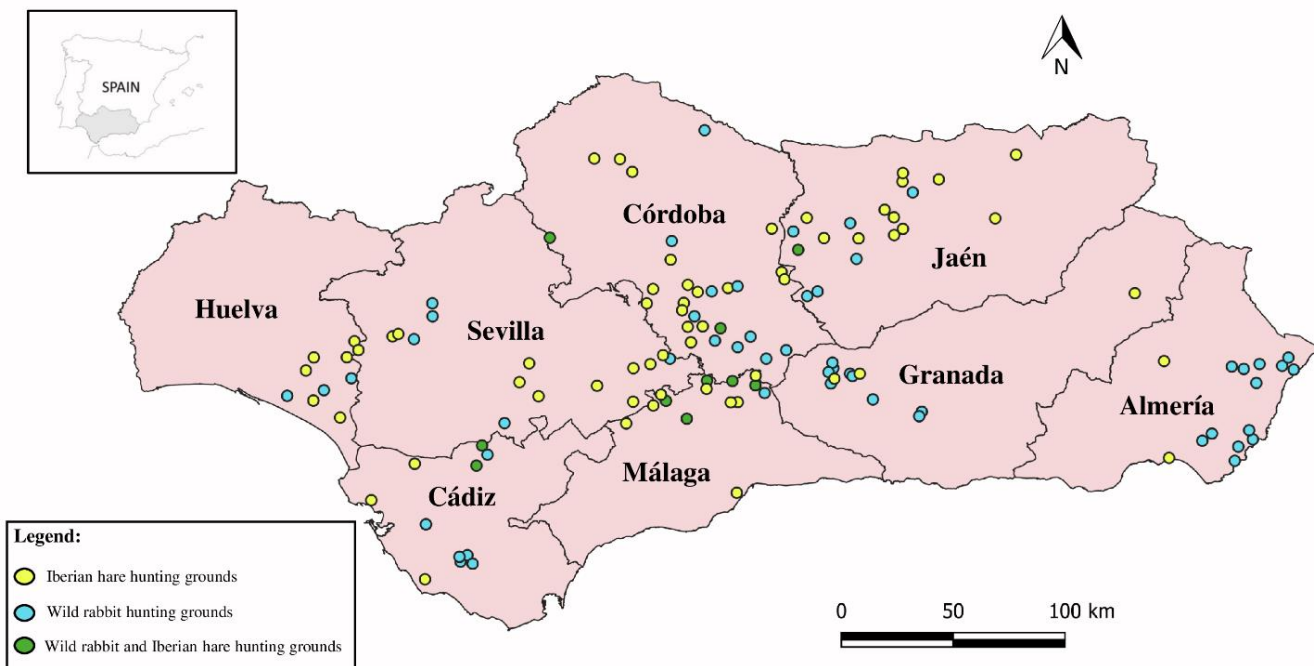
In Europe, the number of human *F. tularensis* cases has considerably increased during the last few years, with wildlife playing an important role in the epidemiology of the bacterium [7, 8]. In fact, the European Centre for Disease Prevention and Control has recently emphasized the need for coordinated One Health surveillance to control tularemia in Europe, highlighting the importance of wildlife monitoring [8]. In this continent, zoonotic transmission from wild lagomorphs has already been reported, and contact with these species is recognized as one of the most significant risk factors for tularemia in humans [9-12]. Despite evidence emphasizing the role of wild lagomorphs as a zoonotic source of *F. tularensis* and their potential as sentinels for this bacterium [13], the information about the role of these species in the epidemiology of tularemia is still scarce or unknown in many European regions. In Spain, since the first tularemia outbreak was reported in 1997, which was related to hunting and handling of hares [14], over 1,000 human cases have been confirmed [15], being currently listed as a notifiable disease and recognized as an emerging pathogen in this country [16]. Tularemia cases have been reported in wild lagomorphs in Spain, with almost all instances confined to the north-central regions of the country. Human outbreaks have already been linked to the presence of *F. tularensis* in these wild species in this country [17]. However, information regarding the circulation of this zoonotic bacterium in wild lagomorph species in southern Spain is very

limited. Therefore, the aim of this study was to molecularly investigate the occurrence of *F. tularensis* in European wild rabbit (*Oryctolagus cuniculus*) and Iberian hare (*Lepus granatensis*) populations inhabiting Iberian Mediterranean ecosystems.

## Material and Methods

### *Study area and sampling collection*

Between the 2017/2018 and the 2022/2023 hunting seasons, a cross-sectional epidemiological study was conducted in Andalusia, southern Spain (87,300km<sup>2</sup>, 36°N-38°60'N, 1°75'W-7°25'W) (Figure 1). This region exhibits a Mediterranean climate characterized by hot and dry summers and mild winters. The western region presents higher mean humidity and less extreme mean temperatures than the central and eastern regions [18]. Andalusia is the Spanish region with the second highest number of wild lagomorphs hunted annually, with 1.4 million wild rabbits and 251 thousand hares harvested each year [19].



**Figure 1.** Spatial distribution of sampled hunting grounds in the study region (Andalusia, southern Spain).

Sample size was calculated to ensure a 99% probability of detecting at least one positive animal, assuming a minimum prevalence of 0.5% in the study area [20]. We analyzed a total of 952 animals, including 774 European wild rabbits and 178 Iberian

hares, from 135 hunting grounds. Samples were distributed across the eight provinces of Andalusia and provided by hunters from each of the hunting grounds during the study period. All animals were legally harvested during the hunting seasons (August-February). Spleens were removed from these animals aseptically and stored in individually labelled plastic tubes. The samples were kept refrigerated until arrival at the laboratory and immediately froze at -80°C until molecular analysis were undertaken.

During sampling, an epidemiological questionnaire was completed by directly interviewing the gamekeepers of the hunting grounds. Information on each animal was recorded, including species, location, year of sampling, age (determined by body weight and body length according to [21]) and sex.

#### *Molecular detection*

Total DNA from spleen was extracted with the commercial NucleoSpin Tissue<sup>®</sup> kit (Macherey-Nagel, Germany), following the manufacturer's instructions. Concentration and quality of all DNA elutions were measured using the NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Values of 260/280 of all extractions were within the expected range and the DNA presented a mean concentration of 407.7 ng/μl. The presence of *F. tularensis* DNA was first tested by a broad-spectrum PCR which targets de 16S ribosomal gene of the *Francisella* genus using the Bio-Rad T100 Thermal Cycler (Hercules, CA, USA), the DreamTaq Green PCR master mix (2x) kit (Thermo Fisher Scientific<sup>™</sup>, Waltham, MA, USA), the primer set F5: 5'- CCT TTT TGA GTT TCG CTC C -3' and F11: 5'- TAC CAG TTG GAA ACG ACT GT -3' which targets 1140bp within the 16S rRNA gene and 8.0 μl of the extracted DNA [22]. Positive control was obtained after DNA extraction of positive *Hyalomma lusitanicum* ticks already published in [23] (GenBank Accession Number: MT386092). The PCR amplification conditions included initial denaturation at 95 °C for 2 min, followed by 35 cycles, each consisted of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 and extension at 72 °C for 1 min, with a final extension of 5 min at 72 °C. The PCR products were subjected to electrophoresis on a 1.5% agarose gel, stained with RedSafe<sup>™</sup> Nucleic Acid Staining Solution (iNtRON Biotechnology, Gyeonggi, South Korea). This diagnostic technique has been previously used in numerous studies to screen *Francisella* spp, including *F. tularensis* [24-27].

#### **Results and discussion**

None of the 774 (0.0%; 95%CI: 0.0-0.5) wild rabbits and 178 (0.0%; 95%CI: 0.0-2.1) Iberian hares analyzed showed *F. tularensis* DNA. Although the complementary use of qPCR could have strengthened the data we present, our results indicate the absence or very low circulation of the bacterium in wild lagomorph populations in the study area.

Of the 952 lagomorphs sampled, excluding missing values, 683 (68.3%) were adults, 231 (24.7%) subadults, and 65 (7.0%) juveniles. Furthermore, the sampling was balanced in terms of sex, with 362 (47.0%) males and 409 (53.0%) females among the European wild rabbits. As for the Iberian hares, 89 (53.0%) were males and 79 (47.0%) were females.

To the best of the author's knowledge, this is the first large-scale study assessing *F. tularensis* circulation in wild lagomorphs in Andalusia. In this Iberian region, the European wild rabbit and the Iberian hare are one of the major small game species and a significant food source for humans, often consumed without veterinary inspection [28]. These wild lagomorph species can act as reservoirs for vector-borne zoonotic pathogens [29-31]. In this regard, both the European wild rabbit and the Iberian hare have been suggested to play a role in the sylvatic cycle of several zoonotic pathogens in southern Spain, such as *Coxiella burnetii*, *Sarcoptes scabiei* and *Leishmania infantum* [32-34]. Despite not finding *F. tularensis* DNA in the present study, endemic distribution of *F. tularensis* was found in these wild lagomorph species in northwest Spain, with prevalences of 1.4% (21/1492) for European wild rabbits and 9.6% (135/1403) for Iberian hares [17]. These findings point to differences in the geographical distribution of the bacterium across the Iberian Peninsula. Similarly, a heterogeneous distribution of *F. tularensis* was found in European brown hare (*Lepus europaeus*) in France [35]. Differences in the spatial distribution of *F. tularensis* might be due to the abundance of natural reservoir species or environmental factors enhancing the transmission of the bacterium in specific epidemiological scenarios [13]. In this regard, it has been described that large tularemia outbreaks in humans in northwestern Spain presented an association with common vole (*Microtus arvalis*) abundance, which favors disease transmission and spillover contamination in the environment likely affecting Iberian hare populations of that region [36-39]. In this sense, *F. tularensis* has a high survival capacity in humid and cold environments, with water being a key factor in the spread of the disease. The study region is characterized mainly by dry, warm summers and mild winters, which do not favor the bacterium's survival in the environment [18].

The results obtained in the present study, together with those observed by Minguéz-González et al. [17] in northwest Spain, denote spatial variations in the risk of zoonotic transmission of *F. tularensis* from wild lagomorphs throughout the Iberian Peninsula. Of note, these findings echo with the geographical distribution of human tularemia cases in Spain, where most outbreaks have been recorded in the northwest with no cases reported in southern Spain so far [16]. Human outbreaks are often preceded by the appearance of the disease in animals [8]. Thus, it has already been described that the multiannual cyclicity of vole outbreaks may serve as a basis for predicting the risk of human tularemia in Spain [36]. In this sense, the European brown hare has been shown to be a useful sentinel species for *F. tularensis* surveillance in France [13]. However, while this species is found only in the north of Spain, the Iberian hare is present in almost the entire Iberian Peninsula and the European wild rabbit is the most abundant lagomorph species in this European region. Further epidemiological studies are warranted to assess circulation of this bacterium in wild lagomorph species and to estimate the risk of exposure for humans from these species in the Iberian Peninsula [7].

### **Conclusions**

In summary, our findings indicate the absence or very low circulation of *F. tularensis* in European wild rabbit and Iberian hare populations, denoting a limited risk of transmission of *F. tularensis* from these lagomorphs to other sympatric species, including humans, in Mediterranean ecosystems of southern Spain. However, given the potential usefulness of wild lagomorphs as sentinel species of this zoonotic bacterium, large-scale monitoring programs should be implemented and maintained in wild lagomorph populations in the Iberian Peninsula.

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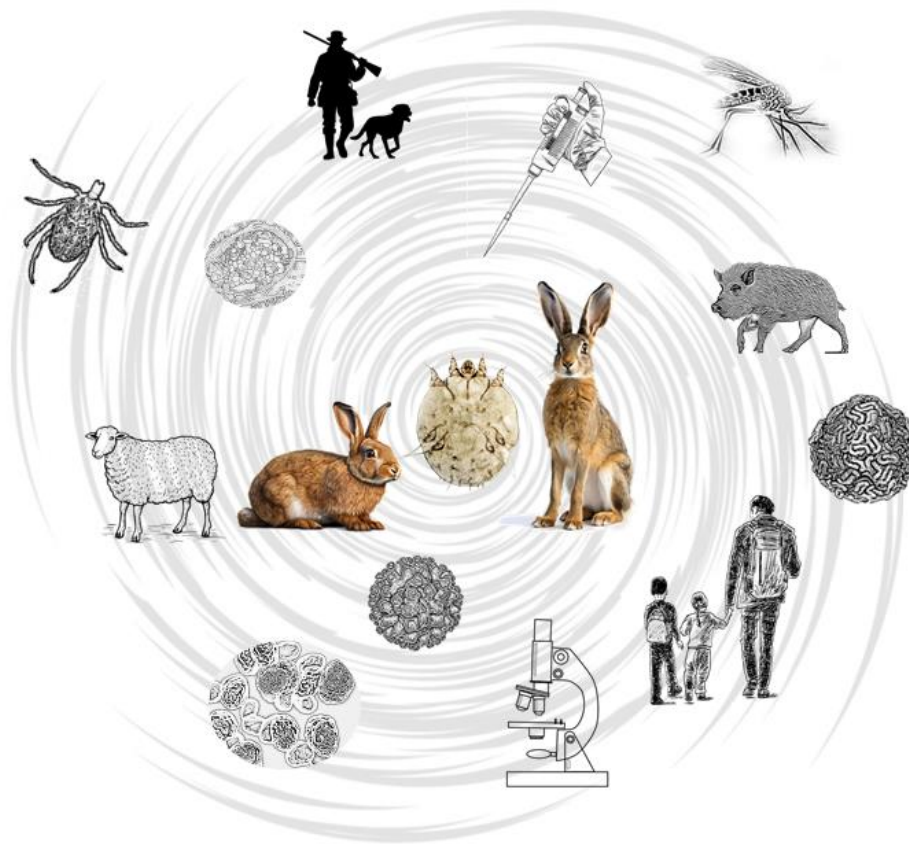
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# CHAPTER 3

## Parasitic pathogens





## Chapter 3.1.

### **Epidemiological survey and risk factors associated with *Sarcoptes scabiei* in wild lagomorphs in Spanish Mediterranean ecosystems.**



Castro-Scholten, S., García-Bocanegra, I., Iglesias, N., Casais, R., Cano-Terriza, D., Jiménez-Martín, D., Barbero-Moyano, J., Camacho-Sillero, L., Rouco, C., & Caballero-Gómez, J. (2024). **Epidemiological survey and risk factors associated with *Sarcoptes scabiei* in wild lagomorphs in Spanish Mediterranean ecosystems.** *Research in Veterinary Science*, 177, 105344

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**Abstract**

Although mortality caused by *Sarcoptes scabiei* has been reported in European wild rabbit (*Oryctolagus cuniculus*) and Iberian hare (*Lepus granatensis*), there is a lack of detailed information regarding the exposure of wild lagomorph species to this parasite. Here, we aimed to determine the seroprevalence and potential risk factors associated with *S. scabiei* exposure in European wild rabbits and Iberian hares in Mediterranean ecosystems of southern Spain. Between 2018/2019 and 2021/2022 hunting seasons, serum samples from 464 wild rabbits and 132 Iberian hares were collected from 100 hunting grounds in Andalusia (southern Spain). Sera were tested using an in-house indirect ELISA to detect specific anti-*S. scabiei* antibodies based on the immunodominant protein Ss $\lambda$ 20 $\Delta$ B3. The overall apparent individual seroprevalence was 15.9% (95/596; 95%CI: 13.0-18.9). Antibodies against *S. scabiei* were detected in 11.6% (54/464; 95%CI: 8.7-14.5) of the European wild rabbits and 31.1% (41/132; 95%CI: 23.2-39.0) of the Iberian hares. Species (Iberian hare), age (adults) and geographical area (western Andalusia) were identified as risk factors potentially associated with *S. scabiei* exposure using generalized estimating equation analysis. By applying spatial analysis, two significant cluster of high seropositivity were detected in western and central Andalusia, respectively. The seroprevalence values obtained provide evidence of endemic, widespread and heterogeneous exposure to *S. scabiei* among wild lagomorph populations in Spanish Mediterranean ecosystems. Our findings underscore the importance of implementing integrated surveillance programs for sarcoptic mange in wild lagomorphs as well as in other sympatric species.

**Keywords:** *S. scabiei*, *Oryctolagus cuniculus*, *Lepus granatensis*, zoonoses, surveillance, sarcoptic mange.



## Introduction

*Sarcoptes scabiei*, the causative agent of sarcoptic mange, is a highly contagious parasite with a worldwide distribution (Gortázar et al., 2007). Beside humans, the disease has been reported in 39 different families comprising 148 species of wild and domestic mammals, leading to significant economic, health and conservation issues (Escobar et al., 2022; Menzano et al., 2007; Tarigan and Huntley, 2005). Since the first description of sarcoptic mange in red fox (*Vulpes vulpes*) (Fürstenberg, 1861), the host range of *S. scabiei* has exponentially increased, expanding its distribution among wildlife across six continents (Escobar et al., 2022). This expansion, together with the high severity of the disease in some species, has led to recognize sarcoptic mange as a current emerging panzootic in wildlife (Escobar et al., 2022).

In Spain, *S. scabiei* infection has been reported in a wide range of wild mammal species, including ungulates, carnivores, and lagomorphs, with certain species demonstrating particular sensitivity to this parasite (Rossi et al., 2019). The first reported epizootic outbreak of sarcoptic mange affecting the Iberian ibex (*Capra pyrenaica*) occurred in 1987 in the Cazorla Natural Park (Andalusia, southern Spain). This outbreak resulted in a population decline of over 95% affecting an estimated population of nearly 9500 individuals (León-Vizcaíno et al., 1999). Since then, sarcoptic mange has caused high rates of morbidity and mortality in this wild Caprinae species, especially among populations with no previous exposure to the parasite (Rossi et al., 2019). In Spain, infection by *S. scabiei* has been documented in several wild ungulate species, including Cantabrian Chamois (*Rupicapra pyrenaica parva*) (Fernández-Morán et al., 1997), Barbary sheep (*Ammotragus lervia*) (González-Candela et al., 2004), roe deer (*Capreolus capreolus*) (Oleaga et al., 2008a), red deer (*Cervus elaphus*) (Oleaga et al., 2008b), wild boar (*Sus scrofa*) (Valldeperes et al., 2021), and European mouflon (*Ovis areis musimon*) (León-Vizcaíno et al., 1999). Furthermore, clinical cases and mortality attributed to *S. scabiei* have also been reported in wild carnivore species, including the Iberian wolf (*Canis lupus*) (Oleaga et al., 2013), the threatened European wild cat (*Felis silvestris silvestris*) (Nájera et al., 2021), and the Iberian lynx (*Lynx pardinus*) (Oleaga et al., 2019).

Regarding wild lagomorphs, mortality outbreaks by sarcoptic mange were initially observed in European wild rabbit (*Oryctolagus cuniculus*) populations in 2008 (Millán, 2010), with the first fatal case reported in the Iberian hare (*Lepus granatensis*) in 2019 (Cardells et al., 2021). Despite the significance of these events, only a single

serosurvey on *S. scabiei* has been conducted worldwide in wild rabbits, dating back 14 years (Millán et al., 2012). Additionally, no studies have assessed the circulation of this pathogen in hare species so far. Given the susceptibility of wild rabbit and Iberian hare to *S. scabiei* and the paucity of information regarding their exposure to this parasite, the present study aims to determine the seroprevalence, potential risk factors and spatial distribution of *S. scabiei* in wild lagomorphs in Mediterranean ecosystems of southern Spain.

## **Material and Methods**

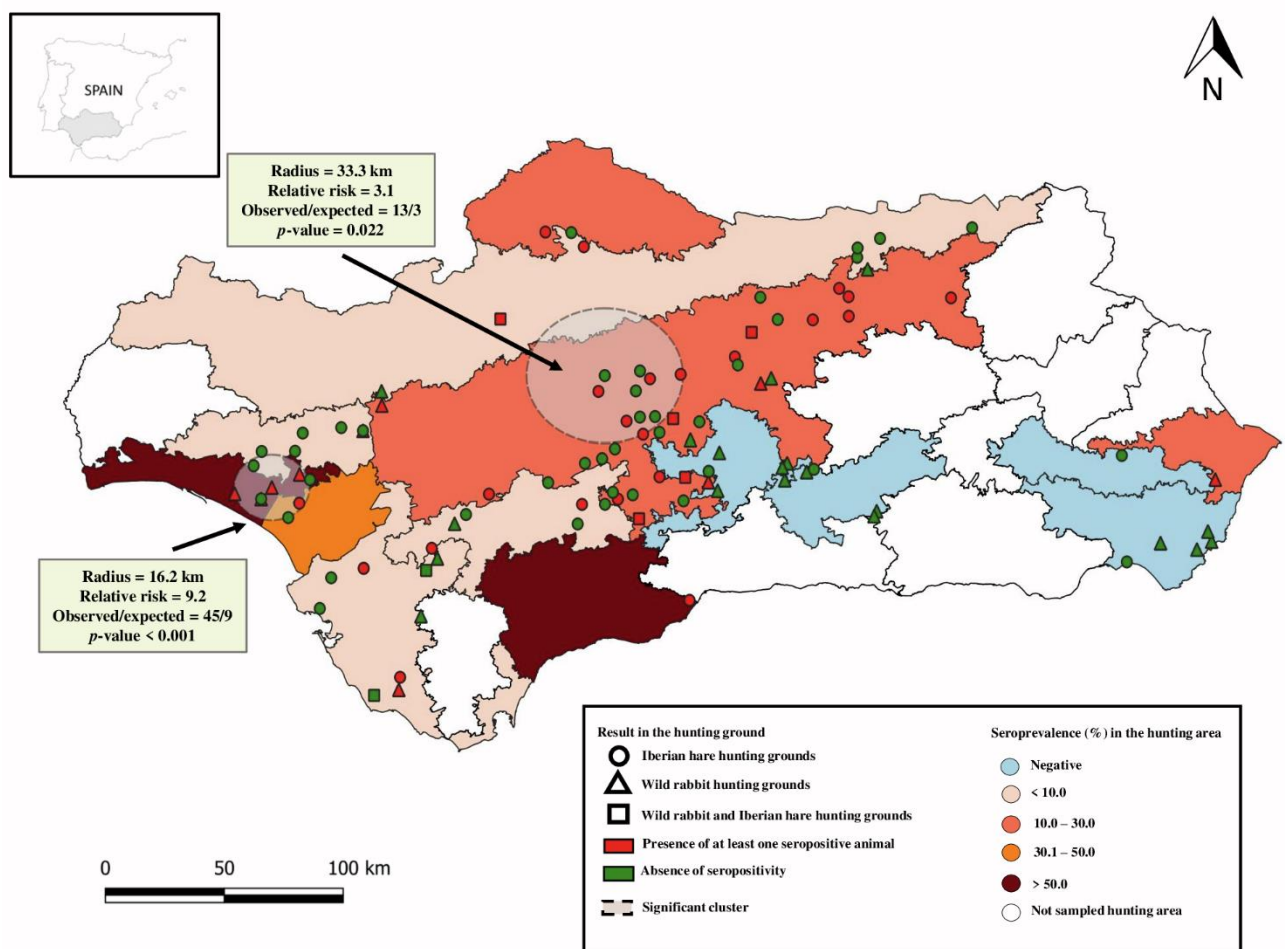
### *Study design and sampling*

A cross-sectional epidemiological study was carried out in Andalusia (36°N-38°60'N, 1°75'W-7°25'W) (southern Spain) between 2018/2019 and 2021/2022 hunting seasons. This region exhibits a Mediterranean climate characterised by hot and dry summers and mild winters. The western region is noted for its higher humid levels and less extreme mean temperatures compared to the central and eastern regions. As a part of the epidemiological surveillance program, the Regional Government of Andalusia has divided the area into 23 hunting areas (Figure 1) based on biological, environmental, and epidemiological factors, including the presence and abundance of large and small game populations (CAPMA, 2013).

Sampling was stratified by hunting area based on the presence and density of wild rabbits in each area (CAPMA, 2013). Random selection of sampling hunting grounds was carried out within each hunting area. Hunters contributed between 1 and 33 (mean:12.9) European wild rabbits per hunting ground for sampling (Figure 1). A total of 464 European wild rabbits were sampled from 36 hunting grounds distributed across 11 hunting areas over the course of four consecutive hunting seasons. Additionally, 132 Iberian hares were opportunistically sampled from 72 hunting grounds across 12 hunting areas within the same study area and period. Blood samples were collected from all animals, extracted from the hearth or thoracic cavity, and centrifuged at 400x *g* for 10 min. The resulting serum was stored -80°C until serological analysis was conducted.

Whenever possible, information on each animal was recorded, including species, location, date of sampling, age, sex, body condition (based on the kidney fat index) (Flux, 1971) and external and internal lesions (Table 1). Three groups of age were considered according to bodyweight and body length (Morris, 1972): young (<40 days old), subadult

(from 40 days to 8 months), or adult (over 8 months). Epidemiological data related to hunting grounds were also gathered through personal interviews with the gamekeepers using a standardized questionnaire. The information obtained included the characteristics of the hunting ground, the presence of disease and control measures, management practices, and presence of other domestic and wild sympatric species (Table 1). In addition, meteorological information for each sampling area [mean and maximum annual temperatures ( $^{\circ}\text{C}$ ), humidity ( $\text{g}/\text{m}^3$ ), and mean annual rainfall ( $\text{mm}$ )] was collected from the closest official meteorological station (AEMET, 2023).



**Figure 1.** Spatial distribution of the seroprevalence of *Sarcptes scabiei* in wild lagomorphs in Andalusia (southern Spain).

### *Laboratory analysis*

Sera were tested to detect specific anti-*S. scabiei* antibodies using an in-house indirect enzyme-linked assay (ELISA) based on the antigen Ssλ20ΔB3 an immunodominant recombinant protein derivates from the polypeptide Ssλ20 (accession number AM286740) (Casais et al., 2007). Ssλ20ΔB3 consists of the 263 amino acid residues from the carboxy terminus of the Ssλ20 polypeptide containing only one complete amino acid repeat present in Ssλ20. The ELISA was performed as previously described for wild rabbits (Millán et al., 2012) with minor modifications. In this study, protein G horseradish peroxidase conjugate, diluted 2000 times in blocking solution, was used as secondary antibody, and TMB as substrate solution to visualize the reaction. The results were expressed as a percentage of the relative optical density (% relative OD<sub>450nm</sub>), calculated using the following formula:

$$OD = [(OD_{\text{sample}} - OD_{\text{negative control}}) / (OD_{\text{positive control}} - OD_{\text{negative control}})] \times 100$$

Serum from an experimentally infested rabbit with *S. scabiei*, collected prior to infestation and at week 11 post-challenge (Casais et al., 2014), served as negative and positive controls, respectively, for all plates. The cut-off level for this ELISA test was calculated as the mean of the % relative OD<sub>450nm</sub> from 17 scabies-free wild-caught rabbits (Millán et al., 2013) plus three times the standard deviation (Bomstein and Wallgren, 1997; Hollanders et al., 1997).

### *Statistical analyses*

The apparent prevalence of antibodies against *S. scabiei* was determined from the proportion of positive samples to the total number of examined, using the two-sided exact binomial test, with 95% confidence intervals (95%CI). Coefficients and standard error values generated by an intercept only generalized estimating equation (GEE) binomial logistic regression model, with hunting ground as subject variable, were used to adjust the estimated seroprevalence and 95%CI for clustering at hunting ground level (Dohoo et al., 2003). Associations between seroprevalence with explanatory variables were screened using the Pearson's Chi-square or Fisher's exact test, as appropriate. Cut-off points for continuous variables were determined at the 33<sup>rd</sup> and 66<sup>th</sup> percentiles, to homogenize the scales of the explanatory variables. All variables with a  $p < 0.05$  in the bivariate analysis were selected for further analysis. Collinearity between pairs of variables was then tested by Cramer's V coefficient, and finally a GEE analysis was

carried out to study the effect of the variables selected from the bivariate analysis. The number of positive animals was assumed to follow a binomial distribution, and 'hunting ground' was included as the subject variable. Forward selection was used for introduction of variables, starting with the variable with the lowest  $p$ -value in the bivariate analysis. The model was re-run until all remaining variables showed statistically significant values ( $p < 0.05$ ). For the choice of the best model, the quasi-likelihood under the independence model criterion (QIC) was considered (Table S1). SPSS 25.0 software (Statistical Package for Social Sciences, Inc., Chicago, IL, USA) was used for all statistical analyses.

#### *Spatial cluster analysis*

The Bernoulli spatial scan statistic was applied to detect the presence of aggregations of hunting grounds with significantly higher *S. scabiei* seropositivity (Kulldorff et al., 2006), using SaTScan™ v10.1.2 software. The number of Monte Carlo simulations was set to 1000 for the cluster scan statistic. SaTScan was used to estimate relative risk (RR), representing the relative frequency of seropositive individuals compared to baseline, for each cluster. Clusters were considered significant at  $p < 0.05$ .

### **Results**

Antibodies against *S. scabiei* were found in 95 out of the 596 (15.9%; 95%CI: 13.0-18.9) wild lagomorphs tested. The apparent individual seroprevalence was 11.6% (54/464; 95%CI: 8.7-14.5) in European wild rabbit, and 31.1% (41/132; 95%CI: 23.2-39.0) in Iberian hare. After adjustment for clustering, the estimated individual seroprevalences were 11.8% (95%CI: 5.3-24.3) and 29.1% (95%CI: 21.6-38.0) in European wild rabbit and Iberian hare, respectively. None of the analysed animals showed lesions compatible with sarcoptic mange.

At least one seropositive animal was found in 38.0% (38/100) and 71.4% (10/14) of the sampled hunting grounds and hunting areas, respectively. The Bernoulli model identified two statistically significant cluster in the study area. One was in western Andalusia with a radius of 16.2 km and included 7 hunting grounds (RR) = 9.2;  $p < 0.001$ ). The second cluster was located in central Andalusia with a radius of 33.3 km and included 10 hunting grounds (RR: 3.1;  $p = 0.022$ ) (Figure 1). Seroprevalence varied among hunting season, ranging from 6.3% (27/432; 95%CI: 4.0-8.5) in the 2020-2021 hunting season to 64.4% (47/63; 95%CI: 53.4-75.4) during the 2021-2022 season (Table 1).

**Table 1.** Distribution of the seroprevalence of *Sarcoptes scabiei* in wild lagomorphs in Andalusia (southern Spain) according to animal and hunting ground variables.

<b>Variable</b>	<b>Categories</b>	<b>No. positives/ Overall*</b>	<b>Seroprevalence (%)</b>
<b>Data recorded from the sampled animals</b>			
Species	European wild rabbit	54/464	11.6
	Iberian hare	41/132	31.1
Age	Adult	86/415	20.7
	Subadult	6/135	4.4
	Young	2/37	5.4
Sex	Male	32/278	11.5
	Female	61/310	19.7
Kidney fat index	0	35/107	32.7
	1	17/148	11.5
	2	11/108	10.2
	3	13/99	13.1
Bodyweight (kg)	0.4-1.0	6/212	2.8
	1.1-1.2	4/129	3.1
	1.3-3.1	25/98	25.5
Body length (cm)	19-37	2/144	1.4
	38-40	7/153	4.6
	41-59	24/115	20.9
Hunting season	2018/2019	13/54	24.1
	2019/2020	8/37	21.6
	2020/2021	27/432	6.3
	2021/2022	47/73	64.4
<b>Hunting ground's characteristics</b>			
Geographical area	Western	56/206	27.2
	Central	27/177	15.3
	Eastern	12/213	5.6
Burrow density	High	51/413	12.3

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	Medium	21/61	34.4
	Low	2/30	6.7
Predominant age group	Adult animals	39/189	20.6
	Young animals	9/177	5.1
	Similar	24/121	19.8
High abundance of ticks in the hunting ground	Yes	20/313	6.4
	No	10/140	7.1
High abundance of fleas in the hunting ground	Yes	19/279	6.8
	No	11/174	6.3
Last restocking (months)	No	32/293	10.9
	≤6	8/50	16.0
	6-12	18/91	19.8
	≥12	16/70	22.9
Origin of the restocking	Same game estate	13/139	9.4
	Another game estate	15/49	30.6
Fenced hunting ground	Yes	2/36	5.6
	No	72/468	15.4
Distance to urban areas (Km)	< 10	71/459	15.5
	10-20	1/28	3.6
Presence of rabbit feeders	Yes	47/303	15.5
	No	27/201	13.4
Feed supplementation in rabbits	Yes	22/215	10.2
	No	52/289	18.0
Presence of swamps	Yes	7/34	20.6
	No	67/470	14.3
Presence of waterholes	Yes	32/198	16.2
	No	42/306	13.7
Presence of sources	Yes	3/142	2.1
	No	71/362	19.6
Presence of troughs	Yes	57/430	13.3
	No	17/74	23.0

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Presence of streams	Yes	39/258	15.1
	No	35/246	14.2
Crops intended for hunting	Yes	44/236	18.6
	No	30/268	11.2
<b>Detection of clinical cases of other infectious diseases</b>			
Outbreaks of myxomatosis in the last year	Yes	74/470	15.7
	No	0/34	0.0
Outbreaks of RHD** in the last year	Yes	66/382	17.3
	No	8/122	6.6
Outbreaks of myxomatosis in the last month	Yes	60/324	18.5
	No	14/180	7.8
Outbreaks of RHD** in the last month	Yes	23/93	24.7
	No	51/411	12.4
<b>Presence of other sympatric species in the hunting ground</b>			
Presence of wild boar ( <i>Sus scrofa</i> )	Yes	56/334	16.8
	No	38/244	15.6
Presence of red deer ( <i>Cervus elaphus</i> )	Yes	14/45	31.1
	No	80/533	15.0
Presence of European wildcat ( <i>Felis silvestris silvestris</i> )	Yes	28/155	18.1
	No	46/349	13.2
Presence of badger ( <i>Meles meles</i> )	Yes	71/436	16.3
	No	3/68	4.4
Presence of Iberian lynx ( <i>Lynx pardinus</i> )	Yes	45/86	52.3
	No	29/418	6.9
Presence of mongoose ( <i>Herpestes ichneumon</i> )	Yes	67/372	18.0
	No	1/125	0.8
Presence of genet ( <i>Genetta genetta</i> )	Yes	70/441	15.9
	No	4/63	6.3
Presence of polecat ( <i>Mustela putorius</i> )	Yes	45/246	18.3
	No	29/258	11.2



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Presence of domestic cat ( <i>Felis silvestris catus</i> )	Yes	66/456	14.5
	No	8/48	16.7
Presence of dog ( <i>Canis familiaris</i> )	Yes	62/389	15.9
	No	12/115	10.4
Presence of cattle ( <i>Bos taurus</i> )	Yes	2/47	4.3
	No	27/389	6.9
Presence of goat ( <i>Capra aegagrus hircus</i> )	Yes	17/171	9.9
	No	12/265	4.5
Presence of sheep ( <i>Ovis aries</i> )	Yes	15/223	6.7
	No	14/213	6.6
Presence of farmed rabbit ( <i>Oryctolagus cuniculus</i> )	Yes	32/54	59.3
	No	42/450	9.3
Presence of domestic pig ( <i>Sus scrofa domesticus</i> )	Yes	3/38	7.9
	No	26/398	6.5
<b>Climate characteristics of the hunting ground</b>			
Mean temperature (°C)	12.3-16.9	23/220	10.5
	17.0-17.5	15/116	12.9
	17.6-18.5	5/159	3.1
Max temperature (°C)	18.9-23.0	13/186	7.0
	23.1-24.0	15/147	10.2
	24.1-27.7	15/154	9.7
Mean annual rainfall (mm)	273.3-586.7	12/201	6.0
	586.8-600.0	69/202	34.2
	600.1-1135.0	6/143	4.2
Humidity (g/m <sup>3</sup> )	56-64	9/79	11.4
	65-66	13/120	10.8
	67-100	4/64	6.3

\*Missing values omitted

\*\*RHD: rabbit hemorrhagic disease

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The explanatory variables obtained from the epidemiological questionnaire and the results of the bivariate analysis are summarized in Table 1 and Table S2. The final GEE model identified three variables (species, age, and geographical area) as risk factors potentially associated with *S. scabiei* exposure (Table 2). Six explanatory variables were excluded from the GEE analysis due to collinearity (Table S3). Seropositivity in Iberian hares was significantly higher compared to European wild rabbits ( $p = 0.016$ ). Additionally, significantly higher seropositivity was found in adult animals (20.7%; 86/415) compared to young (5.4%; 2/37) and subadult individuals (4.4%; 6/135). The prevalence of anti-*S. scabiei* antibodies increased significantly in western Andalusia (27.2%; 56/206) compared to central (15.3%; 27/177) and eastern (5.6%; 12/213) regions.

**Table 2.** Generalized estimating equations analysis of risk factors associated with exposure to *Sarcoptes scabiei* in wild lagomorphs in Andalusia (southern Spain).

Variable	Categories	<i>p</i> -value	OR 95%CI
Species	Iberian hare	0.016	3.2 (1.2-8.3)
	European wild rabbit	a	a
Age	Adult	0.034	1.8 (1.0-3.1)
	Young	0.786	0.8 (0.2-2.9)
	Subadult	a	a
Geographical area	Western	0.047	2.9 (1.0-8.2)
	Central	0.100	2.2 (0.9-5.7)
	Eastern	a	a

<sup>a</sup>Reference Category

## Discussion

The present large survey provides new epidemiological information regarding the distribution of sarcoptic mange among wildlife in Mediterranean ecosystems. The overall seroprevalence found in this study (15.9%) indicates a moderate exposure of wild lagomorph populations to *S. scabiei*. Specifically, the seroprevalence found in European wild rabbits (11.8%) is consistent with the 13.0% detected in the only serosurvey carried out in this species to date (Millán et al., 2012). To the best of author's knowledge, this is

the first epidemiological study conducted on this parasite in hare species worldwide. The seroprevalence detected in Iberian hare (29.1%) indicates a high circulation of *S. scabiei* in their populations in southern Spain. It should be noted that none of the animals sampled in the present study showed macroscopic lesions compatible with sarcoptic mange, and outbreaks of sarcoptic mange were not observed by the gamekeepers in the hunting grounds sampled. Taking into account the observed seroprevalences in both species, these findings may reflect different situations, including: i) the presence of undetected skin lesions during external inspection, ii) self-limiting infestation (Millán et al., 2013) and/or iii) clinically asymptomatic infestations as previously suggested (Millán et al., 2012). Nevertheless, further studies are needed to clarify this aspect.

Significantly higher seropositivity was found in Iberian hares compared to wild rabbits, indicating that either both species are not equally exposed to *S. scabiei* or present differences in susceptibility to infection. In this respect, while various outbreaks of sarcoptic mange have been reported in wild rabbits (Millán, 2010; Navarro-Gonzalez et al., 2010), only one clinical case has been described in hares to date (Cardells et al., 2021). Additionally, besides species, seropositivity to *S. scabiei* in wild lagomorphs was also age-related. There was a significantly higher seroprevalence observed in adults compared to subadults and young animals. This difference may be indicative of the cumulative likelihood of re-exposure to the parasite throughout the lifetime and/or the prolonged persistence of antibodies. Moreover, although anti-*S. scabiei* antibodies were not detectable after eight weeks in experimentally infested wild rabbits that recovered from the disease (Millán et al., 2013), the antibody response was shown to be stronger and longer after re-infestation (Casais et al., 2014). In natural conditions, wild lagomorphs may be regularly exposed to low doses of the parasite, leading to the production of long-lasting antibodies without actually developing clinical disease.

Seropositive animals were found in all the hunting seasons, including young or subadult animals in each of them. At least one positive animal was also detected in 38.0% and 71.4% of the hunting grounds and hunting areas sampled, respectively. These findings indicate a wide endemic circulation of the parasite among wild lagomorph populations in southern Spain. Nevertheless, the significantly higher seroprevalence detected in western Andalusia, and the two clusters found in this region and central Andalusia revealed that the distribution of the parasite in these species was not homogeneous across the study area (Figure 1). The transmission dynamics of sarcoptic

mange in wildlife has been associated with several factors, such as environmental conditions and animal density (Iacopelli et al., 2020; Millán et al., 2012; Pence and Ueckermann, 2002). Consistent with this, the western region of Andalusia exhibits higher mean humidity, average annual rainfall, less-extreme mean temperatures, and abundance of wild lagomorphs than those observed in central and eastern regions (AEMET, 2024; CAPMA, 2013). These conditions not only favour greater parasite survival in the environment but also increases the number of direct contacts between individuals, thus facilitating the circulation of *S. scabiei* among their populations (Arlian, 1989; Millán et al., 2012).

In conclusion, the results obtained indicate a widespread, endemic, and heterogeneous circulation of *S. scabiei* in wild lagomorphs within the Spanish Mediterranean ecosystems of southern Spain. The seroprevalence values detected, particularly in Iberian hare populations, underscore the importance of implementing integrated surveillance programs for *S. scabiei* focused on wild lagomorphs and other sympatric species involved in the transmission and maintenance of this multi-host parasite. Further studies are required to evaluate the differences between wild lagomorph species and to assess the impact of *S. scabiei* in their populations in the Iberian Mediterranean ecosystems.

### Supplementary material

**Table S1.** Models obtained by generalized estimating equations analysis ranked by QIC.  $\Delta$  means difference of QIC with respect to the best model.

Models	QIC	$\Delta$
Species+Age+ Geographical area	482.2	0.0
Age+ Geographical area	493.3	11.1
Geographical area	516.8	34.6

**Table S2.** Results of bivariate analysis according to animal and hunting ground variables.

Variable	Categories	<i>p</i>
Species	European wild rabbit	< 0.001

Iberian hare		
Age	Adult	<b>&lt; 0.001</b>
	Subadult	
	Young	
Sex	Male	<b>0.004</b>
	Female	
Kidney fat index	0	<b>&lt; 0.001</b>
	1	
	2	
	3	
Bodyweight (kg)	0.4-1.0	<b>&lt; 0.001</b>
	1.1-1.2	
	1.3-3.1	
Body length (cm)	19-37	<b>&lt; 0.001</b>
	38-40	
	41-59	
Hunting season	2018/2019	<b>&lt; 0.001</b>
	2019/2020	
	2020/2021	
	2021/2022	
Geographical area	Western	<b>&lt; 0.001</b>
	Central	
	Eastern	
Burrow density	High	<b>&lt; 0.001</b>
	Medium	
	Low	
Predominant age group	Adult animals	<b>&lt; 0.001</b>
	Young animals	
	Similar	
High abundance of ticks in the hunting ground	Yes	0.454
	No	
	Yes	0.502

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High abundance of fleas in the hunting ground	No	
	No	
Last restocking (months)	≤6	<b>0.029</b>
	6-12	
	≥12	
Origin of the restocking	Same game estate	<b>0.001</b>
	Another game estate	
Fenced hunting ground	Yes	0.077
	No	
Distance to urban areas (Km)	< 10	0.061
	10-20	
Presence of rabbit feeders	Yes	0.304
	No	
Feed supplementation in rabbits	Yes	<b>0.010</b>
	No	
Presence of swamps	Yes	0.218
	No	
Presence of waterholes	Yes	0.265
	No	
Presence of sources	Yes	<b>&lt;0.001</b>
	No	
Presence of troughs	Yes	<b>0.027</b>
	No	
Presence of streams	Yes	0.438
	No	
Crops intended for hunting	Yes	<b>0.013</b>
	No	
Outbreaks of myxomatosis in the last year	Yes	<b>0.004</b>
	No	
	Yes	<b>0.002</b>

Outbreaks of RHD* in the last year	No	
Outbreaks of myxomatosis in the last month	Yes	<b>0.001</b>
	No	
Outbreaks of RHD* in the last month	Yes	<b>0.003</b>
	No	
Presence of wild boar ( <i>Sus scrofa</i> )	Yes	0.395
	No	
Presence of red deer ( <i>Cervus elaphus</i> )	Yes	<b>0.007</b>
	No	
Presence of European wildcat ( <i>Felis silvestris</i> <i>silvestris</i> )	Yes	0.099
	No	
Presence of badger ( <i>Meles meles</i> )	Yes	<b>0.004</b>
	No	
Presence of Iberian lynx ( <i>Lynx pardinus</i> )	Yes	<b>&lt; 0.001</b>
	No	
Presence of mongoose ( <i>Herpestes ichneumon</i> )	Yes	<b>&lt; 0.001</b>
	No	
Presence of genet ( <i>Genetta genetta</i> )	Yes	<b>0.028</b>
	No	
Presence of polecat ( <i>Mustela putorius</i> )	Yes	<b>0.017</b>
	No	
Presence of domestic cat ( <i>Felis silvestris</i> <i>catus</i> )	Yes	0.408
	No	
Presence of dog ( <i>Canis</i> <i>familiaris</i> )	Yes	0.091
	No	
Presence of cattle ( <i>Bos</i> <i>taurus</i> )	Yes	0.374
	No	
	Yes	0.023

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Presence of goat ( <i>Capra aegagrus hircus</i> )	No	
Presence of sheep ( <i>Ovis aries</i> )	Yes	0.551
	No	
Presence of farmed rabbit ( <i>Oryctolagus cuniculus</i> )	Yes	<b>&lt; 0.001</b>
	No	
Presence of domestic pig ( <i>Sus scrofa domestica</i> )	Yes	0.473
	No	
Mean temperature (°C)	12.3-16.9	<b>0.008</b>
	17.0-17.5	
	17.6-18.5	
Max temperature (°C)	18.9-23.0	0.526
	23.1-24.0	
	24.1-27.7	
Mean annual rainfall (mm)	273.3-586.7	<b>&lt;0.001</b>
	586.8-600.0	
	600.1-1135.0	
Humidity (g/m <sup>3</sup> )	56-64	0.529
	65-66	
	67-100	

\*RHD: rabbit hemorrhagic disease

**Table S3.** Variables excluded from the generalized estimating equation analysis due to collinearity with any of the three variables included in the final model for the determination of potential risk factors associated to *S. scabiei* seroprevalence in Andalusia (southern Spain).

Independent variable	Variables removed due to collinearity
Species	Bodyweight (kg)
	Body length (cm)



Hunting season	
Age	--
Presence of mongoose ( <i>Herpestes ichneumon</i> )	
Geographical area	Presence of sources
	Mean temperature (°C)

### Acknowledgements

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*Epidemiological survey and risk factors associated with Sarcoptes scabiei in wild lagomorphs in Spanish Mediterranean ecosystems*

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## SYNTHESIS

Wild lagomorphs are key species in the ecology of Mediterranean ecosystems, acting as landscape modulators, prey for numerous predators, a significant important food source for humans, and primary small-game species. However, in recent decades, these species have faced significant threats, among which infectious diseases stand out. These have severely impacted on wild lagomorph populations in these regions, disrupting ecosystem balance and negatively affecting the rural economy. Besides, they can also act as reservoir or spillover hosts of several zoonotic pathogens, of which some may not have a direct impact on their populations but pose public health concerns. However, and despite the EFSA has recognized the importance of surveillance in wild lagomorphs for zoonotic diseases, there is still very limited information regarding the role these species may play in the epidemiology of zoonotic pathogens. The present doctoral thesis assesses the involvement of the European wild rabbit and the Iberian hare in the transmission and maintenance of viral, bacterial and parasitic diseases of public health interest in Andalusia, southern Spain. This synthesis assembles the most relevant results derived from the research carried out, highlighting the implication of wild lagomorphs, as well as the risk factors associated with exposure and/or infection, to these zoonotic pathogens.

The active surveillance carried out on wild lagomorph populations within the framework of this thesis has enabled the evaluation of the circulation of several viruses of public health importance, including the emerging hepatitis E virus, the leading viral cause of acute hepatitis. In Europe, an increasing number of human cases caused by the emerging and zoonotic HEV-3ra subtype has been reported during the last few years, rabbits serving as its main reservoirs. However, despite circulation of the virus has been detected in lagomorphs in several European countries, their role in the epidemiology of this virus in Mediterranean ecosystems is still under debate. Therefore, in **Chapter 1.1**, we assessed exposure of HEV in wild lagomorph populations in southern Spain. Our result confirms that both species are naturally and equally exposed to HEV, with seroprevalences of 6.8% and 6.7% in European wild rabbit and Iberian hare, respectively. However, the spatial circulation of the virus in these species was not homogeneous, with a significantly higher seropositivity in eastern Andalusia. These findings together with the absence of viral RNA in the fecal samples analyzed pointed that these wild lagomorph species play a limited role in the epidemiology of the virus in southern Spain.

Another emerging virus in Europe is CCHFV, one of the most important tick-borne zoonotic viruses for public health as recently evidenced by the EFSA. Although outbreaks of CCHF have been linked to wild lagomorphs, the main hosts of immature stages of *Hyalomma lusitanicum*, little is known about the role of wild lagomorphs in the epidemiology of this virus in Spain, where human cases, sometimes fatal, has worryingly increased during the last few years. In the **Chapter 1.2**, we investigated both the exposure to this virus in wild lagomorphs and the presence of viral RNA in ticks feeding on them in Mediterranean ecosystems in southern Spain, a CCHFV endemic area. As neither anti-CCHFV antibodies nor CCHFV RNA were detected in the animals and the ticks tested, respectively, a limited risk of CCHFV transmission from these species to other mammals was suggested.

Besides ticks, mosquitoes are the leading arthropod vectors of pathogens for humans and animals. Flaviviruses are the most widely distributed mosquito-borne virus, representing other significant threats for public health. Of these, WNV is considered the leading cause of arboviral encephalitis in humans and is the most geographically widespread flavivirus. Southern Spain is considered a hotspot for WNV circulation with endemic circulation and epidemic outbreaks reported in human and horses since 2010. As wild lagomorphs are considered useful sentinel species for some vector-borne diseases, in **Chapter 1.3**, we assessed exposure to WNV in European wild rabbit and Iberian hare populations of Spanish Mediterranean ecosystems. Seropositivity to WNV antibodies were confirmed in 4.8% and 0.7% of the wild rabbits and Iberian hares analyzed, respectively, and only in animals from western Andalusia sampled between 2020 and 2022, echoing with the spatiotemporal distribution of WNV outbreaks detected in human and horses during those years. Therefore, while wild lagomorphs do not seem to play a significant role in the epidemiology of the virus, they could act as sentinel species for monitoring this mosquito-borne virus.

Not only viral but also zoonotic bacteria, such as *C. burnetii*, are an important threat to human and animal health and European wild rabbits have been suggested to play a major epidemiological role in the maintenance and transmission of this bacterium. Actually, *C. burnetii*, the causative agent of Q fever, has recently been listed by the EFSA as a priority disease for coordinated surveillance under a One Health approach. Nevertheless, there is very little information on these species and their associated ticks. In this context, Chapters **2.1 and 2.2** aimed to assess *C. burnetii* circulation in these

species, to determine potential risk factors associated to the bacterium exposure and to assess the presence of this pathogen in ticks that feed on them in Andalusia, the region with the second highest number of reported Q fever cases in Spain. The widespread exposure, the high frequency of animals with active *C. burnetti* infection, and the high prevalence and diversity of positive tick species observed along these studies highlight the importance of both species as natural reservoirs of this zoonotic bacterium, as well as the role of ticks in the epidemiological cycle of *C. burnetii*, with the potential risk of transmission to sympatric species.

Despite their importance in public health, the pathogens mentioned above do not appear to cause symptomatology in lagomorphs. However, there are other pathogens found in wild lagomorphs that have public health significance and also impact on lagomorph populations. Such is the case of *F. tularensis*, a highly pathogenic zoonotic bacteria which causes tularemia. In northwest Spain, tularemia is considered an endemic disease since 1997, and tularemia cases have been reported in wild lagomorphs in this region. However, the information regarding the circulation of this zoonotic bacterium in southern Spain is very limited. In **Chapter 2.3**, we confirmed the absence or very low active circulation of *F. tularensis* in both European wild rabbit and Iberian hare inhabiting Iberian Mediterranean ecosystems. This indicates a limited risk of transmission of the bacterium from these lagomorphs to other sympatric species, including humans, in Mediterranean ecosystems of southern Spain.

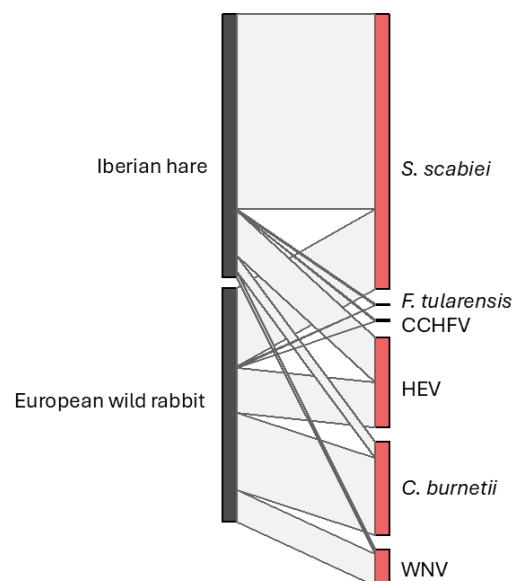
Mortality caused by the zoonotic parasite *S. scabiei*, the causative agent of sarcoptic mange, has also been described in wild lagomorph populations in Spain. The increasing host range and the high severity of the disease in some species has led to recognize sarcoptic mange as an emerging panzootic in wildlife. Despite this, only a single serosurvey has assessed *S. scabiei* exposure in wild rabbits globally and no studies have been conducted on hare species so far. In **Chapter 3.1**, we reported a seroprevalence of 11.8% in European wild rabbits and 29.1% in Iberian hares, confirming for the first time that Iberian hares are naturally exposed to this parasite, which could be of public and animal health concern. Our results provide evidence of endemic, widespread but inter-species, age-related and geographically uneven *S. scabiei* circulation in wild lagomorph populations from southern Spain.

Table 1 summarizes the results obtained from wild lagomorph populations in Andalusia in relation to the different pathogens analyzed in this doctoral thesis.

**Table 1.** Number and type of samples analyzed by species, test used, and results obtained for the different pathogens analyzed.

<b>Pathogen</b>	<b>Species</b>	<b>Sample (n° of samples)</b>	<b>Test used</b>	<b>Frequency of positivity (%)</b>
Hepatitis E virus	European wild rabbit	Serum (370)	ELISA	6.8
		Fecal (212)	RT-PCR	0.0
	Iberian hare	Serum (60)	ELISA	6.7
		Fecal (30)	RT-PCR	0.0
Crimean-Congo hemorrhagic fever virus	European wild rabbit	Serum (473)	ELISA	0.0
		Tick (66)	RT-PCR	0.0
	Iberian hare	Serum (162)	ELISA	0.0
		Tick (54)	RT-PCR	0.0
West Nile virus	European wild rabbit	Serum (394)	Micro-VNT	4.8
	Iberian hare	Serum (141)	Micro-VNT	0.7
<i>Coxiella burnetii</i>	European wild rabbit	Serum (471)	ELISA	11.3
		Spleen (453)	qPCR	16.3
		Tick (450)	qPCR	33.0
	Iberian hare	Serum (167)	ELISA	2.4
		Spleen (121)	qPCR	24.0
		Tick (63)	qPCR	10.3
<i>Francisella tularensis</i>	European wild rabbit	Spleen (774)	PCR	0.0
	Iberian hare	Spleen (178)	PCR	0.0
<i>Sarcoptes scabiei</i>	European wild rabbit	Serum (464)	ELISA	11.6
	Iberian hare	Serum (132)	ELISA	31.1

Figure 1 shows the different exposure levels to the pathogens analyzed in the present doctoral thesis of the European wild rabbit and Iberian hare populations in the southern Spain. The role of both wild lagomorph species in the epidemiology of these pathogens is not homogeneous. Differences between species were observed in three of the six pathogens analyzed. Thus, our results suggest that while the European wild rabbit plays a more prominent role as a reservoir of *C. burnetii* and as spillover host for WNV, the Iberian hare may be particularly important in the maintenance of *S. scabiei*. However, similar exposure levels were observed for the remaining three infectious diseases. In this respect, wild lagomorphs in southern Spain do not appear to play a significant role in the maintenance of CCHFV and *F. tularensis*, and both species are equally exposed to HEV. However, since exposure level is not indicative of clinical susceptibility, potential differences in the vulnerability between these species to the infection by the analyzed pathogens cannot be ruled out for pathogens associated with clinical signs and mortality, such as *S. scabiei*. In this regard, integrate monitoring programs in the wild lagomorph species present in Andalusia might provide valuable information on this issue.



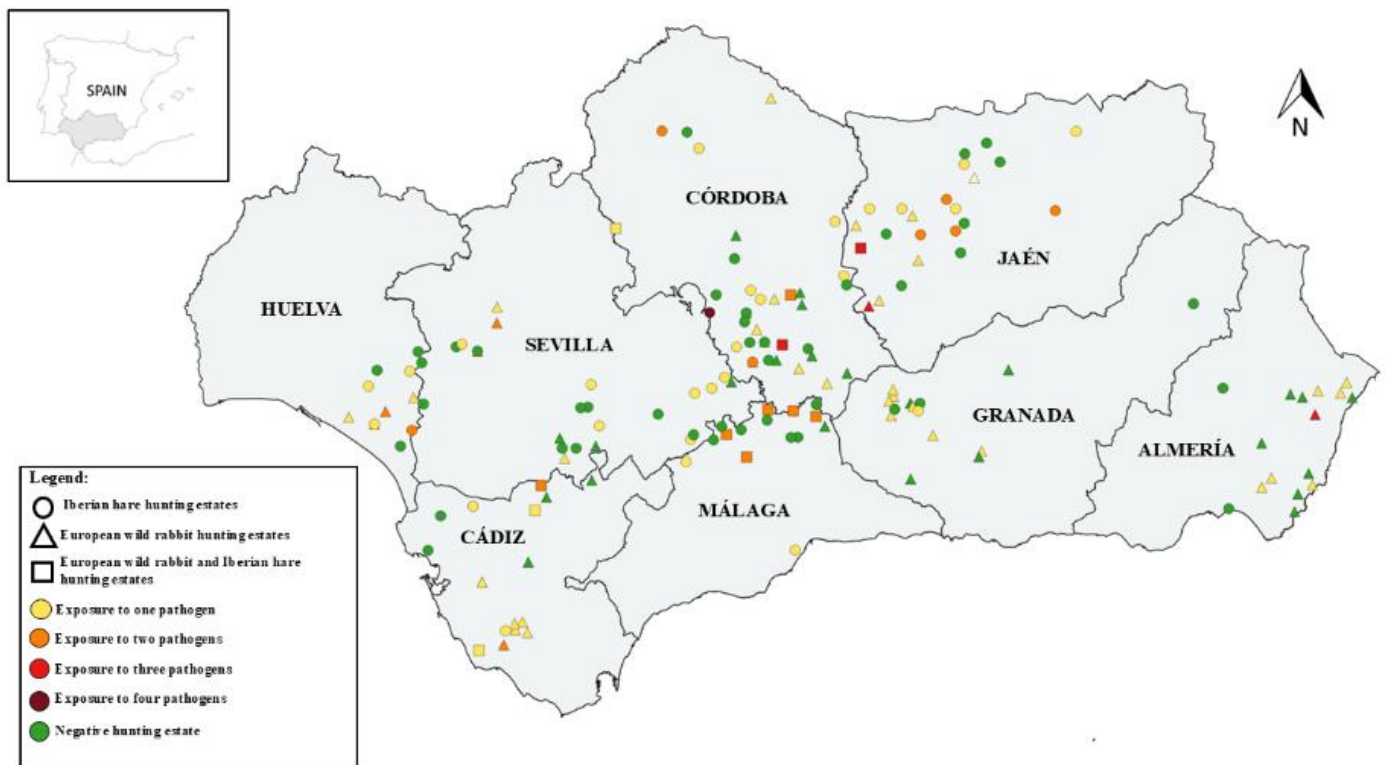
**Figure 1.** Bipartite network representing the exposure to the pathogens included in the doctoral thesis in relation to the wild lagomorph species analyzed.

In the present PhD thesis, a total of 164 hunting estates have been sampled (Figure 2). Exposure to at least one of the pathogens analyzed was detected in 61.0% (50/82) and 46.8% (44/94) of European wild rabbit and Iberian hare hunting estates, respectively (Figure 3). This result indicates widespread spatial distribution of zoonotic pathogens

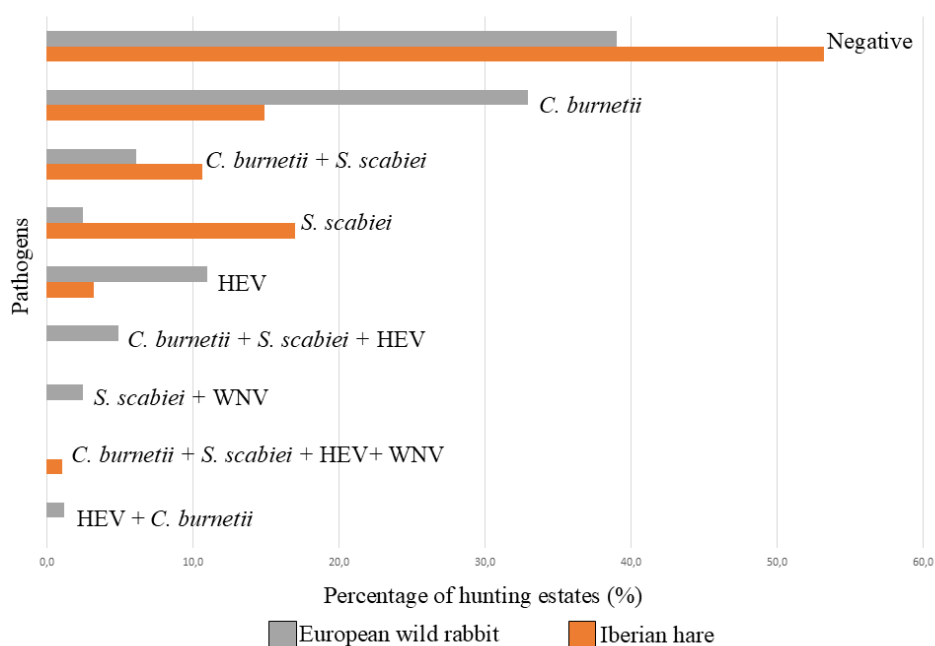


## Synthesis

among wild lagomorph populations in southern Spain. Co-circulation of different pathogens in the same hunting estate was moderate, as 14.6% (12/82) and 11.7% (11/94) of the states where European wild rabbits and Iberian hares were sampled, respectively, presented animals exposed to two or more zoonotic pathogens. Remarkably, one hunting estate located in Central Andalusia presented Iberian hares exposed to four of the pathogens analyzed. Of note, a young Iberian hare with antibodies anti-WNV, *S. scabiei* and HEV was detected in this hunting estate, indicating circulation of these three pathogens in the same year.



**Figure 2.** Spatial distribution of the hunting estates analyzed.



**Figure 3.** Percentage of hunting estates with wild lagomorphs exposed to the different zoonotic pathogens analyzed.

The results obtained in the present doctoral thesis give important insights into the role of European wild rabbits and Iberian hares in the epidemiology of pathogens with conservation, animal and public health importance. This information is valuable for establishing surveillance and control strategies in lagomorphs and can serve as a guide for international institutions. Thus, it has been confirmed absence of circulation of CCHFV (**Chapter 1.2**) and *F. tularensis* (**Chapter 2.3**) and limited exposure to HEV (**Chapter 1.1**), indicating that wild lagomorphs play a limited role in the epidemiology of these pathogens in southern Spain. Nevertheless, the prevalence and seroprevalence values obtained for *C. burnetii* (**Chapters 2.1** and **2.2**) and *S. scabiei* (**Chapter 3.1**) highlight the role of European wild rabbits and Iberian hares in the epidemiological cycle of these pathogens, serving as potential sources of transmission for sympatric species, including humans. These findings have also expanded the known host range of both pathogens, as they had never been previously described in Iberian hares. It is also highlighted that lagomorphs inhabiting western Andalusia presented a significantly higher exposure to both *C. burnetii* and *S. scabiei*. Lastly, the seroprevalence obtained for WNV (**Chapter 1.3**) suggests that, although the European wild rabbit and the Iberian hare do not seem to play a primary role in the epidemiology of this virus, these species

## *Synthesis*

could act as sentinels in Mediterranean ecosystems of Spain, particularly in years with high circulation of this flavivirus.

This doctoral thesis highlights the importance of epidemiological surveillance of zoonotic diseases in wild lagomorphs, providing valuable information on risk factors associated with pathogen circulation, which can enhance control measures for diseases of public health importance. Integrated monitoring programs for zoonotic pathogens in wild lagomorphs across the Iberian Peninsula are justified to gain a deeper and broader understanding of the role of wild lagomorphs in the epidemiology of zoonotic pathogens in different epidemiological scenarios.

## CONCLUSIONS

1. The HEV seroprevalence found in wild lagomorphs (6.7%) inhabiting Andalusia indicates natural exposure to this pathogen, with widespread and heterogeneous viral circulation. Nevertheless, the absence of active HEV infection and the low seropositivity point that the European wild rabbit and the Iberian hare play a limited role in the epidemiology of the virus in this region.
2. The absence of anti-CCHFV antibodies in the wild lagomorphs analyzed, as well as the dearth of CCHFV ARN in their ticks, indicate a limited risk of transmission of this pathogen from European wild rabbits and Iberian hares to other sympatric species, including humans, in southern Mediterranean ecosystems.
3. The detection of WNV seropositive wild lagomorphs in Andalusia constitutes the first report of WNV exposure in wild rabbit in Spain and in Iberian hare worldwide. Although the seroprevalence value found in these species was low (3.7%), the spatiotemporal distribution of seropositive wild lagomorphs overlaps with the largest outbreak of WNV in Spain, suggesting that this species could serve as sentinel for monitoring WNV in Iberian Mediterranean ecosystems.
4. The prevalence of antibodies (8.9%) and *C. burnetii* DNA (17.9%) found in both wild lagomorph species analyzed, as well as the high prevalence (27.5%) and diversity of positive tick species observed point an endemic circulation of *C. burnetii* in wild lagomorph population inhabiting southern Spain and increase the range of susceptible species to this zoonotic pathogen. Our results indicate widespread but heterogeneous exposure to this bacterium and the risk factors identified should be prioritized in future risk-based surveillance programs for *C. burnetii*. In addition, we confirm that wild lagomorphs can act as natural reservoirs of this bacterium in Mediterranean ecosystems of southern Spain, potentially acting as sources of *C. burnetii* infection for other sympatric species, including humans.
5. The lack of *F. tularensis* DNA in the wild lagomorphs analyzed indicates absence or very low circulation of the bacterium in European wild rabbit and Iberian hare populations in Mediterranean ecosystems of southern Spain.
6. The seropositivity to *S. scabiei* found in European wild rabbit and Iberian hare populations (11.6% and 31.1%, respectively) in Andalusia indicates moderate exposure

## *Conclusions*

to this parasite and point that exposure to the parasite is age and species related. Likewise, our results indicate endemic but not homogeneous circulation of the parasite among wild rabbit and Iberian hare populations in this Spanish region, highlighting the need for integrated surveillance programs targeting sarcoptic mange in wild lagomorphs and other sympatric species.

7. The results obtained in this doctoral thesis underline the importance of epidemiological surveillance for a better understanding of the spatiotemporal distribution of pathogens shared between wild lagomorphs and humans providing relevant information to improve control measures against transmissible diseases of public health concern.

## **APPENDIX**

### **Appendix I. Scientific communications derived from this doctoral thesis in international or national meetings.**

Sabrina Castro Scholten is the first author or co-author of three and two scientific communications related with zoonotic pathogens in wild lagomorphs presented in international and national meeting, respectively.

#### **I.A. International meetings**

**1. Title:** Exposición al virus West Nile en lagomorfos silvestres del sur de España.

**Congress:** III Congreso internacional de Sanidad y Bienestar Animal.

**Place:** Madrid, Spain.

**Date:** 14-16<sup>th</sup> November 2024.

**Organizing institution:** Consejo General de Colegios de Veterinarios de España.

**Type of communication:** Poster.

**Authors:** Castro Scholten, S., Caballero Gómez, J., Bravo Barriga, D., Llorente, F., Cano Terriza, D., Jiménez Clavero, M. A., Jiménez Martín, D., Camacho Sillero, L., García Bocanegra, I.

**2. Title:** Epidemiología de *Coxiella burnetii* en lagomorfos silvestres y en las garrapatas que les parasitan en Andalucía.

**Congress:** II Congreso internacional de Sanidad y Bienestar Animal.

**Place:** Córdoba, Spain.

**Date:** 21-23<sup>rd</sup> September 2023.

**Organizing institution:** Consejo General de Colegios de Veterinarios de España.

**Type of communication:** Oral.

**Authors:** Nadales Pérez, B. J., Castro Scholten, S., Caballero Gómez, J., Martínez, R., Cano Terriza, D., Jiménez Martín, D., Remesar, S., Jiménez Ruíz, S., Gómez Guillamón, F., García Bocanegra, I.

**3. Title:** Sarna sarcóptica en liebre Ibérica (*Lepus granatensis*) y conejo silvestre (*Oryctolagus cuniculus*) en el sur de España.

**Congress:** 39èmes rencontres du Groupe d'Etudes sur l'Eco-pathologie de la Faune Sauvage de Montagne (GEEFSM).

**Place:** Córdoba, Spain.

**Date:** 12-15<sup>th</sup> October 2022.

**Organizing institution:** GEEFSM.

**Type of communication:** Oral.

**Authors:** Castro Scholten, S., Casáis, R., Cano Terriza, D., Martínez Martín, D., Gómez Guillamón, F., Barbero Moyano, J., Beato Benítez, A., Martínez, R., Fajardo, T., Muñoz Fernández, L., García Bocanegra, I.

#### **I.B. National meetings**

**1. Title:** Sarna sarcóptica en liebre Ibérica (*Lepus granatensis*) y conejo silvestre (*Oryctolagus cuniculus*) en Andalucía.

**Congress:** IV Congreso de Veterinaria y Ciencia y Tecnología de los Alimentos.

Appendix

**Place:** Córdoba, Spain.

**Date:** 22-23<sup>rd</sup> November 2023.

**Organizing institution:** Facultad de Veterinaria, Universidad de Córdoba.

**Type of communication:** Poster.

**Authors:** Castro Scholten, S., Iglesias, N., Casáis, R., Caballero Gómez, J., Cano Terriza, D., Jiménez Martín, D., Jiménez Ruiz, S., Gómez Guillamón, F., García Bocanegra, I.

**2. Title:** Exposición a *Coxiella burnetii* en lagomorfos silvestres del sur de España\*.

**Congress:** XXIX Congreso Sociedad Científica de Salud Pública Veterinaria (AVESA).

**Place:** Córdoba, Spain.

**Date:** 16-17<sup>th</sup> November 2023.

**Organizing institution:** AVESA.

**Type of communication:** Oral.

**Authors:** Castro Scholten, S., Caballero Gómez, J., Cano Terriza, D., Jiménez Martín, D., Jiménez Ruiz, S., Muñoz Fernández, L., Fajardo, T., Rouco, C., Gómez Guillamón, F., García Bocanegra, I.

\*Awarded communication.

## Appendix II. Other scientific publications derived from the research activity during the PhD.

Sabrina Castro Scholten is the first or co-first author of four scientific articles related with the epidemiology of infectious diseases. In addition, the PhD candidate is co-author of 17 scientific articles, the vast majority of those dealing with the epidemiology, diagnosis or control of infectious diseases of public and animal health concerns. Sabrina Castro Scholten is also the first author or co-author of 17 and 12 scientific communications presented in international and national meetings, respectively, as well as author of two dissemination articles.

### II.A. Research articles

- 1. Authors:** Jiménez Martín, D., Caballero Gómez, J., Cano Terriza, D., Jiménez Ruiz, S., Paniagua, J., Prieto Yerro, P., Castro Scholten, S., García Bocanegra, I.  
**Title:** Seroepidemiology of *Coxiella burnetii* in Domestic and Wild Ruminant Species in Southern Spain.  
**Journal:** Animals (2024). Doi: 10.3390/ani14213072
- 2. Authors:** Castro Scholten, S., Vasinioti, V. I., Caballero Gómez, J., García Bocanegra, I., Pellegrini, F., Salvaggiulo, A., Odigie, A. E., Diakoudi, G., Camero, M., Decaro, N., Martella, V., Lanave, G.  
**Title:** Identification and characterization of a novel circovirus in Iberian lynx in Spain  
**Journal:** Research in Veterinary Science (2024). Doi: 10.1016/j.rvsc.2024.105336
- 3. Authors:** Barbero Moyano, J., Castro Scholten, S., González, M., Moreno, I., Domínguez, M., Cano Terriza, D., Jiménez Martín, D., Camacho Sillero, L., Martínez, R., García Bocanegra, I.  
**Title:** Epidemiological surveillance of *Leishmania infantum* in wild lagomorphs in Spanish Mediterranean ecosystems.  
**Journal:** Preventive Veterinary Medicine (2024). Doi: 10.1016/j.prevetmed.2024.106231
- 4. Authors:** Frías, M., Fischer, K., Castro Scholten, S., Bost, C., Cano Terriza, D., Risalde, M. A., Acevedo, P., Jiménez Ruiz, S., Sadeghi, B., Groschup, M. H., Caballero Gómez, J., García Bocanegra, I.  
**Title:** Epidemiologic Survey of Crimean-Congo Hemorrhagic Fever Virus in Suids, Spain.  
**Journal:** Emerging Infectious Diseases (2024). Doi: 10.3201/eid3005.240074
- 5. Authors:** Rios Muñoz, L., González, M., Caballero Gómez, J., Castro Scholten, S., Casares Jimenez, M., Agulló Ros, I., Corona Mata, D., García Bocanegra, I., Lopez Lopez, P., Fajardo, T., Mesquita, J. R., Risalde, M. A., Rivero Juarez, A., Rivero, A.  
**Title:** Epidemiologic Survey of Crimean-Congo Hemorrhagic Fever Virus in Suids, Spain.  
**Journal:** Emerging Infectious Diseases (2024). Doi: 10.3201/eid3004.231629



**6. Authors:** Ávalos, G., Caballero Gómez, J., Mata Méndez, P., Castro Scholten, S., Jiménez Martín, D., Koster, P. C., Santín, M., Bailo, B., Cano Terriza, D., González Barrio, D., Mateo, M., García Bocanegra, I., Dashti, A., Nájera, F., Carmena, D.

**Title:** Detection and genotyping of zoonotic microsporidia in the endangered Iberian lynx (*Lynx pardinus*)

**Journal:** Medical Mycology (2024). Doi: 10.1093/mmy/myae027

**7. Authors:** Bost, C., Castro Scholten, S., Sadeghi, B., Cano Terriza, D., Frías, M., Jiménez Ruiz, S., Groschup, M. H., García Bocanegra, I., Fischer, K.

**Title:** Approaching the complexity of Crimean-Congo hemorrhagic fever virus serology: A study in swine

**Journal:** Journal of Virological Methods (2024). Doi: 10.1016/j.jviromet.2024.114915

**8. Authors:** Matas Méndez, P., Ávalos, G., Caballero Gómez, J., Dashti, A., Castro Scholten, S., Jiménez Martín, D., González Barrio, D., Muñoz de Mier, G. J., Bailo, B., Cano Terriza, D., Mateo, M., Nájera, F., Xiao, L., Koster, P. C., García Bocanegra, I., Carmena, D.

**Title:** Detection and Molecular Diversity of *Cryptosporidium* spp. and *Giardia duodenalis* in the Endangered Iberian Lynx (*Lynx pardinus*), Spain

**Journal:** Journal of Virological Methods (2024). Doi: 10.3390/ani14020340

**9. Authors:** Baz Flores, S., Herraiz, C., Peralbo Moreno, A., Barral, M., Cruz Arnal, M., Balseiro, A., Cano Terriza, D., Castro Scholten, S., Cevidanes, A., Conde Lizarralde, A., Cuadrado Matías, R., Escribano, F., Fernández de Luco, D., Fidalgo, E. E., Hermoso de Mendoza, J., Fandos, P., Gómez Guillamón, F., Granados, J. E., Jiménez Martín, D., López Olvera, J. R., Martín, I., Martínez, R., Mentaberre, G., García Bocanegra, I., Ruiz Fons, F.

**Title:** Mapping the risk of exposure to Crimean-Congo haemorrhagic fever virus in the Iberian Peninsula using Eurasian wild boar (*Sus scrofa*) as a model.

**Journal:** Ticks and Tick-borne Diseases (2024). Doi: 10.1016/j.ttbdis.2023.102281

**10. Authors:** González, M., Paniagua, J., Jiménez Martín, D., Cano Terriza, D., Castro Scholten, S., Barbero Moyano, J., Jiménez Ruíz, S., García Bocanegra, I.

**Title:** Monitoring the dynamics of consumption of ungulate game by-products in vulture feeding stations in Iberian Mediterranean ecosystem.

**Journal:** Research in Veterinary Science (2023). Doi: 10.1016/j.rvsc.2023.105026

**11. Authors:** Remesar, S., Castro Scholten, S., Morrondo, P., Díaz, P., Jiménez Martín, D., Muñoz Fernández, L., Fajardo, T., Cano Terriza, D., García Bocanegra, I.

**Title:** Occurrence of *Anaplasma* spp. in wild lagomorphs from Southern Spain: Molecular detection of new *Anaplasma bovis* lineages

**Journal:** Research in Veterinary Science (2023). Doi: 10.1016/j.rvsc.2023.105093

**12. Authors:** Rego, L.\* , Castro Scholten, S.\*, Cano, C., Jiménez Martín, D., Köster, P. C., Caballero Gómez, J., Bailo, B., Dashti, A., Hernández Castro, C., Cano Terriza, D., Vioque, F., Maloney, J. G., Santín, M., García Bocanegra, I., Carmena, D., González Barrio, D.

**Title:** Iberian wild leporidae as hosts of zoonotic enteroparasites in Mediterranean ecosystems of Southern Spain

**Journal:** Zoonoses and public health (2022). Doi: 10.1111/zph.13018

\*Equally contributed.

**13. Authors:** Remesar, S., Castro Scholten, S., Morrondo, P., Díaz, P., Jiménez Martín, D., Rouco, C., Camacho Sillero, L., Cano Terriza, D., García Bocanegra, I.

**Title:** Molecular detection of *Ehrlichia* spp. in ticks parasitizing wild lagomorphs from Spain: characterization of a novel *Ehrlichia* species.

**Journal:** Parasites & Vectors (2022). Doi: 10.1186/s13071-022-05600-4

**14. Authors:** Camacho Sillero, L., Cardoso, B., Beato Benítez, A., Gómez Guillamón, F., Díaz Cao, J. M., Jiménez Martín, D., Caballero Gómez, J., Castro Scholten, S., Cano Terriza, D., García Bocanegra, I.

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

**Journal:** Transboundary and Emerging Diseases (2022). Doi: 10.1111/tbed.14709

**15. Authors:** Caballero Gómez, J., Rivero Juarez, A., Jurado Tarifa, E., Jiménez Martín, D., Jiménez Ruiz, E., Castro Scholten, S., Ulrich, R. G., López López, P., Rivero, A., García Bocanegra, I.

**Title:** Serological and molecular survey of hepatitis E virus in cats and dogs in Spain.

**Journal:** Transboundary and Emerging Diseases (2021). Doi: 10.1111/tbed.14437

**16. Authors:** Remesar, S., Castro Scholten, S., Cano Terriza, D., Díaz, P., Morrondo, P., Jiménez Martín, D., Rouco, C., García Bocanegra, I.

**Title:** Molecular identification of zoonotic *Rickettsia* species in Ixodidae parasitizing wild lagomorphs from Mediterranean ecosystems.

**Journal:** Transboundary and Emerging Diseases (2021). Doi: 10.1111/tbed.14379

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

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

**Journal:** Preventive Veterinary Medicine (2021). Doi: 10.1016/j.prevetmed.2021.105508

\*Equally contributed.

**18. Authors:** Castro Scholten, S., Cano Terriza, D., Jiménez Ruiz, S., Almería, S., Risalde M. A., Vicente, J., Acevedo, P., Arnal, M. C., Balseiro, A., Gómez Guillamón, F., Escribano, F., Puig Ribas, M., Dubey, J., García Bocanegra, I.

**Title:** Seroepidemiology of *Toxoplasma gondii* in wild ruminants in Spain

**Journal:** Zoonosis and Public Health (2021). Doi: 10.1111/zph.12878

**19. Authors:** Almería, S., Cano Terriza, D., Prieto, P., Dubey, J., Jiménez Martín, D., Castro Scholten, S., Paniagua, J., García Bocanegra, I.

**Title:** Seroprevalence and risk factors of *Toxoplasma gondii* infection in wild ungulates that cohabit in a natural park with human–animal interaction in the Mediterranean ecosystem.

**Journal:** Zoonosis and Public Health (2021). Doi: 10.1111/zph.12821

**20. Authors:** Jiménez Martín, D., García Bocanegra, I., Almería, S., Castro Scholten, S., Dubey, J., Amaro López, M., Cano Terriza, D.

**Title:** Epidemiological surveillance of *Toxoplasma gondii* in small ruminants in southern Spain.

**Journal:** Preventive Veterinary Medicine (2020). Doi: 10.1016/j.prevetmed.2020.105137

**21. Authors:** Cano Terriza, D., Almería, S., Caballero Gómez, J., Jiménez Martín, D., Castro Scholten, S., Dubey, J., García Bocanegra, I.

**Title:** Exposure to *Toxoplasma gondii* in zoo animals in Spain.

**Journal:** Preventive Veterinary Medicine (2020). Doi: 10.1016/j.prevetmed.2020.104930

## **II.B. Congress communications**

### **II.B.1. International meetings**

**1. Title:** Escape room: a teaching tool to enhance learning of Infectious Diseases in the Degree in Veterinary Medicine\*.

**Congress:** VII Congreso español y I hispano-portugués de docencia veterinaria.

**Place:** Cáceres, Spain.

**Date:** 05-06<sup>th</sup> June 2024.

**Organizing institution:** Universidad de Extremadura.

**Type of communication:** Oral.

**Authors:** Jiménez Martín, D., Castro Scholten, S., Martínez, R., González, M., García Bocanegra, I., Beato Benítez, A., Fajardo, T., Frias, M., Caballero Gómez, J., Rialde, M. A., Jiménez Ruiz, S., Herrera, J. C., Cano Terriza, D.

\*Awarded communication.

**2. Title:** Interactive learning adventures: enhancing wildlife veterinarian training through escape room methodology.

**Congress:** VII Congreso español y I hispano-portugués de docencia veterinaria.

**Place:** Cáceres, Spain.

**Date:** 05-06<sup>th</sup> June 2024.

**Organizing institution:** Universidad de Extremadura.

**Type of communication:** Oral.

**Authors:** Castro Scholten, S., Jiménez Martín, D., González, M., Martínez, R., Cano Terriza, D., Barbero Moyano, J., Paniagua, J., Bravo Barriga, D., Rejón Segura, S., Agulló Ros, I., Muñoz Fernández, L., García Bocanegra, I.

\*Awarded communication.

**3. Title:** Epidemiological study of hepatitis E virus in wild boar (*Sus scrofa*) in Andalusia, southern Spain.

**Congress:** XIV Reunião de Ungulados Silvestres Ibéricos.

**Place:** Figueira de Castelo Rodrigo, Portugal.

**Date:** 29-30<sup>th</sup> September 2023.

**Organizing institution:** Universidade de Aveiro.

**Type of communication:** Oral.

**Authors:** Jiménez Ruiz, S., García Bocanegra, I., Caballero Gómez, J., Jiménez Martín, D., Castro Scholten, S., Cano Terriza, D., González, M., Martínez Pérez, R., Beato Benítez, A., Talavera, V., Camacho Sillero, L., Gómez Guillamón, F.

**4. Title:** Comparison of different CCHFV serology test formats for swine sera.

**Congress:** 3rd International Conference on Crimean-Congo Hemorrhagic Fever.

**Place:** Thessaloniki, Greece.

**Date:** 19-21<sup>st</sup> September 2023.

**Organizing institution:** Aristotle University of Thessaloniki.

**Type of communication:** Oral.

**Authors:** Bost, C., Castro Scholten, S., Sadeghi, B., Cano Terriza, D., Frías, M., Jiménez Ruiz, S., Groschup, M. H., García Bocanegra, I., Fischer, K.

**5. Title:** Seroprevalencia de Arteritis Vírica Equina en diferentes regiones de Europa.

**Congress:** XXII Congreso internacional de Medicina y Cirugía Equina.

**Place:** Sevilla, Spain.

**Date:** 18-19<sup>th</sup> November 2022.

**Organizing institution:** Consejo Andaluz de Colegios Oficiales de Veterinarios.

**Type of communication:** Poster.

**Authors:** Franco, J. J., Cano Terriza, D., Cunilleras, J. E., González, M., García, J., Jiménez Martín, D., Alguacil, E., Castro Scholten, S., Beato Benítez, A., García Bocanegra, I.

**6. Title:** Estudio del papel epidemiológico de los lagomorfos silvestres como reservorios de *Leishmania Infantum* de Andalucía\*.

**Congress:** I congreso internacional de Sanidad y Bienestar Animal.

**Place:** León, Spain.

**Date:** 15-17<sup>th</sup> September 2022.

**Organizing institution:** Consejo General de Colegios de Veterinarios de España.

**Type of communication:** Oral.

**Authors:** Barbero Moyano, J., Castro Scholten, S., Martínez, R., González, M., Moreno, I., Domínguez, M., Cano Terriza, D., Jiménez Martín, D., Gómez Guillamón, F., García Bocanegra, I.

\*Awarded communication.

**7. Title:** Molecular detection and characterization of *Cryptosporidium* spp. in wild lagomorphs from southern Spain: a preliminary study.

**Congress:** 15<sup>th</sup> International Congress of Parasitology.

**Place:** Danmark, Copenhagen.

**Date:** 21-26<sup>th</sup> August 2022.

**Organizing institution:** World Federation of Parasitologists.

**Type of communication:** Poster.

**Authors:** Cano, C., Castro Scholten, S., Rego, L., Vioque, F., Jiménez Martín, D., Köster, P. C., Bailo, B., Dashti, A., Cano Terriza, D., Caballero Gómez, J., García Bocanegra, I., Carmena, D., González Barrio, D.

**8. Title:** Serosurveillance of the myxoma virus in Iberian hares (*Lepus granatensis*) after the first outbreak.

**Congress:** 6<sup>th</sup> World Lagomorph Conference.

**Place:** Montpellier, France.

**Date:** 04-08<sup>th</sup> July 2022.

**Organizing institution:** Université Sorbonne Paris Nord.

**Type of communication:** Oral.

**Authors:** Cardoso, B., Martínez Haro, M., Gómez Guillamón, F., Rouco, C., Castro Scholten, S., Viñuelas, J. A., Chinchilla, J. M., Jiménez Martín, D., Aguayo, J. A., Acevedo, P., Queirós, J., Alves, P. C., García Bocanegra, I.

**9. Title:** Seroprevalence of *Toxoplasma gondii* in wild and domestic lagomorphs in Spain.

**Congress:** International Conference on Avian, Herpetological and Exotic Mammal Medicine.

**Place:** Budapest, Hungary.

**Date:** 28-30<sup>th</sup> March 2022.

**Organizing institution:** European Association of Avian Veterinarians (EAAV), the Association of Exotic Mammal Veterinarians (AEMV), the Association of Reptile and Amphibian Veterinarians (ARAV), and the European College of Zoological Medicine (ECZM).

**Type of communication:** Oral.

**Authors:** Calero Vázquez, D., Castro Scholten, S., Cano Terriza, D., Aguayo Adán, J. A., Rouco, C., Almería, S., Jiménez Martín, D., Jiménez Ruíz, S., Villena, I., García Bocanegra, I.

**10. Title:** Exposure to Hepatitis E virus in domestic and wild lagomorphs in Andalusia (southern Spain).

**Congress:** International Conference on Avian, Herpetological and Exotic Mammal Medicine.

**Place:** Budapest, Hungary.

**Date:** 28-30<sup>th</sup> March 2022.

**Organizing institution:** European Association of Avian Veterinarians (EAAV), the Association of Exotic Mammal Veterinarians (AEMV), the Association of Reptile and Amphibian Veterinarians (ARAV), and the European College of Zoological Medicine (ECZM).

**Type of communication:** Oral.

**Authors:** Calero Vázquez, D., Caballero Gómez, J., Rivero Juárez, A., Castro Scholten, S., Cano Terriza, D., Aguayo Adán, J. A., Jiménez Martín, D., Beato Benítez, A., Barbero, J., García Bocanegra, I.

**11. Title:** Seroepidemiología de *Toxoplasma gondii* en ungulados silvestres de España.

**Congress:** 38<sup>th</sup> Annual Meeting of the Groupe d'Etudes sur l'Ecopathologie de la Faune Sauvage de Montagne (GEEFSM)

**Place:** Lanslebourg, France.

**Date:** 07-10<sup>th</sup> October 2021.

**Organizing institution:** GEEFSM.

**Type of communication:** Oral.

**Authors:** Jiménez Martín, D., Castro Scholten, S., Cano Terriza, D., Jiménez Ruiz, S., Almería, S., Risalde, M. A., Vicente, J., Acevedo, P., Arnal, M. C., Balseiro, A., Gómez Guillamón, F., Escribano, F., Puig Ribas, M., Dubey, J., García Bocanegra, I.

**12. Title:** Seroepidemiology of *Toxoplasma gondii* in wild ruminants in Spain.

**Congress:** XII RUSI. Reunión de Ungulados Silvestres Ibéricos.

**Place:** Vila Real, Portugal.

**Date:** 01-02<sup>nd</sup> October 2021.

**Organizing institution:** Waves Portugal.

**Type of communication:** Oral.

**Authors:** Jiménez Ruiz, S., Castro Scholten, S., Cano Terriza, D., Almería, S., Risalde, M. A., Vicente, J., Acevedo, P., Arnal, M. C., Balseiro, A., Gómez Guillamón, F., Escribano, F., Puig Ribas, M., Dubey, J., García Bocanegra, I.

**13. Title:** Monitoring of Schmallenberg virus in zoo animals in Spain, 2002-2019.

**Congress:** 69th WDA / 14th EWDA.

**Place:** Cuenca, Spain.

**Date:** 31<sup>st</sup> August-02<sup>nd</sup> September 2021.

**Organizing institution:** Universidad de Castilla la Mancha y Universidad de Córdoba.

**Type of communication:** Poster.

**Authors:** Jiménez Martín, D., Caballero Gómez, J., García Bocanegra, I., Navarro, N., Guerra, R., Gerique, C., Martínez Nevado, E., Soriano, P., Castro Scholten, S., Beato, A., Cano Terriza, D.

**14. Title:** Zoo animals as sentinels for Bluetongue virus monitoring in Spain.

**Congress:** 69th WDA / 14th EWDA.

**Place:** Cuenca, Spain.

**Date:** 31<sup>st</sup> August-02<sup>nd</sup> September 2021.

**Organizing institution:** Universidad de Castilla la Mancha y Universidad de Córdoba.

**Type of communication:** Poster.

**Authors:** Caballero Gómez, J., Cano Terriza, D., Pujols, J., Martínez Nevado, E., Carbonell, M. D., Guerra, R., Recuero, J., Soriano, P., de Castro, N., Castro Scholten, S., Barbero, J., García Bocanegra, I.

**15. Title:** Seroprevalence of *Trichinella* spp. in wild boar (*Sus scrofa*) in south-western Spain.

**Congress:** 28th Internacional conference of the World Association for the Advancement of Veterinary Parasitology.

**Place:** Dublin, Ireland.

**Date:** 19-22<sup>th</sup> July 2021.

**Organizing institution:** World Association for the Advancement of Veterinary Parasitology.

**Type of communication:** Poster.

**Authors:** Buffoni Perazzo, L., Cano Terriza, D., García Bocanegra, I., Jiménez Martín, D., Castro Scholten, S., Gómez Guillamón, F., Martínez Moreno, A.

**16. Title:** First approach to the use of the eBook as a teaching tool in the Degree in Veterinary Medicine.

**Congress:** International Congress on Education in Animal Sciences (ICEAS).

**Place:** Murcia, Spain.

**Date:** 07-08<sup>th</sup> June 2021.

**Organizing institution:** Universidad de Murcia.

**Type of communication:** Poster.

**Authors:** Cano Terriza, D., Jiménez Martín, D., Castro Scholten, S., Caballero Gómez, J., Risalde M. A., Molina Hernández, V., Garrido, F., García Bocanegra, I.

**17. Title:** *Toxoplasma gondii* in wild ungulates that cohabit in a natural park with high human-animal interaction in Spain.

**Congress:** XI Reunión de Ungulados Silvestres Ibéricos (RUSI).

**Place:** Madrid, Spain.

**Date:** 23-24<sup>th</sup> October 2020.

**Organizing institution:** Universidad de Politécnica de Madrid.

**Type of communication:** Oral.

**Authors:** Castro Scholten, S., Almería, S., Cano Terriza, D., Prieto, P., Dubey, J., Jiménez Martín, D., Paniagua, J., García Bocanegra, I.

## II.B.2. National meetings

**1. Title:** Uso del Escape Room como herramienta de gamificación para reforzar el aprendizaje en la docencia universitaria.

**Congress:** Congreso de Innovación Docente en Ciencias (I CIDoC).

**Place:** Córdoba, Spain.

**Date:** 19-20<sup>th</sup> June 2024.

**Organizing institution:** Facultad de Ciencias de la Universidad de Córdoba.

**Type of communication:** Oral.

**Authors:** Castro Scholten, S., Martínez, R., González, M., Cano Terriza, D., García Bocanegra, I., Jiménez Martín, D.

**2. Title:** Estudio epidemiológico del virus de la fiebre hemorrágica de Crimea-Congo en suidos domésticos y silvestres en España\*.

**Congress:** V Congreso Salud Pública Veterinaria.

**Place:** Ayamonte, Spain.

**Date:** 25-27<sup>th</sup> April 2024.

**Organizing institution:** Consejo Andaluz de Colegios Oficiales Veterinarios.

**Type of communication:** Oral.

**Authors:** Jiménez Ruiz, S., Frias, M., Fischer, K., Castro Scholten, S., Bost, C., Cano Terriza, D., Riscalde, M. A., Acevedo, P., Sadeghi, B., Groschup, M. H., Caballero Gómez, J., García Bocanegra, I.

\*Awarded communication.

**3. Title:** Estudio seroepidemiológico de *Leishmania infantum* en conejo doméstico (*Oryctolagus cuniculus*) mascota en la provincia de Málaga.

**Congress:** XXII Congreso de Especialidades Veterinarias de AVEPA.

**Place:** Bilbao, Spain.

**Date:** 21-22<sup>nd</sup> April 2023.

**Organizing institution:** AVEPA.

**Type of communication:** Oral.

**Authors:** Vázquez Calero, D. B., Barbero Moyano, J., Cano Terriza, D., González, M., Martínez, R., Castro Scholten, S., Moreno, I., Domínguez, M., Jiménez Martín, D., García Bocanegra, I.

**4. Title:** Circulación del virus de la Hepatitis E en gatos y perros en la provincia de Córdoba

**Congress:** VI Congreso Nacional de GEHEP de la SEIMC.

**Place:** Granada, Spain.

**Date:** 21-23<sup>rd</sup> September 2021.

**Organizing institution:** SEIMC.

**Type of communication:** Poster.

**Authors:** Caballero-Gómez, J., Rivero-Juárez, A., Jurado-Tarifa, E., Jiménez-Martín, D., Jiménez-Ruiz, E., Castro-Scholten, S., López-López, P., García-Bocanegra, I.

**5. Title:** Seroepidemiología de *Toxoplasma gondii* en lagomorfos silvestres y domésticos de España.

**Congress:** III Congreso Veterinaria y Ciencia y Tecnología de los Alimentos

**Place:** Córdoba, Spain.

**Date:** 22<sup>nd</sup> June 2021.

**Organizing institution:** Facultad de Veterinaria, Universidad de Córdoba.

**Type of communication:** Poster.

**Authors:** Castro Scholten, S., Cano Terriza, D., Aguayo Adán, J. A., Vázquez Calero, D., Almería, S., Jiménez Martín, D., Gómez Guillamón, F., García Bocanegra, I.

**6. Title:** Detección de especies de Rickettsia zoonóticas en garrapatas de lagomorfos silvestres en Andalucía

**Congress:** III Congreso Veterinaria y Ciencia y Tecnología de los Alimentos.

**Place:** Córdoba, Spain.

**Date:** 22<sup>nd</sup> June 2021.

**Organizing institution:** Facultad de Veterinaria, Universidad de Córdoba.

**Type of communication:** Poster.

**Authors:** Remesar Alonso, S., Castro Scholten, S., Cano Terriza, D., Díaz, P., Jiménez Ruiz, S., Caballero Gómez, J., Morrondo, P., García Bocanegra, I.

**7. Title:** Seroepidemiología de la paratuberculosis en pequeños rumiantes domésticos en Andalucía.

**Congress:** III Congreso Veterinaria y Ciencia y Tecnología de los Alimentos.

**Place:** Córdoba, Spain.

**Date:** 22<sup>nd</sup> June 2021.

**Organizing institution:** Facultad de Veterinaria, Universidad de Córdoba.

**Type of communication:** Poster.

**Authors:** Jiménez Martín, D., García Bocanegra, I., Risalde, M. A., Isla, J., Castro Scholten, S., Gortázar, C., Paniagua, J., Cano Terriza, D.

**8. Title:** Exposición al virus de la hepatitis E en lagomorfos domésticos y silvestres en Andalucía.

**Congress:** III Congreso Veterinaria y Ciencia y Tecnología de los Alimentos.

**Place:** Córdoba, Spain.

**Date:** 22<sup>nd</sup> June 2021.

**Organizing institution:** Facultad de Veterinaria, Universidad de Córdoba.

**Type of communication:** Poster.

**Authors:** Gómez Caballero, J., Rivero Juárez, A., Castro Scholten, S., Vázquez Calero, D., Cano Terriza, D., Aguayo Adán, J. A., Jiménez Martín, D., García Bocanegra, I.

**9. Title:** Seroprevalencia y factores de riesgo asociados a la infección por toxoplasma gondii en ungulados silvestres de España.

**Congress:** IX Congreso Científico de Investigadores en Formación de la Universidad de Córdoba.

**Place:** Córdoba, Spain.

**Date:** 03-06<sup>th</sup> May 2021.



**Organizing institution:** Universidad de Córdoba.

**Type of communication:** Oral.

**Authors:** Castro Scholten, S., Cano Terriza, D., García Bocanegra, I.

**10. Title:** Seroprevalencia y factores de riesgo asociados a la infección por toxoplasma gondii en ungulados silvestres de España.

**Congress:** V Congreso científico investigadores noveles.

**Place:** Córdoba, Spain.

**Date:** 11<sup>th</sup> November 2021.

**Organizing institution:** Universidad de Córdoba.

**Type of communication:** Oral.

**Authors:** Jiménez Martín, D., Cano Terriza, D., Risalde M. A., Isla, J., Castro Scholten, S., Gortázar, C., García Bocanegra, I.

**11. Title:** *Toxoplasma gondii* en pequeños rumiantes del sur de España.

**Congress:** IV Congreso científico investigadores noveles.

**Place:** Córdoba, Spain.

**Date:** 07<sup>th</sup> November 2019.

**Organizing institution:** Universidad de Córdoba.

**Type of communication:** Oral.

**Authors:** Jiménez Martín, D., Cano Terriza, D., Díaz, J. M., Almería, S., Castro Scholten, S., Dubey, J., García Bocanegra, I.

**12. Title:** Estudio seroepidemiológico de *Toxoplasma gondii* en ovino y caprino de Andalucía.

**Congress:** XXIV Simposium AVEDILA.

**Place:** La Rioja, Spain.

**Date:** 07-08<sup>th</sup> November 2019.

**Organizing institution:** Asociación de Veterinarios Especialistas en Diagnóstico Laboratorial.

**Type of communication:** Poster.

**Authors:** Jiménez Martín, D., García Bocanegra, I., Díaz Cao, J. M., Almería, S., Castro Scholten, S., Jiménez Ruiz, S., Dubey, J., Franco, J. J., Cano Terriza, D.

## **II.C. Dissemination articles**

**1. Authors:** Rios Muñoz, L., González, M., Castro Scholten, S., Agulló Ros, I., Fajardo, T., Caballero Gómez, J.

**Title:** El cerdo como posible reservorio del virus de la hepatitis E de la rara.

**Journal:** SUIS (2024).

**2. Authors:** Barbero Moyano, J., Castro Scholten, S., Martínez, R., González, M., Moreno, I., Domínguez, M., Cano Terriza, D., Jiménez Martín, D., Gómez Guillamón, F., García Bocanegra, I.

**Title:** Estudio del papel epidemiológico de los lagomorfos silvestres como reservorios de *Leishmania infantum* en Andalucía.

**Journal:** iVeterinaria (2023).

### Appendix III. Projects in which the doctoral student participates

Sabrina Castro Scholten has participated in eight and three research and teaching innovation projects, respectively.

#### III.A. Research projects

**1. Improvement of preventive actions to emerging lagoviruses in the Mediterranean basin: development and optimization of methodologies for pathogen detection and control.** REF: PRIMA-S2-11-PCI2019-103698. IP: Carlos Rouco Zufiaurre. Entidad convocante: PRIMA-Partnership for Research and Innovacion in the Mediterranean Area. Entidad financiadora: Ministerio de Ciencia, Innovación y Universidades. Periodo: 2019-2022. Presupuesto: 902.400€ (subproyecto UCO: 95.000€). **Equipo de trabajo.**

**2. Evaluación epidemiológica de reservorios domésticos y silvestres de *Leishmania spp.* en Andalucía.** REF: UCO-FEDER-1264967. IP: Ignacio García Bocanegra. Entidad financiadora: Universidad de Córdoba/Consejería de Economía y Conocimiento de la Junta de Andalucía. Periodo: 2020-2022. Presupuesto: 34.974€. **Equipo de trabajo.**

**3. Proyecto AgroMIS: ceiA3 instrumento estratégico hacia un tejido productivo Agroalimentario Moderno, Innovador y Sostenible: motor del territorio rural andaluz. Sublínea: SL2423.** REF: A1122062E0\_AGROMIS. IP: David Cano Terriza e Ignacio García Bocanegra. Entidad financiadora: Consejería de economía, conocimiento, empresas y universidad. Junta de Andalucía. Periodo: 2022-2022. Presupuesto: 22.500€. **Investigadora colaboradora.**

**4. Avances en el conocimiento de enfermedades en lagomorfos silvestres en ecosistemas mediterráneos de España desde una perspectiva de salud global (LagoHealth).** REF: PID2019-111080RB-C21. IP: Ignacio García Bocanegra. Entidad financiadora: Ministerio de Ciencia e Innovación. Periodo: 2020-2023. Presupuesto: 181.500€. **Equipo de trabajo.**

**5. Evaluación del papel de los lagomorfos silvestres como reservorios de enfermedades zoonóticas transmitidas por vectores en la península ibérica.** REF: P.P. 2023 Submod. 2.4. IP: David Cano Terriza. Entidad financiadora: Universidad de Córdoba (Plan Propio Galileo 2023). Periodo: 2023-2024. Presupuesto: 10.000€. **Equipo de trabajo.**

**6. Evaluación del papel de mamíferos domésticos y silvestres en la epidemiología del SARS-COV-2 en entornos urbanos en Andalucía.** REF: PPG2021-UCOSOCIAL-02. IP: David Cano terriza. Entidad financiadora: Universidad de Córdoba (UCO-SOCIAL-INNOVA). Periodo: 2021-2022. Presupuesto: 4.460€. **Equipo de trabajo.**

**7. Evaluación de las especies silvestres en la epidemiología de las enfermedades emergentes en España (EpiWild).** REF: PPIT\_2022E\_026489. IP: David Cano terriza e Ignacio García Bocanegra. Entidad financiadora: Universidad de Córdoba. Periodo: 2022-2025. Presupuesto: 1.905,49€. **Investigadora colaboradora.**

**8. Evaluación de técnicas de diagnóstico no oficiales en tuberculosis caprina (GoaTBcontrol).** REF: PPIT\_2022E\_026324. IP: David Cano terriza e M<sup>a</sup> Ángeles Risalde Moya. Entidad financiadora: Universidad de Córdoba. Periodo: 2022-2025. Presupuesto: 15.312,85€. **Investigadora colaboradora.**

### **III.B. Teaching Innovation Projects**

**1. Uso del escape room como herramienta de gamificación para reforzar el aprendizaje de técnicas de identificación microbiológica.** REF: 2024-1-3011. IP: David Cano Terriza. Entidad financiadora: Universidad de Córdoba (Modalidad 1. Proyectos de Innovación Docente). Periodo: 2024-2025. **Equipo de trabajo.**

**2. Escape room: una herramienta para reforzar el aprendizaje en el Grado de Veterinaria.** REF: 2023-2-3002. IP: Ignacio García Bocanegra. Entidad financiadora: Universidad de Córdoba (Modalidad 2. Proyectos de Innovación para formación en Innovación Docente). Periodo: 2023-2024. **Equipo de trabajo.**

**3. Uso del eBook como herramienta docente para el desarrollo de contenidos bilingües aplicados a asignaturas de Grado y Postgrado.** REF: 2020-1-3014. IP: Ignacio García Bocanegra. Entidad financiadora: Universidad de Córdoba (Modalidad 1. Proyectos de Innovación Docente). Periodo: 2020-2021. **Equipo de trabajo.**

**Appendix IV. Outreach activities in which the doctoral student participates**

Sabrina Castro Scholten has participated in nine science outreach activities.

**1. Name of the event:** Programa de Orientación Vocacional para el alumnado de Secundaria, Bachillerato y Formación Profesional Superior (OrientaUCO 2024).

**Place:** Córdoba, Spain

**Date:** 23<sup>rd</sup> April 2024.

**Participation as:** speaker

**2. Name of the event:** Día internacional de la mujer y la niña en la ciencia.

**Place:** Córdoba, Spain

**Date:** 11<sup>th</sup> February 2024.

**Participation as:** speaker

**3. Name of the event:** Hipatia-UCO de mentoría universitaria para alumnado con altas capacidades.

**Place:** Córdoba, Spain

**Date:** 02<sup>nd</sup> May 2023.

**Participation as:** speaker

**4. Name of the event:** Programa de Orientación Vocacional para el alumnado de Secundaria, Bachillerato y Formación Profesional Superior (OrientaUCO 2023).

**Place:** Córdoba, Spain

**Date:** 25<sup>th</sup> April 2023.

**Participation as:** speaker

**5. Name of the event:** Día internacional de la mujer y la niña en la ciencia.

**Place:** Córdoba, Spain

**Date:** 11<sup>th</sup> February 2023.

**Participation as:** speaker

**6. Name of the event:** La Noche Europea de los Investigadores

**Place:** Córdoba, Spain

**Date:** 30<sup>th</sup> September 2022.

**Participation as:** speaker

**7. Name of the event:** La Noche Europea de los Investigadores

**Place:** Córdoba, Spain

**Date:** 24<sup>th</sup> September 2021.

**Participation as:** speaker

**8. Name of the event:** Ingenios en ruta.

**Place:** Córdoba, Spain

**Date:** 05<sup>th</sup> March 2021.

**Participation as:** speaker

**9. Name of the event:** La Noche Europea de los Investigadores

**Place:** Córdoba, Spain

**Date:** 27<sup>th</sup> November 2020.

**Participation as:** speaker



