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SHORT COMMUNICATION

Seroprevalence responses to RHDV variants in wild European rabbits: evidence of resilience in the Iberian Peninsula

Juan Antonio Aguayo-Adán[1] · Simone Santoro[2] · Rafael Villafuerte[3] · Miguel Delibes-Mateos[3] · Francisca Castro[4,5] · Tereza Almeida[6,7] · Joana Abrantes[6,7,8] · Carlos Rouco[9]

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Abstract

The emergence and spread of the main genotypes of *Lagovirus europaeus* (GI.1 and GI.2), the rabbit haemorrhagic disease virus (RHDV) have had severe consequences for the conservation of European rabbit (*Oryctolagus cuniculus*), a keystone species within the Iberian Mediterranean ecosystem. Understanding seroprevalence in wild rabbit populations in response to RHDV outbreaks is crucial for estimating the proportion of individuals capable of surviving such events. This study investigates the serological and demographic responses to the circulation of GI.1 and GI.2 genotypes by analysing data from 18 wild rabbit populations surveyed in 2002 and 2021, approximately a decade after each strain's emergence. Results show a significantly higher seroprevalence during the GI.2 genotypes period (0.58 vs. 0.44). However, our analysis did not reveal any significant relationship between seroprevalence and population abundance after either outbreak. These findings suggest resilience in wild rabbit populations to RHDV, with critical implications for disease management and conservation. The study stresses the need for continued monitoring to mitigate RHDV's impact and support the preservation of rabbit populations and associated ecosystems.

Keywords Antibodies seroprevalence · Calicivirus · Hunting · Lagovirus · Monitoring · *Oryctolagus cuniculus* · Population abundance

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✉ Carlos Rouco
crouco@us.es

Juan Antonio Aguayo-Adán
jaguayoadan@gmail.com

Simone Santoro
simonesantoro77@gmail.com

Rafael Villafuerte
rafael.villafuerte@csic.es

Miguel Delibes-Mateos
mdelibes@iesa.csic.es

Francisca Castro
francisca.castro@uco.es

Tereza Almeida
tjvalmeida@cibio.up.pt

Joana Abrantes
jabrantes@cibio.up.pt

[1] Departamento de Botánica, Ecología y Fisiología Vegetal, Área de Ecología, Universidad de Córdoba, Córdoba, Spain

[2] Departamento de Ciencias Integradas, Facultad de Ciencias Experimentales, Universidad de Huelva, Huelva, Spain

[3] Instituto de Estudios Sociales Avanzados (IESA-CSIC), Campo Santo de los Mártires 7, Córdoba 14004, Spain

[4] Departamento de Didácticas Específicas Avda. San Alberto Magno s/n, Universidad de Córdoba, Córdoba 14071, Spain

[5] UCO-CSIC Unidad Asociada "Sociedad, Ecología y Gestión del Medio Ambiente", Universidad de Córdoba, Córdoba, Spain

[6] CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Campus de Vairão, Universidade do Porto, Vairão 4485-661, Portugal

[7] BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, Vairão 4485-661, Portugal

[8] Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Porto 4099-002, Portugal

[9] Departamento Biología Vegetal y Ecología, Área de Ecología, Universidad de Sevilla, Sevilla, Spain

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Introduction

Rabbit haemorrhagic disease (RHD) is caused by a highly contagious and often lethal virus belonging to the Caliciviridae family, known as rabbit haemorrhagic disease virus (RHDV). It has two distinct pathogenic genotypes, GI.1 and GI.2, each presenting unique challenges to domestic and European rabbit populations (Santoro et al. 2023). In addition, GI.2 constitutes a significant threat to other leporid species worldwide (Rouco et al. 2020; Asin et al. 2024). Continuous viral monitoring in wild animals allows for early detection, understanding prevalence, and assessment of population susceptibility (Jia et al. 2020). This system is crucial for implementing timely and effective control or mitigation measures. Antibodies' prevalence within wild populations is a key variable for studying disease dynamics, providing a straightforward way to assess RHD immunity status in rabbit populations (Artois et al. 2009).

In the Iberian Peninsula, GI.1 was first identified in the late 1980s, causing severe haemorrhagic symptoms in infected rabbits (Abrantes et al. 2012; Aguayo-Adán et al. 2022). GI.2 genotype emerged in the early 2010s in France, displaying distinct genetic, pathogenic and epidemiologic characteristics (G et al. 2011, 2013), and spreading worldwide extremely fast (Rouco et al. 2019). The impact of these two genotypes on rabbit populations in the species native range added significant complexity to the epidemiology of RHD in this area, despite the apparently rapid replacement of GI.1 strains by GI.2 (Lopes et al. 2014; Mahar et al. 2018). In fact, this replacement occurred so swiftly that the two variants scarcely coexisted, and their current circulation patterns are practically independent. It is generally assumed that, since the emergence of GI.2, the circulation of GI.1 in the Iberian Peninsula has either ceased entirely or persists at extremely low levels.

Understanding the prevalence and distribution of GI.1 and GI.2 is crucial for estimating the proportion of individuals capable of surviving an epidemic, therefore, monitoring these variants within the rabbit's native range is essential. The disease poses a significant threat to wild rabbit populations, potentially disrupting ecosystems and affecting predator-prey dynamics (Monterroso et al. 2016). This is especially important in the Iberian Peninsula, where endangered predators, like the Iberian lynx (*Lynx pardinus*), the imperial eagle (*Aquila adalberti*) and more than 30 other predator species rely on wild rabbits (Delibes-Mateos et al. 2008). It is also worth mentioning that the species was reassessed in 2019 as Endangered (EN) within its native range by the International Union for Nature Conservation, principally because of the decline caused by GI.2 emergence (Villafuerte and Delibes-Mateos 2019). Additionally, RHD poses a significant risk to the commercial rabbit farming industry, where outbreaks can lead to substantial economic losses (Dalton et al. 2014). Moreover, RHD raises concerns about the potential transmission of the virus to other susceptible species (Asin et al. 2024). Therefore, increasing our knowledge of outbreaks may aid in the design of effective measures to protect rabbit populations and mitigate the risk of spillover to other wildlife species.

This study utilises data from wild rabbit populations monitored several years after both GI.1 and GI.2 outbreaks (i.e. 2002 and 2021). When GI.1 first emerged, it caused sharp population crashes across the Iberian Peninsula (Abrantes et al. 2012), including local extinctions. As a result, it was only possible to survey medium to high-abundant populations located within hunting estates, because these were more likely to persist through both outbreaks, which ensured consistent monitoring. Through this approach, we aimed to determine whether there were differences in antibody prevalence before and after the GI.2 epidemic within the same rabbit populations, in addition to exploring whether these prevalence rates were related to rabbit abundance.

Material and methods

Study sites

The selection of sampling sites was based on a previous study conducted in 2002 in central-southern Spain, which had been surveyed for rabbit abundance and were chosen for their environmentally favourable conditions for rabbits (see Delibes-Mateos et al. 2008). This means that high-rabbit numbers were expected in those areas before conducting the surveys. In those places, rabbit abundance was estimated through walking surveys in which indexes of rabbit abundance (e.g. rabbit latrines) were counted (see below). In addition, when possible, biological samples were collected from rabbits killed by hunters (see below). For this study, we selected 18 localities among those surveyed in 2002, before the GI.2 outbreak. Our selection was mostly based on the availability of rabbit samples from the 2002 survey. These localities were resurveyed in 2021, approximately 10 years after the GI.2 outbreak (Fig. 1). Given the epidemiological context described in the Introduction, particularly regarding the progression of GI.1 and GI.2 outbreaks in the Iberian Peninsula, it is reasonable to associate the seroprevalence data from 2002 with GI.1, and that from 2021 with GI.2. All selected localities were characterised by small game hunting grounds holding medium to high-abundant rabbit populations. The habitat consisted of a mixture of Mediterranean vegetation with some nearby agro-systems. In all these localities, 15–25 rabbits shot during regular hunting game activity were collected on each site during the surveyed

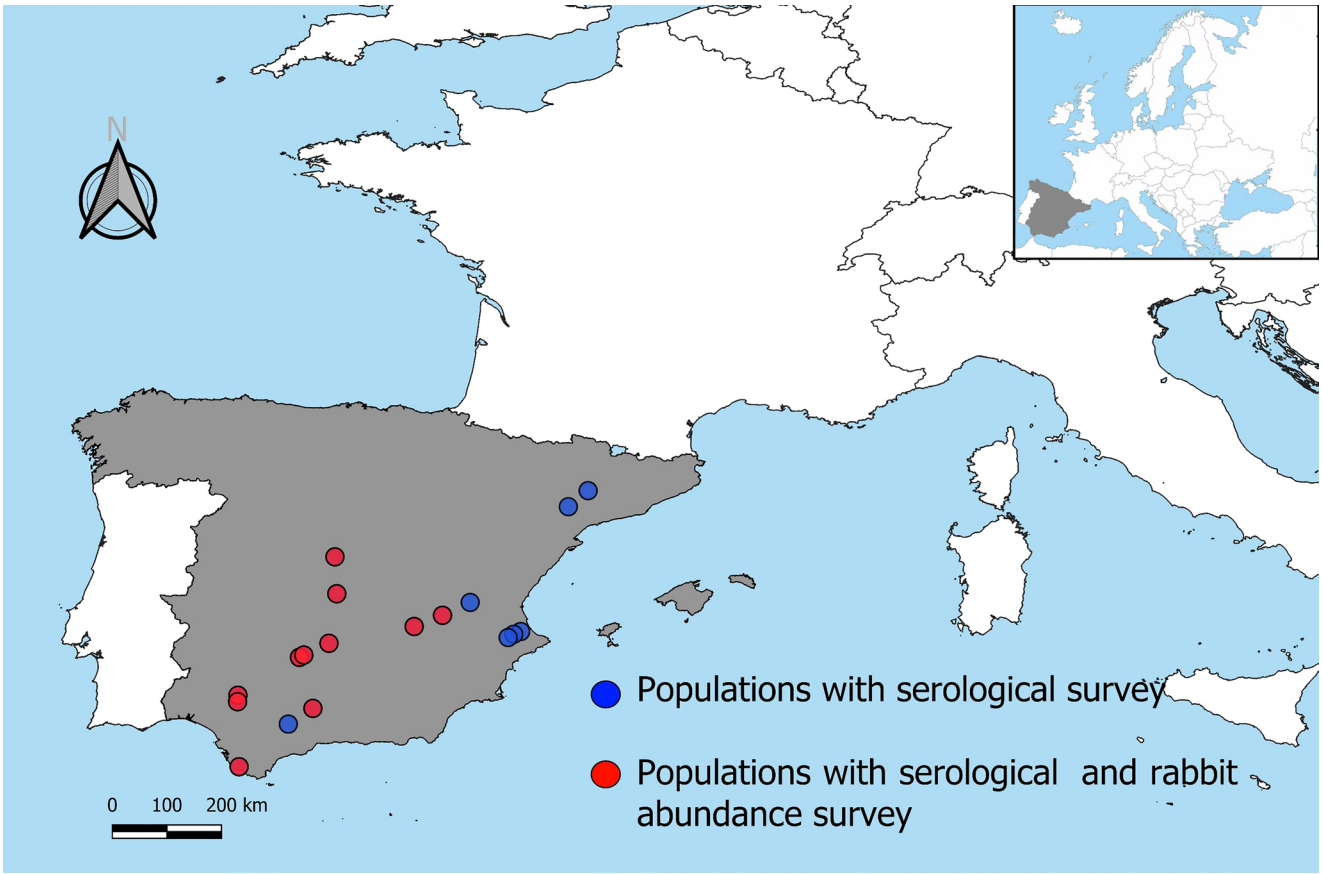

Fig. 1 Locations of wild rabbit populations sampled in 2002 and 2021 in Spain. Rabbit populations where seroprevalence surveys were conducted are marked in blue, while populations where both serological surveys and abundance assessments were performed are marked in red

year. All animals were sexed, weighed, and aged in two age classes, when possible. Rabbits were classified as adults when their distal epiphysis of the ulna was completely ossified and their body mass was > 800 g (> 7 months; Rouco et al. 2018). Blood samples were taken from the thoracic cavity (1 mL in Eppendorff tubes), left to coagulate at room temperature, and centrifuged for 5 min at 700 G. The sera were extracted and frozen (−20 °C) until further analysis.

Rabbit abundance

Four-kilometre transects were walked by two observers in areas favourable to rabbits, mainly those ecotones between Mediterranean scrubland and pastureland or cropland (Delibes-Mateos et al. 2008). We used the total number of latrines counted per 4 kilometres transect as the abundance index. This is a standard method for estimating the abundance of European wild rabbits and assessing the potential impact of RHD (Rouco et al. 2021; Cabezas-Díaz and Virgós 2022). Rabbit latrines abundance could only be recorded in 11 independent localities in central-southern Spain during summer (i.e. June and July) 2002 and again in summer 2021 (Fig. 1).

Serological analysis

The serological analysis of the rabbit sera was performed using an indirect ELISA assay, which works to find anti-RDHV antibodies from both viral variants. GI.1 or GI.2 derived virus-like particles (VLPs) (Bárcena et al. 2015), were diluted in PBS buffer (pH 9.5) and incubated overnight at 4 °C in Nunc MaxiSorp ELISA plates at 100 ng/well. We blocked the plates with 5% non-fat milk in PBS, which was then incubated with the plasma at a dilution of 1/200. After that we used a goat anti-rabbit IgG-HRP antibody at dilution 1/4,000. All incubation steps were carried out for 1 h at 37 °C, followed by washing three times with PBS-0.05% Tween. We then added the substrate solution (3,3',5,5'-tetramethylbenzidine; Abcam) and the reaction was stopped using 50 µL of 1 M phosphoric acid and read at 450 nm after 5–8 minutes. We considered that the sera were positive when the value of the absorbance was higher than 0.2 OD units above background.

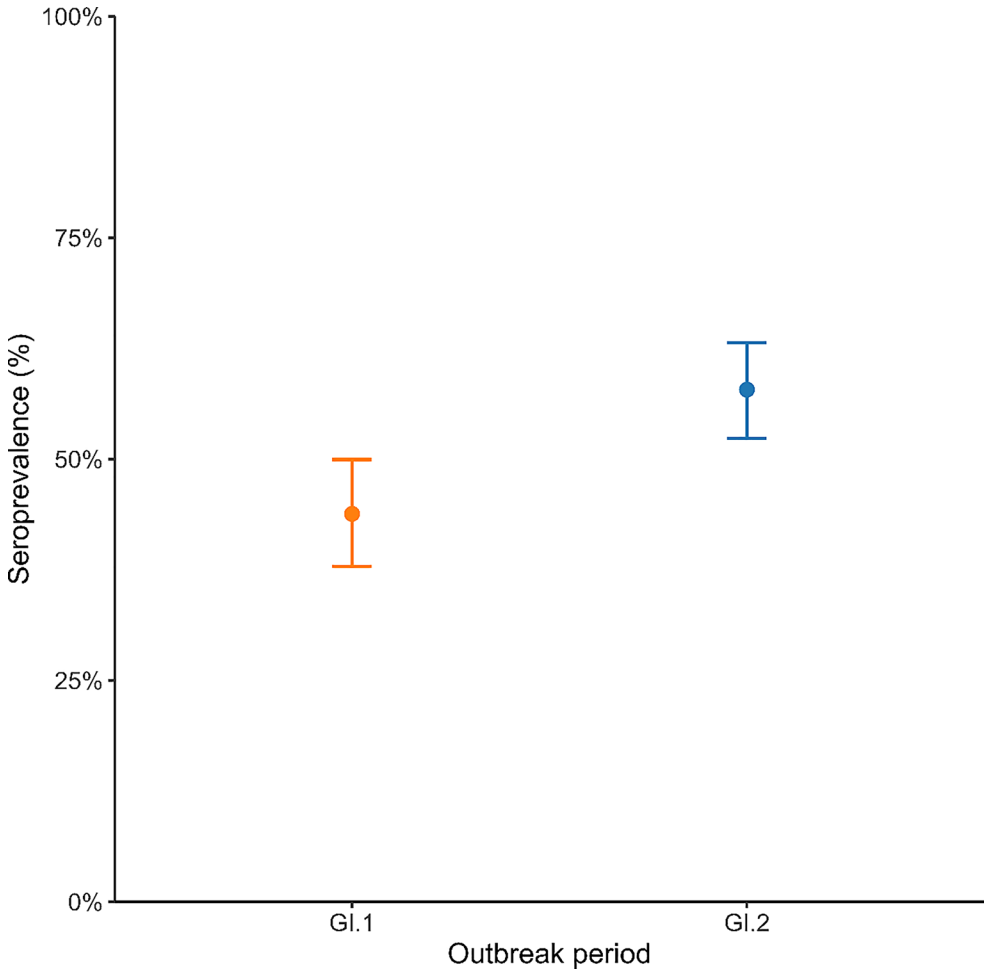

Fig. 2 Estimated seroprevalence in rabbits against RHDV in 2002 (i.e. GI.1) and 2021 (i.e. GI.2). Estimates are marginal predictions from a binomial model that includes period and location as fixed effects. Error bars represent 95% confidence intervals

Statistical analysis

Statistical analyses were carried out using the statistical software R, version 4.3.3 (R Core Team 2024). The R code is available as Supporting Information (Appendix 1). We used abundance and seroprevalence data collected from rabbit populations in 18 locations during the GI.1 outbreak (2002) and the GI.2 outbreak (2021) to assess differences in antibody prevalence between outbreaks and to explore the relationship between seroprevalence and rabbit abundance. For that purpose, we applied generalised linear models (GLMs) using the *glmmTMB* function in R (package glmmTMB, Brooks et al. 2017). For both rabbit abundance and seroprevalence, we modelled the effect of the outbreak period (categorical predictor: GI.1 and GI.2) while controlling for location. Abundance was modelled using a lognormal distribution (log link, n = 21), and seroprevalence (the proportion of individuals with antibodies) was modelled using a binomial distribution (logit link, n = 35). Marginal means for the period effect were obtained using the emmeans package, back-transformed to their respective scales, and pairwise comparisons were conducted to assess differences between the outbreak periods. Finally, to examine whether abundance affected seroprevalence, separate binomial GLMs were fitted for each outbreak period (n = 10 for GI.1 and n = 11 for GI.2). Residual diagnostics were performed using the *simulateResiduals* function from the DHARMa package (Hartig 2024) to ensure model fit.

Results

The average abundance (including 95% CI) of wild rabbits 14 years after GI.1outbreak (19.6 latrines/km, 11.9–32.1) was similar (log scale $\beta = 0.05$, SE = 0.25, $p = 0.83$) to that estimated 10 years after GI.2 outbreak (18.1 latrines/km, 13.3–25.7). In contrast, the average seroprevalence during the GI.2 period (mean: 0.58, 95% CI: 0.52–0.63) was significantly higher than during the GI.1 period (0.44, 0.38–0.5) (logit scale $\beta = -0.56$, SE = 0.17, $p < 0.001$) (Fig. 2). The association between abundance and seroprevalence was not statistically significant during either the GI.1 ($\beta = 0.002$, SE = 0.002, $p = 0.14$; Fig. 2) or GI.2 ($\beta = 0.002$, SE = 0.003, $p = 0.59$) periods.

Discussion

Our results show a significantly higher seroprevalence of rabbits to RHDV during the GI.2 sampling period than during the GI.1 sampling period. Still, no significant difference in rabbit abundance was observed between both surveys. The observed variation in seroprevalence between the two sampling periods may reflect the wild European rabbit's resilience and capacity to develop resistance more than intrinsic differences between the viral strains themselves. Both GI.1 and GI.2 variants have significantly impacted wild rabbit populations, although the severity of their effects may have varied geographically. During the first wave of GI.1, a widespread population decline was reported (Santoro et al. 2023), with mortality rates reaching approximately 90% (Abrantes et al. 2012). Notably, Capucci et al. (2017) documented increased pathogenicity of GI.2 strains isolated in 2014 and 2015, with mortality rates around 80%, approaching those typically attributed to GI.1. This evidence underscores that despite the presence of two distinct variants, both have exerted strong selective pressures on wild rabbit populations.

This increase in seroprevalence during the GI.2 period could also reflect a stronger immune profile among surviving adults, given that GI.2 more severely affects young rabbits (Dalton et al. 2012). Those surviving early exposure may maintain antibodies longer, thus raising overall seroprevalence without implying lower virulence.

In relation to the serological assay used (Bárcena et al. 2015), although partial cross-reactivity exists, it can distinguish between antibodies against GI.1 and GI.2. In particular, Bárcena et al. (2015) demonstrated that serological responses were consistently stronger for the homologous than for the heterologous antigen, supporting the assay's capacity to discriminate between GI.1 and GI.2 antibodies. However, the assay does not include virus-like particles (VLPs) for non-pathogenic lagoviruses and therefore cannot reliably distinguish or differentiate antibodies induced by these strains. While non-pathogenic lagoviruses often induce cross-reactive antibodies that may bind to GI.1 or GI.2 VLPs due to shared epitopes (G et al. 2011), the resulting signals are typically weak and should be interpreted with caution. Cross-protection conferred by non-pathogenic strains has been shown to vary significantly, ranging from complete protection with RCV (Capucci et al. 1996), to partial with RCV-A1 (Strive et al. 2010), and no protection with strain 06–11 (G et al. 2011). Therefore, the role of non-pathogenic strains in the epidemiology of RHDV in wild rabbit populations, particularly in the Iberian Peninsula, remains complex and incompletely understood.

Regarding the potential relationship between seroprevalence and rabbit abundance, previous studies on GI.1 suggested that RHD tended to become endemic in high-density populations. In such populations, the virus circulated among young rabbits, which were also not killed up to two months of age, leading to high seroconversion rates, thereby helping maintain population numbers. Conversely, in lower-density populations, delayed epidemic outbreaks among older rabbits could drive populations down to a lower equilibrium level (Calvete 2006). Interestingly, the most abundant populations in the Iberian Peninsula have remained relatively numerous, albeit at lower levels than before disease outbreaks (Delibes-Mateos et al. 2008). It has been suggested that larger populations are more likely to contain individuals that develop antibodies and thus resistance to the disease. For example, in Australia, consistently high seroprevalence (around 60%) against GI.1 was observed during the summer, coinciding with the period of rabbit population growth (Mutze et al. 2015). Similarly, in Spain, a positive correlation between GI.1 antibody prevalence and wild rabbit abundance was documented (Cotilla et al. 2010). Such effects of abundance are important considerations when interpreting disease impact from seroprevalence data. Cotilla et al. (2010) further suggested that serological monitoring during late spring and summer could help track these processes, observing that rabbit populations increased when seroprevalence exceeded 40%, but declined when it was lower. However, our study did not reveal a significant relationship between rabbit abundance and seroprevalence, so we cannot confirm or refute whether higher abundance favours increased seroprevalence. The lack of a relationship between abundance and seroprevalence in our study may be due to several non-exclusive reasons. For example, the nature of the sampled areas, primarily hunting grounds, where rabbit populations were relatively abundant. Areas with high rabbit abundance may recover more effectively from the impact of the virus than areas with lower abundance (Calvete 2006). This was consistent with the observation that in several low-abundance hunting areas where samples were collected in 2002, no rabbits were available for hunting in 2021, indicating local population depletion (C. Rouco, pers. obs.). Moreover, the small sample size considered in our study may have influenced the results. Our aim was to compare seroprevalence in a larger number of locations. Still, in many of these hunting grounds sampled more than 20 years ago, we could not collect rabbit samples during the second period because rabbit populations had collapsed. Thus, we resampled only medium to high abundant populations, leading to a bias in the data and overrepresenting areas with higher rabbit abundance. This could also explain the lack of a significant difference in abundance between the two periods. This brings us to another issue: saturation in latrine counts at high abundance may further complicate matters. As rabbit abundance increases, the variability in

the number of latrines also rises, likely influenced by factors such as habitat type and environmental conditions. For example, Palomares (2001) found that latrine counts were less reliable in Mediterranean scrubland habitats, where latrine numbers were unexpectedly low compared to rabbit abundance, highlighting the importance of considering habitat characteristics when using this method to estimate population size. This variability may make difficult comparing rabbit abundances across different dates or locations using latrine counts. Cabezas-Díaz and Virgós (2022) noted that "the relationship between the number of latrines and abundance reached a plateau when the number of latrines exceeded 70 per km.". This issue affects over 50% of the sites surveyed by those authors. Additionally, extrinsic factors, such as other diseases like myxomatosis, variations in hunting pressure among years, or weather conditions (e.g., Rödel and Dekker 2012), may cause short-term fluctuations in rabbit abundance, potentially blurring or masking any association between current rabbit abundance and seroprevalence.

One inherent limitation of our study is the reliance on seroprevalence data from single sampling years (2002 and 2021) to represent the antibody response before and after the emergence of GI.2. Viral circulation and outbreak intensity in wild rabbit populations can vary considerably among years due to environmental conditions, host population dynamics, and other epidemiological factors. Consequently, seroprevalence values from individual years may not fully capture this temporal variability. However, due to the scarcity of continuous long-term data, these years were selected to broadly reflect the epidemiological context: 2002 represents the period when GI.1 was predominantly circulating, while 2021 corresponds to a time when GI.2 had become the dominant genotype in the Iberian Peninsula (e.g. Rouco et al. 2019). Thus, while acknowledging the limitations of single-year sampling, these time points may provide a reasonable approximation of the predominant RHDV variant circulating during each period. Nonetheless, future studies incorporating multi-year longitudinal data would be invaluable to characterise interannual fluctuations in virus circulation and host immunity more precisely.

In conclusion, rabbit abundance in the sampled hunting areas was similar ~10 years after each RHDV outbreak, likely due to the populations' ability to partially recover. Initiatives such as the ongoing IBERCONEJO Life project (https://www.iberconejo.eu/en/home/) may provide valuable long-term and large-scale ecological and serological data collection and analyses. The continuation of this evidence-based approach will be essential for mitigating the impact of future outbreaks, thereby supporting the conservation of affected rabbit populations.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42991-025-00532-9.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required, as no animals were killed specifically for this study. Samples were collected from wild rabbits legally hunted during the official hunting season in full compliance with Spanish regulations. No ethical approval by an Institutional Animal Care and Use Committee was deemed necessary.

Conflict of interest Authors declare no conflicts of interests. One of the authors of this article, C. Rouco, is a member of the editorial board of Mammalian Biology.

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