European Journal of Wildlife Research (2025) 71:90
https://doi.org/10.1007/s10344-025-01966-9

RESEARCH

Toxoplasmosis in the European brown hare: pathology, strain genotyping and population exposure within the Iberian distribution range

Josep Estruch[1] · Martha Y. Salas-Fajardo[2] · Rafael Calero-Bernal[2] · Sabrina Castro-Scholten[3] · Ignacio García-Bocanegra[3,4] · Vanesa Alzaga[5] · Santiago Lavín[1] · Carlos Rouco[6] · Roser Velarde[1]

Received: 20 January 2025 / Revised: 11 June 2025 / Accepted: 25 July 2025

Abstract

The European brown hare (*Lepus europaeus*), widely distributed across Eurasia, is a key prey species for many vertebrates. *Toxoplasma gondii*, a globally distributed zoonotic protist, can infect all homoeothermic animals, including lagomorphs. Hares are highly susceptible, with sporadic toxoplasmosis cases reported in central and northern Europe, but its impact on southern populations remains unclear. Lethal toxoplasmosis was confirmed in two European brown hares from Catalonia, northeastern Spain (2018 and 2021). Samples were collected for histopathology and molecular analyses. Necropsy showed good body condition but revealed splenomegaly, liver necrosis, and pulmonary oedema. Microscopically, necrotizing multiorgan lesions with protozoan-like structures were observed. *Toxoplasma gondii* DNA was detected in spleen, with microsatellites-genotyping identifying a type II specific variant of the ToxoDB#3 genotype. In addition, 231 serum samples from passive and active surveillance (2013–2023) from Catalonia ($n=205$) and other northern Spanish regions ($n=28$) were tested for anti-*T. gondii* IgG antibodies using an indirect ELISA kit, showing a seroprevalence of 1.73% (4/231; 95% CI: 0.05–3.41). These are the first clinical toxoplasmosis cases reported in European brown hares from southern Europe, highlighting their unusual susceptibility. The low seroprevalence, consistent with Mediterranean reports, raises questions about exposure frequency. Further studies are needed to assess the impact of *T. gondii* on European brown hare populations.

Keywords Apicomplexa · Lagomorphs · Mortality · Seroprevalence · *Toxoplasma gondii*

✉ Josep Estruch
josep.estruch@uab.cat

Martha Y. Salas-Fajardo
marthays@ucm.es

Rafael Calero-Bernal
r.calero@ucm.es

Sabrina Castro-Scholten
sabrina1996cs@gmail.com

Ignacio García-Bocanegra
nacho.garcia@uco.es

Vanesa Alzaga
valzagag@gan-nik.es

Santiago Lavín
santiago.lavin@uab.cat

Carlos Rouco
crouco@us.es

Roser Velarde
roser.velarde@uab.cat

[1] Wildlife Ecology & Health group (WE&H) and Servei d'Ecopatologia de Fauna Salvatge (SEFaS), Departament de Medicina i Cirurgia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona (UAB), Bellaterra 08193, Spain

[2] Animal Health Department, Faculty of Veterinary Sciences, SALUVET, Complutense University of Madrid, Ciudad Universitaria s/n, Madrid 28040, Spain

[3] Departamento de Sanidad Animal, Grupo de Investigación en Sanidad Animal y Zoonosis (GISAZ), UIC Zoonosis y Enfermedades Emergentes ENZOEM, Universidad de Córdoba, Córdoba 14071, Spain

[4] CIBERINFEC, ISCIII– CIBER de Enfermedades Infecciosas, Instituto de Salud Carlos III, Madrid 28029, Spain

[5] Gestión Ambiental de Navarra (GAN-NIK), Pamplona 31015, Spain

[6] Departamento Biología Vegetal y Ecología, Área de Ecología, Universidad de Sevilla, Sevilla 41012, Spain

Published online: 06 August 2025

Introduction

Toxoplasma gondii (phylum Apicomplexa) is a zoonotic protozoan distributed worldwide and capable of causing clinical infection and mortality to a wide range of animal species. Wild and domestic felids act as definitive hosts, shedding environmentally resistant oocysts in their faeces, whilst all homoeothermic animals are virtually susceptible to infection. Ingestion of water or food contaminated with the sporulated oocysts, consumption of cysts present in prey's tissues or vertical transmission from mother to the foetus may represent the three major vias of acquiring the infection (Dubey 2021). Humans can also get infected by consumption of uncooked or undercooked meat and unpasteurised milk, direct contact with the parasite, vertical transmission or through blood products and organ transplants. Therefore, toxoplasmosis constitutes a significant Public Health concern (Tenter et al. 2000; Hill and Dubey 2016; Machado et al. 2019; Stelzer et al. 2019). Toxoplasmosis is the second high-ranked parasitic foodborne disease in Europe (van der Giessen et al. 2021). Beyond its role as a major cause of reproductive failure through congenital infection, the disease is associated with characteristic lesions, particularly in immunocompromised individuals, including alterations in the central nervous system or ocular disease (Strang et al. 2020; Arora et al. 2022).

The European brown hare (*Lepus europaeus*) is a lagomorph historically abundant across Europe. It serves as a crucial prey species for many vertebrates and is widely hunted and consumed by humans in various countries (Viviano et al. 2021). The species can be considered the most widespread among hares in the European continent, occupying numerous habitats, including northeastern Spain, its southernmost natural border (Gortazar et al. 2009). In recent decades, the European brown hare has experienced declines throughout its range (Edwards et al. 2000; Hacklander and Schai-Braun 2019). Contributing factors include habitat loss and fragmentation, predation, poor hunting management, climatic factors, and transmissible diseases (Lavazza and Cooke 2018; Smith et al. 2005; Treml et al. 2007; Wibbelt and Frölich 2005). In the Iberian Peninsula, the European brown hare coexists with two other hare species that occupy distinct geographical niches, although occasionally overlapping. These are the Iberian hare (*Lepus granatensis*), the most abundant and widespread species, and the Broom hare (*Lepus castroviejoi*), which has a very restricted range in northern Spain (Estonba et al. 2006).

Hares are known to be highly susceptible to primary infection by *T. gondii* (Gustafsson et al. 1997; Sedlák et al. 2000; Lindsay and Dubey 2020). In fact, even if related literature is scarce, some natural fatal systemic toxoplasmosis cases have been described in European brown hares from northern and central Europe (Jokelainen et al. 2011; Račka et al. 2021). Regarding the Mediterranean countries, although the circulation of the parasite has been demonstrated in European brown hares from France, Italy, and Greece (Aubert et al. 2010; Ebani et al. 2016; Tsokana et al. 2019), no evidence of clinical infection associated with mortality has been recorded to date. Just one survey focusing on the prevalence of *T. gondii* in the Iberian hare has been conducted so far, showing differences among years and age groups (Fernández-Aguilar et al. 2013).

In our study, we describe clinical *T. gondii* infections in two wild European brown hares from northeastern Spain, providing insights into the associated pathology and the genotype of the infecting strain. Additionally, we present a comprehensive serosurvey to assess the exposure of local hare populations to the parasite.

Materials and methods

Necropsy and sample collection

Between 2013 and 2023, a total of 123 dead-found European brown hares, collected by hunters and Rural Agents, were studied as part of the Wildlife Passive Surveillance Program in Catalonia. A systematic necropsy and histopathological investigation were performed, conducting also bacteriological, virological and other laboratory analyses when necessary for the final diagnosis. Toxoplasmosis was then confirmed in two individuals recovered from Montferrer i Castellbò (LE18003; Lleida, August 2018) and Santa Coloma de Farners (LE21038; Girona, November 2021). As part of the diagnosis routine, tissue samples from the spleen, liver, lung, brain, heart, skeletal muscle, and kidney were collected and deposited in 4% neutral buffered formaldehyde for 24 h. After fixation, the tissues were trimmed, embedded in paraffin, and stained with haematoxylin and eosin for histological examination. Samples from the spleen, brain, heart or skeletal muscle, and blood were frozen at -20 °C for subsequent analyses.

Simultaneously, 231 blood samples were collected from European brown hare carcasses for serological survey, either directly from the heart or thoracic fluid, by using sterile equipment. These included samples from individuals found dead due to causes unrelated to toxoplasmosis as part of the Wildlife Passive Surveillance Program in Catalonia ($n=121$), those obtained in hunting campaigns during the study period ($n=84$), and samples from neighbouring northern regions, comprising Navarre ($n=27$) and Castile and Leon ($n=1$), collected through passive surveillance in 2020 and 2022 (Fig. 1, Appendix S1: Table S1). Hare carcasses were previously weighed, sexed and categorised into two

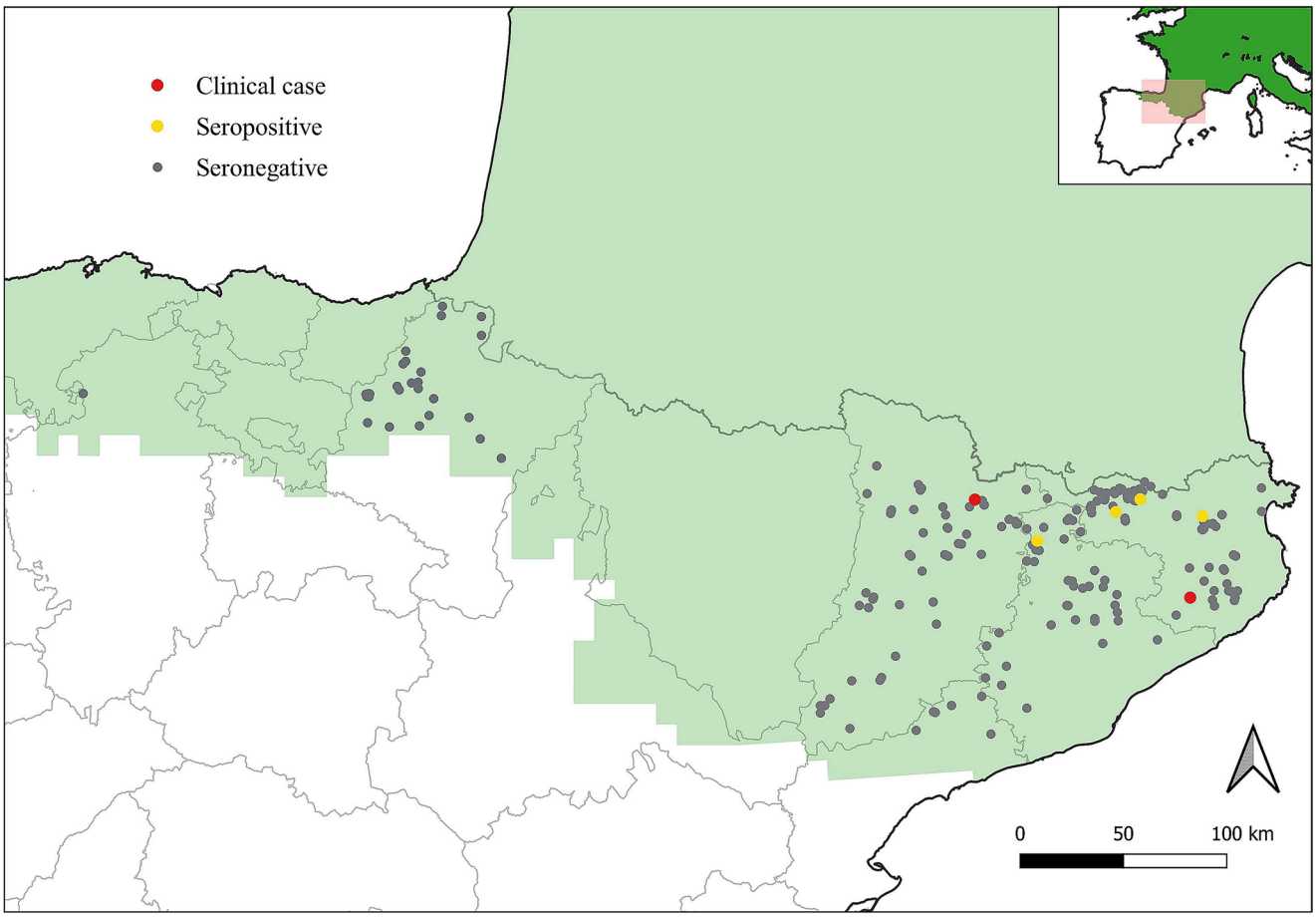

Fig. 1 Geographic location of the studied European brown hares (*Lepus europaeus*) for the detection of *T. gondii* exposure within the northern regions of Catalonia, Navarre and Castile and Leon of Spain, during 2013 and 2023. The green-shadowed area informs on the habitat distribution of the studied species in the Iberian Peninsula and Europe (Inset). Each dot represents a sampled individual hare

age groups (adults and juveniles), following radium-ulna ossification patterns (Broekhuizen and Maaskamp 1981) (Appendix S1: Table S1). Blood samples were centrifuged at 3500 rpm to obtain serum, which was subsequently frozen at -20 °C for further analysis.

Parasite direct detection and genotyping

Each tissue sample ($n=6$, from 0.6 to 4.5 g) from the suspected toxoplasmosis cases was artificially digested using acid-pepsin method (Dubey 1998) and a portion (50 mg) of subsequent pellets were transferred to Maxwell® 16 Mouse Tail DNA Purification Kit (Promega, WI, USA) to obtain *T. gondii* DNA. Then, *Toxoplasma*-DNA detection was performed by single-tube nested-PCR, targeting the ribosomal internal transcriber spacer 1 (ITS-1) region (Castaño et al. 2014). PCR products of 227 bp size were visualized under UV light by 1.8% (w/v) agarose gel electrophoresis stained with GelRed® Nucleic Acid Stain (Biotium, CA, USA). Amplicon identity was confirmed after Sanger sequencing at the Center for Genomic Technologies, Complutense University of Madrid (Spain). Then, sequences were edited and aligned using Clustal Omega from BioEdit v.7.0.5.3 (Hall 1999). Consensus sequences were compared to NCBI database entries via the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To detect the parasite load in the samples that tested positive to ITS-1 and to select those with the highest values for genotyping, a duplex qPCR reaction targeting the 529 repetitive element (529RE) and an internal amplification control (IAC) (Homan et al. 2000) was performed using a 7500 FAST real-time PCR system (Applied Biosystems, CA, USA). RH strain DNA (10 ng/ml) and nuclease-free water were used as positive and negative controls, respectively.

Aiming genetic characterization of the involved strains, two *T. gondii*-positive DNA samples yielding optimal low Ct values by qPCR (Ct = 21.4, spleen of LE21038; Ct = 19.4, muscle of LE18003) were selected and subjected to a multinested PCR-RFLP based on 11 genetic markers (SAG1, SAG2, altSAG2, BTUB, L358, PK1, Apico, GRA6, C22-8,

C29-2, and SAG3)(Su et al. 2010) and to microsatellite length fragment analysis, based on 15 genetic markers (N61, B18, M33, M48, TUB2, N83, XI.1, N82, TgM-A, W35, IV.1, B17, N60, M102, AA) (Ajzenberg et al. 2010; Joeres et al. 2023). Fragment analysis was carried out using capillary electrophoresis on an ABI 3730 DNA Analyzer (Applied Biosystems, MA, USA) at the Center for Genomic Technologies, Complutense University of Madrid (Spain). Fragment sizes were determined with PeakScanner v.1.0 software (ABI PRISM, Applied Biosystems, USA).

In addition, multilocus sequencing typing (MLST) of *CS3* and *GRA7* markers, which are informative about the potential virulence of the strain (Bottós et al. 2009; Pena et al. 2008), were amplified and subjected to bidirectional Sanger sequencing using internal primers at the Center for Genomic Technologies, Complutense University of Madrid (Spain); sequence curation and allele identification was carried out as previously reported (Fernández-Escobar et al. 2022).

Serological analysis

All sera samples were thawed, refrigerated overnight at 4 °C and tested the following day using a commercial ID Screen® Toxoplasmosis Indirect Multi-species ELISA kit (IDVet, Grabels, France), following the manufacturer's instructions. Optical densities (OD) were measured at 450 nm using a spectrophotometer. Samples with an OD value greater than 0.45 were considered positive, according to the manufacturer's guidelines.

Results

Post-mortem exam of the two cases, including macroscopic and histopathological analyses, revealed lesions compatible with a protozoan systemic infection, likely consistent with *T. gondii*. The first hare, LE18003, was a lactating adult female weighing 4.00 kg, with depleted fat stores but no signs of muscular atrophy. The second animal, LE21038, was an adult male in good body condition, weighing 3.10 kg (Fig. 2). Externally, LE21038 exhibited bilateral epistaxis and mild icterus/jaundice of the subcutaneous tissue (Fig. 2). No external alterations were observed in LE18003.

The most significant internal macroscopic findings in both cases included irregular areas of lung congestion and haemorrhage, a substantial amount of serohaemorrhagic foam in the trachea, extreme congestion and enlargement of the spleen, slight diffuse liver pallor with randomly distributed miliary necrotic foci, mesenteric lymphadenomegaly with multifocal pinpoint necrotic foci, and marked renal congestion (Fig. 2). LE21038, which was better preserved, showed additional findings, including up to 15 ml of serohaemorrhagic pleural effusion, haemorrhagic urine (Fig. 2), distended intestines filled with clear mucoid fluid, and petechial haemorrhages on the gastric mucosa. In contrast, LE18003 was frozen before the necropsy, and even if it had reddish contents in the intestines, its significance was unclear.

Histopathological lesions were similar in both animals, although freezing artefacts and autolysis hindered tissue evaluation in LE18003. Multifocal randomly distributed areas of coagulative and often lytic necrosis with haemorrhage, accompanied by lymphoplasmacytic and histiocytic inflammatory infiltrates, were observed in multiple tissues. These lesions were associated with intralesional

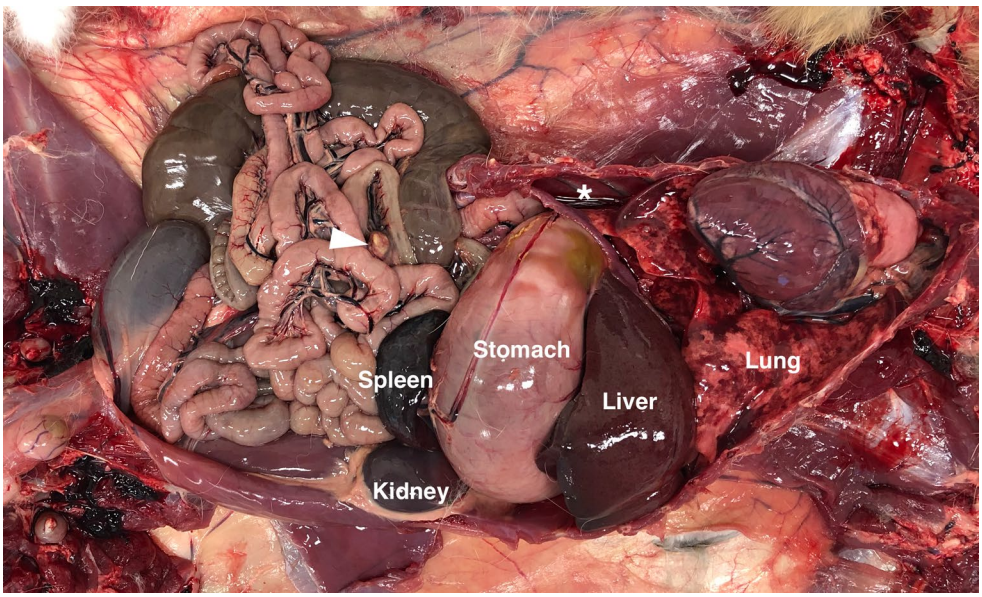

Fig. 2 European brown hare (*Lepus europaeus*), LE21038, with systemic toxoplasmosis. In the thoracic cavity note the voluminous lungs with multifocal haemorrhages, along with serohemorrhagic fluid (*). In the abdominal cavity, the most striking changes include a congested, enlarged spleen (splenomegaly) and yellow areas of necrosis in the central mesenteric lymphnode (arrowhead). Good body condition is evident from the presence of perirenal fat stores, as well as icterus/jaundice of the subcutaneous tissue

apicomplexan immature tissue cyst or parasite zoites enclosed within parasitophorous vacuoles in multiple organs, including the liver, spleen, mesenteric lymph nodes, lungs, heart, brain, and bone marrow (Fig. 3). The tissue cysts measured approximately 15 × 20 µm.

In the liver, parasites were observed within Kupffer cells and hepatocytes. In addition to necrotic foci, an increased number of leucocytes, particularly heterophils, was noted in the hepatic sinusoids (Fig. 3). The spleen showed marked hyperplasia of the lymphoid follicles and periarteriolar sheaths, along with congestion, and fibrin deposits in necrotic areas. The mesenteric lymph nodes exhibited extensive areas of necrosis and haemorrhage, as well as increased lymphocyte and macrophage activity in the subcapsular and medullary sinuses.

The lungs were characterised by severe interstitial pneumonia with alveolar oedema, thickened alveolar septa due to increased inflammatory cells, predominantly mononuclear, and numerous foamy macrophages and lymphoplasmacytic cells within the alveoli. Parasites were frequently seen within the cytoplasm of those intralveolar macrophages (Fig. 3). Myocardial lesions included scattered foci of myofibrillar degeneration and mononuclear interstitial inflammatory infiltrates with intrasarcoplasmic protozoan parasites (Fig. 3). In the brain, multifocal areas of malacia with gitter cells and gemistocytic astrocytes were associated with intralesional immature cysts and free zoites, most likely multiplying tachyzoites (Fig. 3). Additionally, protozoan-like structures were identified within macrophages in the bone marrow.

Toxoplasma gondii DNA was successfully detected in both hares via conventional nested PCR (*ITS-1*) and qPCR (529RE). Positive tissues in LE18003 included the brain, heart and spleen, while in LE21038 included the brain, heart and skeletal muscle (Appendix S1: Table S2). Regarding strain genotyping, although complete profiles were not obtained through Mn-PCR-RFLP (Appendix S1: Fig. S1), the two samples analyzed predominantly exhibited type II alleles, but also a type I allele in the *Apico* marker (LE18003), suggesting that the strain profile detected corresponded to a PRU type II variant (ToxoDB#3) (Table 1). More discriminatory results were detected by MS-typing, resulting in full allele profiles and indicating the same type II variant of *T. gondii* (Table 1). Both hares harboured the same strain, and their microsatellite profile did not match to previous type II variant detected in the *T. gondii* isolates

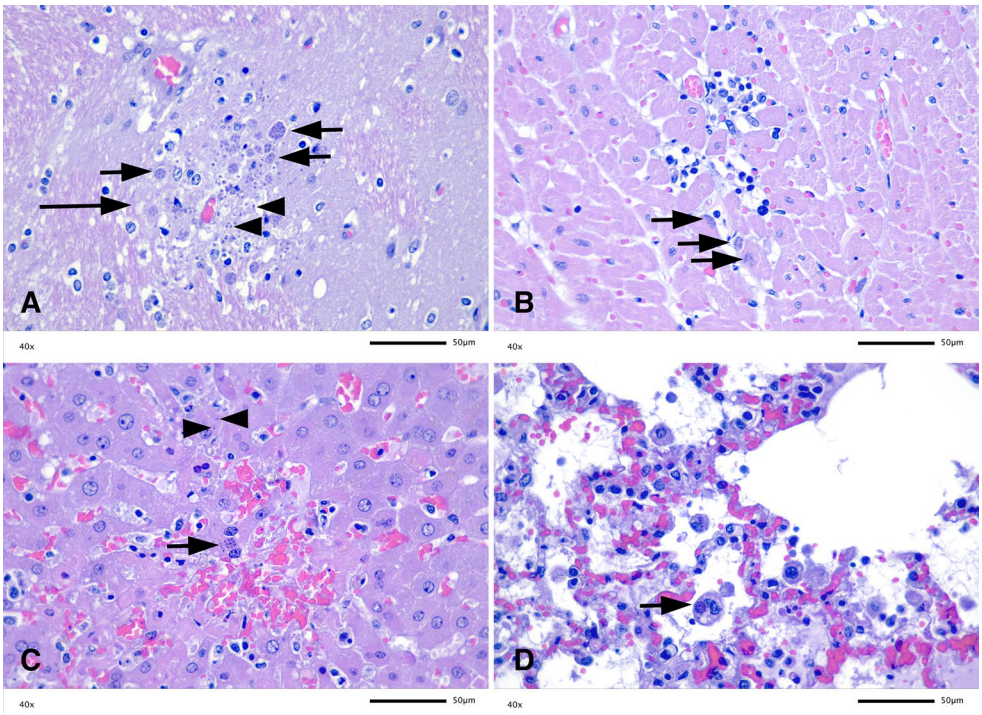

Fig. 3 European brown hare (*Lepus europaeus*), LE21038. Inflammation and necrosis associated with *Toxoplasma gondii* in multiple tissues. **A**) Brain. Cluster of apicomplexan-like cysts in an area of necrosis (long arrow), with numerous, most likely multiplying tachyzoites (arrowheads). Intact tissue cysts or parasitophorous vacuoles are indicated by short arrows. **B**) Myocardium. Foci of interstitial mononuclear inflammation adjacent to several myofibers containing intrasarcoplasmic apicomplexan-like zoites in a cyst or parasitophorus vacuole (short arrows). **C**) Liver. Area of hepatocellular necrosis and haemorrhage associated with single intracytoplasmic tachyzoites (arrowheads) or clusters (short arrow). **D**) Lung. Interstitial pneumonia with severe alveolar oedema with numerous macrophages, some containing apicomplexan-like cysts (short arrow), along with thickened alveolar septa and fibrinous fibrillar exudate. Hematoxilin & Eosin stain

Table 1 Genotyping of *Toxoplasma gondii* strains from European brown hare (*Lepus europaeus*) positive DNA samples. For LE18003, the processed sample was the skeletal muscle, whereas for LE21038 was the spleen

PCR-RFLP		Marker													
Sample ID	Genotype ToxoDB#	SAG1	3'SAG2	5'SAG2	altSAG2		SAG3	BTUB	GRA6		L358	c22-8	c29-2	PK1	Apico
LE 18,003	#3	II	II	II	II		II	II	II		II	II	II	NA	I
LE 21,038	ND	II	II	II	NA		II	II	II		II	II	II	NA	NA
Reference RH	#10	I	I/III	I/II	I		I	I	I		I	I	I	I	I
Reference Me49	#1	II/III	II	I/II	II		II	II	II		II	II	II	II	II
Reference PRU	#3	II/III	II	I/II	II		II	II	II		II	II	II	II	I
Reference NED	#2	II/III	I/III	III	III		III	III	III		III	III	III	III	III
Microsatellites		**Markers**													
Sample ID	**Type**	**N61**	**B18**	**M33**	**M48**	**TUB2**	**N83**	**XI.1**	**N82**	**TgM-A**	**W35**	**IV.1**	**B17**	**N60**	**M102**	**AA**
LE 18,003	II	103	158	169	225	289	310	356	119	207	242	274	336	140	174	277
LE 21,038	II	103	158	169	225	289	310	356	119	207	242	274	336	140	174	277
Reference RH	I	87	160	169	209	291	306	358	119	209	248	274	342	145	166	265
Reference Me49	II	91	158	169	215	289	310	356	111	207	242	274	336	142	174	265
Reference PRU	II	123	158	169	209	289	310	356	117	207	242	274	336	142	176	265
Reference NED	III	91	160	165	209	289	312	356	111	205	242	278	336	147	188	267

NA: Not amplified; ND: Not determined

derived from chicken, pigs and seagull available to date in the Iberian Peninsula (Appendix S1: Fig. S2).

The *GRA7* marker of LE18003 (skeletal muscle) showed type II alleles and presented 100% homology with other type II sequences from Spain (sheep, acc. no.: MT361127.1) and Italy (dolphin, acc. no.: ON982166.1). The *CS3* marker of LE18003 (skeletal muscle) and LE21038 (spleen) also showed type II alleles not related to virulent profiles and presenting a 100% homology with other type II sequences from Spain (sheep, acc. no.: MW727456.1; pig, acc. no.: MW132601.1).

Antibodies against *T. gondii* were found in 4 out of 231 European brown hares tested (excluding both clinical cases, that also resulted seropositive), obtaining a seroprevalence of 1.73% (95% CI: 0.05–3.41). All seropositive animals were adults from the Catalonia region, collected in 2013, 2015, 2016, and 2021 (Appendix S1: Table S1), with no lesions consistent with systemic or acute toxoplasmosis.

Discussion

The present study reports, to our knowledge, the first two cases of fatal clinical toxoplasmosis in European brown hares in southern Europe. The macroscopic and microscopic lesions described, such as severe splenomegaly, mesenteric lymphadenomegaly, liver necrosis, and pulmonary congestion and oedema, are consistent with previously published records in this species (Sedlák et al. 2000; Jokelainen et al. 2011; Račka et al. 2021). This pattern of alterations, microscopically characterised by acute disseminated multiorgan necrosis in well-nourished individuals, supports the notion that hares may be highly susceptible hosts for *T. gondii* infection, as shown in experimental infections (Gustafsson et al. 1997; Sedlák et al. 2000).

Several hypotheses have been proposed to explain this high susceptibility, mainly related to harsh climatic conditions and stress (Sedlák et al. 2000; Jokelainen et al. 2011; Račka et al. 2021). Even though no clear consensus exists among investigators. The genotype of *T. gondii* involved in the infection could also be considered of interest to understand susceptibility and disease severity in hares, although data for comparison in the European brown hare are limited. In Europe, the Type II clonal lineage predominates (Fernández-Escobar et al. 2022) and was identified in clinical cases of toxoplasmosis in hares from Finland (Jokelainen et al. 2011) and the Czech Republic (Račka et al. 2021). The Type II PRU variant (or ToxoDB#3), identified herein, has been detected in domestic species such as sheep and pigs in Spain (Fernández-Escobar et al. 2020), but might also affect other species. While *T. gondii* type II, including PRU variant, is generally considered of low virulence in experimental

infections in mice (Sedlák et al. 2000; Fernández-Escobar et al. 2021), it has demonstrated remarkable virulence in other hosts, such as sheep and New World primates (Sánchez-Sánchez et al. 2019; Salas-Fajardo et al. 2023). This suggests that susceptibility and virulence induced by *T. gondii* may strongly depend on the host (Mukhopadhyay et al. 2020), as illustrated in the case of the European brown hare.

The observed seroprevalence of anti-*Toxoplasma* antibodies in the European brown hare populations analysed is low (1.73%), similar to other Mediterranean countries, such as Italy (1.3%, 3/222) (Ebani et al. 2016), and Greece (5.7%, 6/105) (Tsokana et al. 2019). In contrast, significantly higher seropositivity values were found in European brown hares in Central Europe, such as Germany (46%, 147/318) (Frölich et al. 2003) and the Czech Republic (21%, 71/333) (Bártová et al. 2010). Recent studies have also documented variable antibody prevalence in hare species globally, ranging from 0 to 21% (reviewed by Almeria et al. 2021). These variations can be partially attributed to differences in methodology, as numerous serological techniques have been employed, including the Modified Agglutination Test (MAT), Indirect Fluorescent Antibody Test (IFAT), Indirect Hemagglutination Assay (IHA) or, in our case, Enzyme-Linked Immunosorbent Assay (ELISA). The specificity, sensitivity and cutoff values of serological tests have not been evaluated in many animal species (Almeria and Dubey 2021), highlighting the need for standardization and consensus to facilitate accurate comparisons. Nevertheless, differences in the epidemiology of the parasite across regions or other additional influencing factors cannot be ruled out.

Considering high susceptibility and low seroprevalence, we hypothesise that *T. gondii* infection in European brown hare populations in southern Europe may be sporadic. One possible explanation could be a low exposure rate of hares to the parasite. Interestingly, serological results from a study conducted on European rabbits (*Oryctolagus cuniculus*) in the same area demonstrated a much higher seroprevalence (53.8%) (Almería et al. 2004), even if the technique used (MAT) was different. Toxoplasmosis in rabbits generally presents a subclinical course (Dubey 2021), so higher seropositivity compared to hares is not surprising. However, given the ecological similarities and overlapping habitats of rabbits and hares (Lush et al. 2017), T. *gondii* exposure rates between both species should be theoretically comparable. Previous studies on *T. gondii* in Iberian hares from south of Navarra, close to our study area, found a seroprevalence of 11.4% by using MAT, with a significantly higher seropositivity rate in juveniles compared to adults (Fernández-Aguilar et al. 2013). They suggested that the parasite might be affecting hare survival and consequently restricting the detection of antibodies in adult individuals. Although it is challenging to find young dead animals in the field, or even to obtain hunted carcasses from young animals (Fernández-Morán et al. 1997; Speth 2013), future sampling efforts that include a higher proportion of juveniles could offer clearer insights into parasite dynamics and potential impact on hare populations.

While this study provides novel insights into fatal toxoplasmosis in European brown hares in southern Europe, certain considerations should be acknowledged. The number of confirmed clinical cases was based on passive disease surveillance, which can be subject to bias such as underreporting, delayed detection, or limited geographic coverage, thereby restricting broader generalisations regarding disease frequency and impact (Ryser-Degiorgis 2013; Tomaselli 2022). Additionally, the sampling, as previously mentioned, included a relatively low proportion of juvenile individual, which may have influenced seroprevalence estimates. The lack of standardization across serological methods also remains an important challenge in toxoplasmosis research for these species, potentially affecting cross-study comparability. Finally, although beyond the scope of this work, future studies integrating environmental and ecological data would contribute to a more comprehensive understanding of exposure patterns, potential species-specific differences and risk factors. Nevertheless, despite these limitations, our findings offer a valuable contribution to the limited knowledge on *T. gondii* infection in wild hares and underscore the importance of continued surveillance and research in this field.

The European brown hare, while classified as a game species and not widely considered threatened, has experienced declines in many areas across Europe during the last decades. Information on the species dynamics in northeastern Spain, its southernmost limit, is still scarce. Mortality outbreaks by infectious diseases such as rabbit haemorrhagic disease and European brown hare syndrome have been reported in this area (Estruch et al. 2025), along with occasional cases of brucellosis (*Brucella suis*), pseudotuberculosis (*Yersinia pseudotuberculosis*), pasteurellosis (*Pasteurella multocida*), and intestinal coccidiosis (*Eimeria* sp.) (data from the Wildlife Passive Surveillance Program in Catalonia). The combined impact of these transmissible diseases, including toxoplasmosis, together with the other factors known to decimate European brown hare populations may affect the viability of the species south to the Pyrenees.

In conclusion, our study underscores the importance of considering the potential impact of toxoplasmosis on European brown hare populations and highlights its susceptibility to the parasite. Although *T. gondii* infection in the species may be rare in our study area, it appears to lead to acute fatal disease when it occurs, so surveillance programs should be maintained. Additionally, our findings provide insights into the genotype responsible for the infection in

hares in this European region, providing a foundation for future investigations into the epidemiology of toxoplasmosis in wild lagomorphs.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10344-025-01966-9.

Acknowledgements We thank the Rural Agents of the Cos d'Agents Rurals from the Catalan Government and hunters for their invaluable collaboration in the wildlife passive surveillance programme in Catalonia and for reporting and transporting cases to SEFaS at the Veterinary Faculty. This programme is supported by the Department of Agriculture, Livestock, Fishing and Food of the Catalan Government. Special thanks to Maria Josep Vargas, Montserrat Rubió and Albert Sanz, technical managers of the Catalan authorities linked to this surveillance programme. We also acknowledge the Navarre Regional Government for their sampling support and collaboration in the project.

Author contributions JE and RV conceptualized the study, conducted the pathology work, and collected the majority of the samples. MYSF and RCB designed and carried out the molecular analyses. IGB and SCS were responsible for the serological analyses. VA contributed samples for the serological studies and assisted with figure preparation. SL oversaw the broader project that encompassed this study. CR provided project supervision and conducted a thorough review of the manuscript. JE prepared the initial draft of the manuscript. All authors critically reviewed and approved the final manuscript.

Funding Open Access Funding provided by Universitat Autonoma de Barcelona. Open Access Funding provided by Universitat Autonoma de Barcelona. This work has benefited from the financial aid of research grants funded by the Spanish Ministry of Science and Innovation: Iber-LagoHealth project, REF: PID2023-151954NB-I00; and REF: PID2022-138673OB-C21. This work was partially financed by CIBER - Consorcio Centro de Investigación Biomédica en Red - (CB2021/13/00083), Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación and Unión Europea–NextGenerationEU. Sabrina Castro-Scholten is supported by FPU grants of the Spanish Ministry of Science, Innovation and Universities (FPU19/06026). Martha Ynés Salas-Fajardo is granted by the program of predoctoral grants from the Peruvian Government (PRONABEC).

Data availability No datasets were generated or analysed during the current study.

Declarations

Ethis approval No approval of research ethics committees was required for this study because procedures were conducted using samples taken from European brown hare carcasses submitted either for post-mortem examination at the Servei d'Ecopatologia de Fauna Salvatge (SEFaS) from the Autonomous University of Barcelona (UAB) within the Wildlife Passive surveillance programme of Catalonia (Spain), or during sampling campaings carried out by the regional government of Navarre.

Competing interests The authors declare no competing interests.

References

Ajzenberg D, Collinet F, Mercier A et al (2010) Genotyping of *Toxoplasma gondii* isolates with 15 microsatellite markers in a single multiplex PCR assay. J Clin Microbiol 48:4641–4645. https://doi.org/10.1128/JCM.01152-10

Almeria S, Dubey JP (2021) Foodborne transmission of *Toxoplasma gondii* infection in the last decade. An overview. Res Vet Sci 135:371–385. https://doi.org/10.1016/j.rvsc.2020.10.019

Almeria S, Murata FHA, Cerqueira-Cézar CK et al (2021) Epidemiological and public health significance of *Toxoplasma gondii* infection in wild rabbits and hares: 2010–2020. Microorganisms 9:1–12. https://doi.org/10.3390/microorganisms9030597

Almería S, Calvete C, Pagés A et al (2004) Factors affecting the Seroprevalence of *Toxoplasma gondii* infection in wild rabbits (*Oryctolagus cuniculus*) from Spain. Vet Parasitol 123:265–270. https://doi.org/10.1016/j.vetpar.2004.06.010

Arora N, Kotwani S, Chhabra M, H M (2022) Hemorrhagic lesions in the central nervous system: toxoplasmosis in a person living with human immunodeficiency virus infection and acquired immunodeficiency syndrome. Cureus 14:e24827. https://doi.org/10.7759/cureus.24827

Aubert D, Ajzenberg D, Richomme C et al (2010) Molecular and biological characteristics of *Toxoplasma gondii* isolates from wildlife in France. Vet Parasitol 171:346–349. https://doi.org/10.1016/j.vetpar.2010.03.033

Bártová E, Sedlák K, Treml F et al (2010) *Neospora Caninum* and *Toxoplasma gondii* antibodies in European brown hares in the Czech Republic. Slovakia Austria Vet Parasitol 171:155–158. https://doi.org/10.1016/j.vetpar.2010.03.002

Bottós J, Miller H, Belfort RN R, et al (2009) Bilateral retinochoroiditis caused by an atypical strain of *Toxoplasma gondii*. 93:1546–1550. https://doi.org/10.1136/bjo.2009.162412.BILATERAL

Broekhuizen S, Maaskamp F (1981) Annual production of young in European hares (*Lepus europaeus*) in the Netherlands. J Zool 193:499–516. https://doi.org/10.1111/j.1469-7998.1981.tb01500.x

Castaño P, Fuertes M, Ferre I et al (2014) Placental thrombosis in acute phase abortions during experimental *Toxoplasma gondii* infection in sheep. Vet Res 45. https://doi.org/10.1186/1297-9716-45-9

Dubey JP (1998) Refinement of Pepsin digestion method for isolation of *Toxoplasma gondii* from infected tissues. Vet Parasitol 74:75–77. https://doi.org/10.1016/S0304-4017(97)00135-0

Dubey JP (2021) Toxoplasmosis of animals and humans. CRC, Boca Raton

Ebani VV, Poli A, Rocchigiani G et al (2016) Serological survey on some pathogens in wild brown hares (*Lepus europaeus*) in central Italy. Asian Pac J Trop Med 9:465–469. https://doi.org/10.1016/j.apjtm.2016.03.032

Edwards PJ, Fletcher MR, Berny P (2000) Review of the factors affecting the decline of the European brown hare, *Lepus Europaeus* (Pallas; 1778) and the use of wildlife incident data to evaluate the significance of Paraquat. Agric Ecosyst Environ 79:95–103. https://doi.org/10.1016/S0167-8809(99)00153-X

Estonba A, Solís A, Iriondo M et al (2006) The genetic distinctiveness of the three Iberian hare species: *Lepus europaeus, L. granatensis*, and *L. castroviejoi*. Mamm Biol 71:52–59. https://doi.org/10.1016/j.mambio.2005.08.010

Fernández-Aguilar X, Alzaga V, Villanúa D et al (2013) Epidemiology and prevalence of *Toxoplasma gondii* infection in the Iberian hare (*Lepus granatensis*). Vet Parasitol 196:194–198. https://doi.org/10.1016/j.vetpar.2013.01.061

Fernández-Escobar M, Calero-Bernal R, Benavides J et al (2020) Isolation and genetic characterization of *Toxoplasma gondii* in Spanish sheep flocks. Parasit Vectors 13:1–14. https://doi.org/10.1186/s13071-020-04275-z

Fernández-Escobar M, Calero-Bernal R, Regidor-Cerrillo J et al (2021) In vivo and in vitro models show unexpected degrees of virulence among *Toxoplasma gondii* type II and III isolates from sheep. Vet Res 52:1–15. https://doi.org/10.1186/s13567-021-00953-7

Fernández-Escobar M, Schares G, Maksimov P et al (2022) *Toxoplasma gondii* genotyping: a closer look into Europe. Front Cell Infect Microbiol 12:842595. https://doi.org/10.3389/fcimb.2022.842595

Fernández-Morán J, Gómez S, Ballesteros F et al (1997) Epizootiology of sarcoptic mange in a population of Cantabrian chamois (*Rupicapra pyrenaica parva*) in Northwestern Spain. Vet Parasitol 73:163–171. https://doi.org/10.1016/s0304-4017(97)00061-7

Frölich K, Wisser J, Schmüser H et al (2003) Epizootiologic and Ecologic investigations of European brown hares (*Lepus europaeus*) in selected populations from Schleswig-Holstein, Germany. J Wildl Dis 39:751–761. https://doi.org/10.7589/0090-3558-39.4.751

Gortazar C, Milla J, Acevedo P et al (2009) A large-scale survey of brown hare *Lepus Europaeus* and Iberian hare *L. granatensis* populations at the limit of their ranges. Wildl Biol 12:244–250. https://doi.org/10.2981/0909-6396(2007)13

Gustafsson K, Uggla A, Jarplid B (1997) *Toxoplasma gondii* infection in the mountain hare (*Lepus timidus*) and domestic rabbit (*Oryctolagus cuniculus*). J Comp Pathol 117:351–360. https://doi.org/10.1016/S0021-9975(97)80082-5

Hacklander K, Schai-Braun S (2019) *Lepus europaeus*. The IUCN Red List of Threatened Species 2019: e.T41280A45187424. 8235: https://doi.org/http://dx.doi.org/10.2305/IUCN.UK.2019- 1.RLTS.T41280A45187424.en

Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symp 41:95–98

Hill DE, Dubey JP (2016) *Toxoplasma gondii* as a parasite in food: analysis and control. Microbiol Spectr 4. https://doi.org/10.1128/microbiolspec.PFS-0011-2015

Homan WL, Vercammen M, De Braekeleer J, Verschueren H (2000) Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. Int J Parasitol 30:69–75. https://doi.org/10.1016/S0020-7519(99)00170-8

Joeres M, Cardron G, Passebosc-Faure K et al (2023) A ring trial to harmonize *Toxoplasma gondii* microsatellite typing: comparative analysis of results and recommendations for optimization. Eur J Clin Microbiol 42:803–818. https://doi.org/10.1007/s10096-023-04597-7

Jokelainen P, Isomursu M, Näreaho A, Oksanen A (2011) Natural *Toxoplasma gondii* infections in European brown hares and mountain hares in finland: proportional mortality rate, antibody prevalence, and genetic characterization. J Wildl Dis 47:154–163. https://doi.org/10.7589/0090-3558-47.1.154

Lavazza A, Cooke BD (2018) Diseases of lagomorphs. In: Smith AT, Johnston CH, Alves PC, Hackländer K (eds) Lagomorphs: pikas, rabbits, and hares of the world, 1st edn. John Hopkings University, USA, pp 18–21

Lindsay DS, Dubey JP (2020) Toxoplasmosis in wild and domestic animals. In: Weiss LM, Kim K (eds) *Toxoplasma gondii*: the model apicomplexan. Perspectives and methods, 3rd edn. Elsevier, USA, pp 293–320

Lush L, Ward AI, Wheeler P (2017) Dietary niche partitioning between sympatric brown hares and rabbits. J Zool 303:36–45. https://doi.org/10.1111/jzo.12461

Machado FP, Kmetiuk LB, Teider-Junior PI et al (2019) Seroprevalence of anti-*Toxoplasma gondii* antibodies in wild boars (*Sus scrofa*), hunting dogs, and hunters of Brazil. PLoS ONE 14. https://doi.org/10.1371/journal.pone.0223474

Mukhopadhyay D, Arranz-Solís D, Saeij JPJ (2020) Influence of the host and parasite strain on the immune response during *Toxoplasma* infection. Front Cell Infect Microbiol 10. https://doi.org/10.3389/fcimb.2020.580425

Pena HFJ, Gennari SM, Dubey JP, Su C (2008) Population structure and mouse-virulence of *Toxoplasma gondii* in Brazil. Int J Parasitol 38:561–569. https://doi.org/10.1016/j.ijpara.2007.09.004

Račka K, Bártová E, Juránková J et al (2021) Fatal toxoplasmosis in wild European brown hares (*Lepus europaeus*) in tularaemia endemic areas of the Czech republic: poses risk of infection for humans? Transbound Emerg Dis 68:1774–1778. https://doi.org/10.1111/TBED.13925

Ryser-Degiorgis M-P (2013) Wildlife health investigations: needs, challenges and recommendations. BMC Vet Res 9. https://doi.org/10.1186/1746-6148-9-223

Salas-Fajardo MY, Benavides J, Azevedo A et al (2023) Fatal toxoplasmosis in a captive squirrel monkey (*Saimiri boliviensis*) in Portugal. Vet Res Commun 47:2363–2370. https://doi.org/10.1007/s11259-023-10179-x

Sánchez-Sánchez R, Ferre I, Regidor-Cerrillo J et al (2019) Virulence in mice of a *Toxoplasma gondii* type II isolate does not correlate with the outcome of experimental infection in pregnant sheep. Front Cell Infect Microbiol 8. https://doi.org/10.3389/fcimb.2018.00436

Sedlák K, Literák I, Faldyna M et al (2000) Fatal toxoplasmosis in brown hares (*Lepus europaeus*): possible reasons of their high susceptibility to the infection. Vet Parasitol 93:13–28. https://doi.org/10.1016/S0304-4017(00)00325-3

Smith RK, Jennings NV, Harris S (2005) A quantitative analysis of the abundance and demography of European hares *Lepus Europaeus* in relation to habitat type, intensity of agriculture and climate. Mamm Rev 35:1–24. https://doi.org/10.1111/j.1365-2907.2005.00057.x

Speth JD (2013) Thoughts about hunting: some things we know and some things we don't know. Quat Int 297:176–185. https://doi.org/10.1016/j.quaint.2012.12.005

Stelzer S, Basso W, Benavides Silván J et al (2019) *Toxoplasma gondii* infection and toxoplasmosis in farm animals: risk factors and economic impact. Food Waterborne Parasitol 15. https://doi.org/10.1016/j.fawpar.2019.e00037

Strang AGGF, Ferrari RG, do Rosário DK et al (2020) The congenital toxoplasmosis burden in brazil: systematic review and meta-analysis. Acta Trop 211:105608. https://doi.org/10.1016/j.actatropica.2020.105608

Su C, Shwab EK, Zhou P et al (2010) Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. Parasitology 137:1–11. https://doi.org/10.1017/S0031182009991065

Tenter AM, Heckeroth AR, Weiss LM (2000) *Toxoplasma gondii*: from animals to humans. Int J Parasitol 30:1217–1258. https://doi.org/10.1016/S0020-7519(00)00124-7

Tomaselli M (2022) In: Stephen C (ed) Participatory epidemiology and surveillance for wildlife health BT - wildlife population health.

Springer International Publishing, Cham, pp 49–63. https://doi.org/10.1007/978-3-030-90510-1_5

Treml F, Pikula J, Bandouchova H, Horakova J (2007) European brown hare as a potential source of zoonotic agents. Vet Med (Praha) 52:451–456. https://doi.org/10.17221/2050-VETMED

truch J, Cavadini P, Lavazza A, Capucci L, Abrantes J, Lopes AM, Almeida T, Neimanis A, Lavín S, Rouco C, Serrano E, Velarde R (2025) Pathological and serological insights into Lagovirus diseases dynamics in the European brown hare (*Lepus europaeus)*: A nine-year longitudinal study. Vet Microbiol 304:110478. https://doi.org/10.1016/j.vetmic.2025.110478

Tsokana CN, Sokos C, Giannakopoulos A et al (2019) Serological and molecular investigation of selected parasitic pathogens in European brown hare (*Lepus europaeus*) in Greece: inferring the ecological niche of *Toxoplasma gondii* and *Leishmania infantum* in hares. Parasitol Res 118:2715–2721. https://doi.org/10.1007/S00436-019-06388-6/FIGURES/2

van der Giessen J, Deksne G, Gómez-Morales MA et al (2021) Surveillance of foodborne parasitic diseases in Europe in a one health approach. Parasite Epidemiol Control 13. https://doi.org/10.1016/J.PAREPI.2021.E00205

Viviano A, Mori E, Fattorini N et al (2021) Spatiotemporal overlap between the European brown hare and its potential predators and competitors. Animals 11:1–11. https://doi.org/10.3390/ani11020562

Wibbelt G, Frölich K (2005) Infectious diseases in European brown hare (Lepus europaeus). Wildl Biology Pract 1. https://doi.org/10.2461/wbp.2005.1.11