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Seroepidemiology of *Toxoplasma gondii* in wild ruminants in Spain

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Abstract

Toxoplasmosis is a parasitic zoonosis caused by *Toxoplasma gondii* which infects warm-blooded species worldwide. Humans can be infected through ingestion of tissue cysts from raw or undercooked meat, including game meat. A nationwide large-scale cross-sectional study was conducted to assess exposure to *T. gondii* in seven wild ruminant species in Spain. A total of 2,040 serum samples from 77 sampling sites randomly distributed in the five bioregions (BRs) covering mainland Spain were tested for antibodies against *T. gondii* using the modified agglutination test. The overall seroprevalence was 22.0% (449/2,040). Seroprevalence by species in decreasing order was as follows: 39.6% (141/356) in roe deer (*Capreolus capreolus*), 37.1%

(138/372) in fallow deer (*Dama dama*), 16.6% (92/553) in red deer (*Cervus elaphus*), 14.0% (26/186) in Southern chamois (*Rupicapra pyrenaica*), 11.5% (24/209) in mouflon (*Ovis aries musimon*), 7.8% (27/346) in Iberian wild goat (*Capra pyrenaica*) and 5.6% (1/18) in Barbary sheep (*Ammotragus lervia*). Seropositivity was detected in 74.0% (57/77) of the sampling sites. Results indicate widespread but not homogeneous exposure to *T. gondii* in wild ruminant populations in Spain during the last two decades and highlight differences related to animal species and spatial distribution of these species in this country; this implies potential consequences of *T. gondii* for animal health, conservation and public health.

KEYWORDS

food-borne pathogens, Spain, *Toxoplasma gondii*, wild ruminants, zoonosis

1 | INTRODUCTION

Toxoplasmosis is a zoonosis caused by *Toxoplasma gondii*, an intracellular protozoan parasite of worldwide distribution. Its lifecycle involves species of the Felidae family as the definitive host, and virtually all warm-blooded species as intermediate hosts (Dubey, 2010). *Toxoplasma gondii* is the most widespread zoonotic parasite in human beings, who become infected by the ingestion of tissue cysts from undercooked or raw meat, consumption of food or water contaminated with *T. gondii* oocysts, or by congenital transmission (Dubey, 2010; Hill & Dubey, 2014). Most infections appear to be asymptomatic; however, it can lead to abortion in women and severe neuromuscular complications or even death in immunocompromised people and newborns (Robert-Gangneux & Dardé, 2012). Additionally, *T. gondii* infection has also been associated with both subtle behavioural changes and, in some cases, severe neuropsychiatric disorders (Milne et al., 2020).

During the last few decades, most of the wild ruminant species in Spain have spatially expanded due to different factors: the intensification of game management practices, changes in land use, decrease of natural predators or introduction of individuals outside their native range (Acevedo et al., 2011; Carpio et al., 2021). This situation led to overabundance of wild ruminant species in some areas (Caughley, 1981), which overlaps with a socio-economical context where demand for eco-friendly and sustainable game meat has significantly increased (Carpio et al., 2021; Navarro-González et al., 2016). These factors imply an increase in the risk of food-borne pathogen transmission associated with the consumption of game meat and game products (Santoro et al., 2019). In fact, the meat of large game species has been identified as an important zoonotic source for *T. gondii* (EFSA, 2007). Hunters and consumers of raw meat, as well as people who process meat, are at higher risk for *T. gondii* infection (Fecková et al., 2020). Importantly, clinical toxoplasmosis and even *T. gondii* outbreaks have been reported after consumption of

Impacts

- *Toxoplasma* seroprevalence was widespread among wild ungulates in Spain.
- Highest seroprevalence was found in roe deer and fallow deer compared with the five remaining species.
- Animals living in northern Spain, with an Atlantic climate, had significantly higher risk of *T. gondii* exposure.

raw or undercooked venison (Gaulin et al., 2020; Ross et al., 2001; Schumacher et al., 2020).

Toxoplasma gondii exposure has been shown to be widespread in wild ruminant species in different areas and regions of Spain (Almería et al., 2018, 2021; García-Bocanegra et al., 2013; Panadero et al., 2010). However, no large nationwide scale studies have been carried out involving a representative sample size, both at bioregion and species levels, to get a deeper and broader understanding of the epidemiological situation of this zoonotic pathogen in wild ruminant populations. Hence, the aim of the present study was to determine the nationwide seroprevalence and risk factors associated with *T. gondii* exposure in all the bioregions and wild ruminant species present in mainland Spain.

2 | MATERIALS AND METHODS

### 2.1 | Study design and sampling

Between 1999 and 2020, a nationwide survey was performed and samples from the seven wild ruminant species present in Spain, red deer (*Cervus elaphus*), fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), Iberian wild goat (*Capra pyrenaica*), mouflon (*Ovis aries musimon*), Southern chamois (*Rupicapra pyrenaica*) and Barbary

sheep (*Ammotragus lervia*). Samples were collected throughout the five bioregions (BRs) of mainland Spain (Figure 1). These bioregions were defined by the Spanish National Wildlife Disease Surveillance Plan, based on the ecosystems, presence and abundance of wild ungulates, climatological and epidemiological criteria of the wildlife communities present (for more details see Muñoz et al., 2010; PNVSFS, 2020). Briefly, BR1 has an Atlantic climate, with mild temperatures and high average annual rainfall. Bordering this BR to the south lies BR2, with a Continental Mediterranean climate and predominance of cereal crops. The south-central extends BR3, a region with low altitude mountains and cold winters, hot and dry summers, and rainy autumns and springs. The BR4 covers inland mountains with a Continental Mediterranean climate. Finally, along the south and east coast extends BR5 with warm, rainy winters and hot, dry summers.

Sample size was calculated assuming a prevalence of 50%, with a 95% confidence interval ($CI_{95\%}$) and a desired precision of ±5%, resulting in 385 specimens per BR to be sampled (1,925 samples in total). The sampling was then stratified within BRs according to the distribution and density representativeness of the wild ruminant species present. Whenever possible, 59 animals of each of the species present in a BR were sampled to ensure a 95% probability of detecting seropositivity for an assumed minimum prevalence of 5% in each BR. In addition, for each BR, several sampling sites were randomly selected, corresponding to hunting states or game reserves. In each of these sampling sites between 15 and 20, animals were randomly sampled (simple random sampling) when feasible (Figure 1).

A total of 2,040 ruminants were finally sampled from 77 sampling sites, including 553 red deer, 372 fallow deer, 356 roe deer, 346 Iberian wild goats, 209 mouflon, 186 Southern chamois and 18 Barbary sheep (Table 1). All animals were legally harvested by hunters or culled as part of population control programmes on game reserves. Blood samples were obtained by puncture of the endocranial venous sinuses or from the thoracic cavity (Jiménez-Ruiz et al., 2016). Sera were collected after centrifugation and kept frozen at −20°C until analysis. Data on sampled populations, BR, sampling
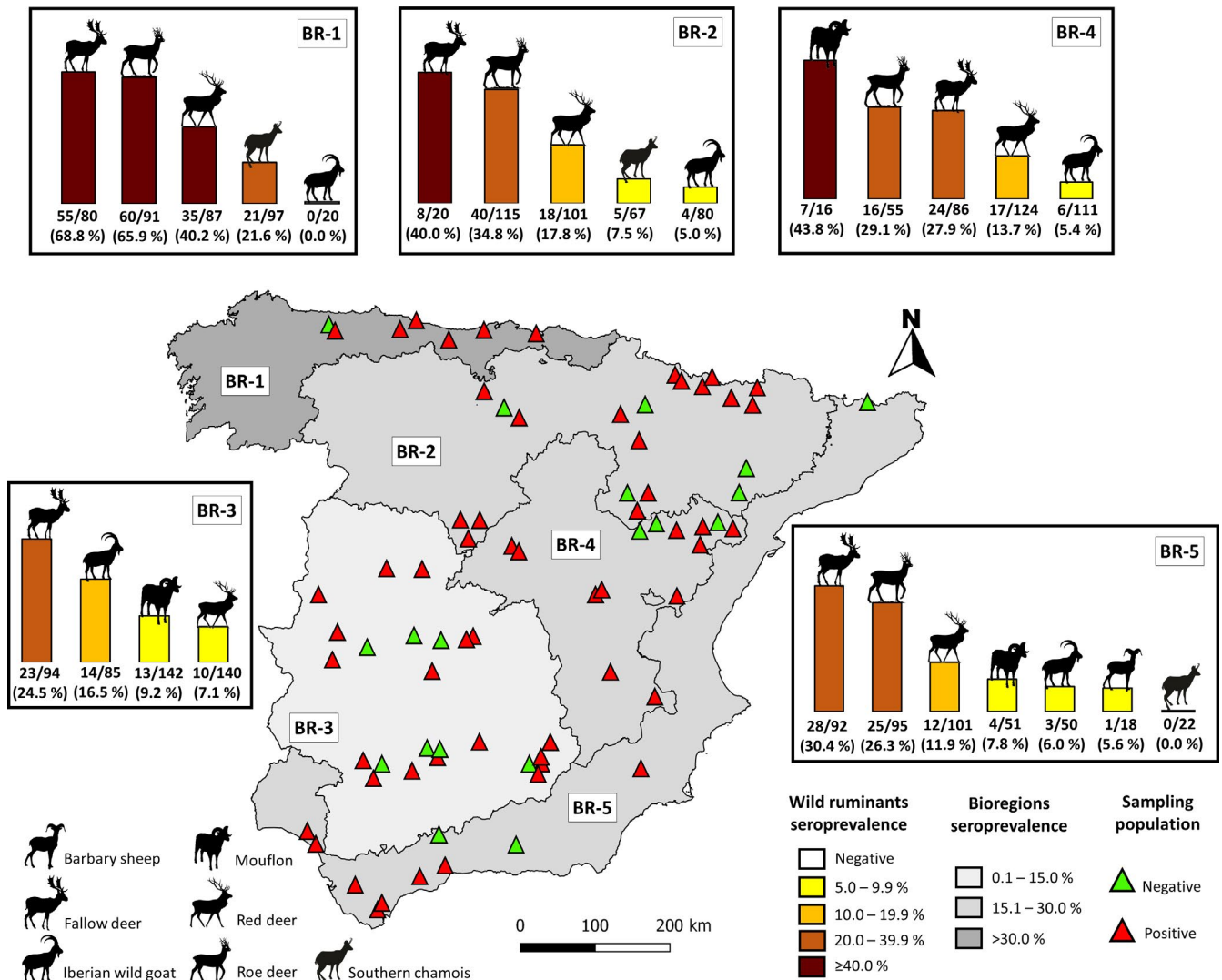
FIGURE 1 Seroprevalence of *Toxoplasma gondii* in the wild ruminant species present in mainland Spain. Triangles represent *T. gondii* exposure as determined by presence of antibodies at each sampling site

Variable	Category	No. positives/overall[a]	Seroprevalence (%)	p-Value
Species	Barbary sheep	1/18	5.6	<.001
	Fallow deer	138/372	37.1	
	Iberian wild goat	27/346	7.8	
	Mouflon	24/209	11.5	
	Red deer	92/553	16.6	
	Roe deer	141/356	39.6	
	Southern chamois	26/186	14.0	
Bioregion	BR1	171/375	45.6	<.001
	BR2	75/383	19.6	
	BR3	60/461	13.0	
	BR4	70/392	17.9	
	BR5	73/429	17.0	
Period	1999–2012	227/740	37.4	<.001
	2013–2015	107/732	14.6	
	2016–2020	65/568	10.0	
Sex	Male	22/207	13.0	.132
	Female	12/150	8.0	
Age	Yearling	4/59	6.4	.107
	Sub-adult	17/104	16.4	
	Adult	17/170	10.0	

TABLE 1 Distribution of the prevalence of antibodies against *Toxoplasma gondii* in wild ruminant populations in Spain by categories and results of the bivariate analysis

[a]Missing values omitted.

year, sex and age (yearlings, sub-adults or adults; according to Sáenz de Buruaga et al., 1991) were recorded for each animal, whenever possible.

2.1.1 | Testing for *T. gondii* antibodies

The presence of *T. gondii* antibodies was determined using the modified agglutination test (MAT) following the protocol described by Dubey and Desmonts, (1987). Samples with titres of 1:25 or higher were considered positive. Positive sera were titrated at dilutions: 1:25, 1:50, 1:100 and 1:500. MAT has been extensively used for the serological diagnosis of *T. gondii* in domestic and wild ruminant species (Almería et al., 2018; Aubert et al., 2010; Gamarra et al., 2008; Heddergott et al., 2018b; Heddergott et al., 2018a; Jiménez-Martín et al., 2020; Waap et al., 2016). A comparison of seropositivity and isolation of viable *T. gondii* in white tailed deer (*Odocoileus virginianus*) in the USA attests the efficiency of MAT for the detection of *T. gondii* antibodies in deer (Dubey et al., 2020). Additionally, Shaapan et al., (2008) found the highest sensitivity (96%) by MAT compared with the Enzyme-Linked Immunosorbent Assay (ELISA) (90.1%) and Indirect Fluorescent Antibody Test (IFAT) (80.4%) when using the Sabin–Feldman dye test (SF) as reference test for the detection of *T. gondii* infection in naturally infected sheep.

2.2 | Statistical analysis

The seroprevalence against *T. gondii* was determined from the proportion of positive samples to the total number of examined, with exact binomial $CI_{95\%}$. Three study periods (1999–2012, 2013–2015 and 2016–2020) were established by stratifying of the sampling years into percentiles 33 and 66 as cut points. Firstly, associations between serological results (as binomial response variable) and categorical explanatory variables (species, BR, sampling period, sex and age) were initially screened using the Pearson's Chi-square test or Fisher's test, as appropriate. Barbary sheep was removed from the statistical analysis because of the low number of samples analysed ($n = 18$) and because all barbary sheep samples were collected in BR5. All variables with a p-value <.10 in this bivariate analysis were selected for further analyses. Collinearity between pairs of variables was tested by Cramer's V coefficient. Secondly, 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 seropositive animals was assumed to follow a binomial distribution, and 'sampling site' was included as random effect factor. A logit link function was considered. A forward introduction of variables was used, 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 computing the change in the odd ratios (OR) by more than 30%. The choice of the best model was based on

the quasi-likelihood under independence model criterion. SPSS 22.0 software (IBM Corp.) was used for all statistical analyses.

3 | RESULTS

Antibodies against *T. gondii* were detected in 449 out of the 2,040 (22.0%) ruminants tested, with overall antibody titres of 1:25 in 240 (53.5%), 1:50 in 103 (22.9%), 1:100 in 69 (15.4%) and ≥1:500 in 37 (8.2%). The distribution of seroprevalences by species, BR, sampling period, sex and age is shown in Table 1. By species, the prevalence of antibodies in decreasing order was as follows: 39.6% (141/356; $CI_{95\%}$: 34.5–44.7) in roe deer, 37.1% (138/372; $CI_{95\%}$: 32.2–42.0) in fallow deer, 16.6% (92/553; $CI_{95\%}$: 13.5–19.7) in red deer, 14.0% (26/186; $CI_{95\%}$: 9.0–18.9) in Southern chamois, 11.5% (24/209; $CI_{95\%}$: 7.2–15.8) in mouflon, 7.8% (27/346; $CI_{95\%}$: 5.0–10.7) in Iberian wild goat and 5.6% (1/18; $CI_{95\%}$: 0.0–16.1) in Barbary sheep.

Geographically, seroprevalence values ranged between 45.6% (171/375; $CI_{95\%}$: 40.6–50.6) in BR1 and 13.0% (60/461; $CI_{95\%}$: 9.9–16.1) in BR3. Seropositive animals were detected in 74.0% (57/77) of the sampling sites and in all the species in the five BRs, except for Iberian wild goats sampled in BR1 and the Southern chamois sampled in BR5 (Table 1, Figure 1). Temporally, a decreasing trend in seroprevalence was found over the years. The highest seroprevalence (37.4%; 277/740; $CI_{95\%}$: 33.9–40.9) was detected during the first study period (1999–2012), decreased to 14.6% (107/732; $CI_{95\%}$: 12.1–17.2) during the second study period (2013–2015) and fell to 11.4% (65/568; $CI_{95\%}$: 8.8–14.1) during the last study period (2016–2020).

The categorical explanatory variables 'species', 'BR' and 'sampling period' presented association ($p < .10$) with the dependent variable, so they were initially selected for multivariate analysis. The variable 'sampling period' was excluded due to collinearity with 'species' and 'BR' variables. The final multivariate analysis showed that the main risk factors potentially associated with *T. gondii* exposure in wild ruminants were species and BR. Significantly higher seroprevalences were observed in roe deer and fallow deer compared with the remaining wild ruminant species. The seroprevalence was also significantly higher in BR1 compared with the remainder BRs (Table 2).

4 | DISCUSSION

The present study is the first large-scale, nationwide, cross-sectional survey on exposure to *T. gondii* comprising all free-ranging wild ruminant species present in a country, considering clearly delimited epidemiological units. Our results provide an overview of the seroprevalence and spatial variations of *T. gondii* in wild ruminant populations in Spain. Important variations and increased risk of exposure in certain species and BRs of the country were observed, while at the same time, the study documented widespread environmental contamination with *T. gondii*. The overall seroprevalence observed (22.0%) is within the range of those reported in previous serosurveys

TABLE 2 Generalized estimating equations analysis of risk factors associated with *Toxoplasma gondii* seropositivity in wild ruminants in Spain

Variable	Category	*p*-Value	OR CI$_{95\%}$
Species	Fallow deer	.003	4.51 (1.7–12.1)
	Roe deer	.001	4.29 (1.9–9.9)
	Red deer	.21	1.75 (0.7–4.2)
	Southern chamois	.35	0.61 (0.2–1.7)
	Mouflon	.83	1.17 (0.3–4.8)
	Iberian wild goat	a	a
Bioregion	BR1	<.001	6.13 (3.1–12.3)
	BR2	.28	1.59 (0.7–3.7)
	BR4	.19	1.64 (0.8–3.5)
	BR5	.42	1.47 (0.6–3.8)
	BR3	a	a

Abbreviation: OR, odds ratio.
[a]Reference category.

at regional level, ranging from 5.5% (San Miguel et al., 2016) to 30.2% (Barroso et al., 2020) (Table 3), and underlines the different epidemiological scenarios for this zoonotic pathogen in wild ruminant populations in Spain.

The risk factor analysis showed that the prevalence of *T. gondii* antibodies in wild ruminants was species related. Differences in the seroprevalence ratios among species are consistent with those observed in other European countries (Table 3). Differences in susceptibility, immunoreactivity, diet, feeding behaviour, habitat and/or the composition and abundance of the host community (including definitive hosts) are possible factors implicated in the differences of *T. gondii* seropositivity among species. We found significantly higher seroprevalences in roe and fallow deer compared with the five remaining species. Roe deer is the most abundant wild ruminant species in Europe (Burbaiteė & Csányi, 2009), and the second species after red deer in Spain (Garrido et al., 2019). Previous surveys in roe deer carried out in Europe showed wide variations in the prevalence of *T. gondii* antibodies among countries and between regions within the same country (Table 3; Fanelli et al., 2021). The average seroprevalence detected in the present study (39.6%) is of the same magnitude as those found in previous studies in Spain and other European countries such as Belgium, France, Norway and Sweden. Slightly higher seroprevalence values were observed in other studies in Belgium and Norway. On the contrary, lower seroprevalences were previously detected in some areas of Spain and in other European countries including the Czech Republic, Finland, Germany,

TABLE 3 Seroprevalence of *Toxoplasma gondii* in wild ruminant species in Europe

Species	Country	No. tested	Serological test[a]	% Positive	Reference
Barbary sheep	Czech Republic	24	IFAT	17	Bartova et al., 2017
		24	ELISA	25	Bartova et al., 2017
	Spain	18	MAT	5.6	**Present study**
		61	MAT	1.5	Candela et al., 2009
		10	MAT	10.0	Gauss et al., 2006
Iberian wild goat	Spain	346	MAT	7.8	**Present study**
		101	MAT	13.9	Almeria et al., 2021
		90	MAT	5.6	Almeria et al., 2018
		531	MAT	27.5	García-Bocanegra et al., 2012
		3	MAT	33.3	Gauss et al., 2006
Red deer	Belgium	7	ELISA	0.0	De Craeye et al., 2011
	Czech Republic	24	ELISA	20.8	Lorencova et al., 2015
		377	IFAT	45.0	Bartova et al., 2007
		303	SF	15.0	Hejlíček et al., 1997
	France	24	MAT	4.0	Aubert et al., 2010
	Germany	47	ELISA	6.4	Bier et al., 2020
	Italy	60	IFAT	22.0	Rocchigiani et al., 2016
		81	ELISA	39.5	Formenti et al., 2015
	Ireland	348	LAT	6.6	Halova et al., 2013
	Norway	571	DA	7.7	Vikoren et al., 2004
		99	SF	12.0	Kapperud, 1978
	Poland	552	ELISA	24.1	Witkowski et al., 2015
	Portugal	14	MAT	21.4	Waap et al., 2016
	Spain	553	MAT	16.6	**Present study**
		76	MAT	7.9	Almeria et al., 2021
		423	MAT	30.7	Barroso et al., 2020
		1,063	MAT	10.5	Almeria et al., 2018
		131	IFAT	13.0	San Miguel et al., 2016
		482	MAT	8.0	Gónzalez-Barrio et al., 2015
		441	MAT	15.6	Gauss et al., 2006

TABLE 3 (Continued)

Species	Country	No. tested	Serological test[a]	% Positive	Reference
Roe deer	Belgium	190	ELISA	43.2	Tavernier et al., 2015
		73	ELISA	52.0	De Craeye et al., 2011
	Czech Republic	79	IFAT	24.0	Bartova et al., 2007
		95	SF	14.0	Hejlíček et al., 1997
	Finland	17	DA	17.6	Jokelainen et al., 2010
	France	1,155	MAT	43.7	Gotteland et al., 2014
		222	ELISA	46.4	Candela et al., 2014
		60	MAT	60.0	Aubert et al., 2010
	Germany	125	ELISA	12.8	Bier et al., 2020
		295	MAT	29.0	Heddergott, Steinbach, et al., 2018; Heddergott, Osten-Sacken, et al., 2018
	Italy	207	LAT	13.0	Gaffurri et al., 2006
	Norway	760	DA	33.9	Vikoren et al., 2004
		8	SF	63.0	Kapperud, 1978
	Poland	92	ELISA	30.4	Witkowski et al., 2015
		19	DA	15.8	Sroka et al., 2007
	Portugal	1	MAT	0.0	Lopes et al., 2011
	Spain	356	MAT	39.6	**Present study**
		22	MAT	13.6	Almeria et al., 2018
		84	ELISA	25.0	Morrondo et al., 2017
		228	IFAT	2.0	San Miguel et al., 2016
		135	ELISA	43.7	Sevila et al., 2014
		160	DA	13.7	Panadero et al., 2010
		278	MAT	39.2	Gamarra et al., 2008
		33	MAT	21.8	Gauss et al., 2006
	Sweden	199	DA	34.0	Malmsten et al., 2011
Fallow deer	Belgium	4	ELISA	0.0	De Craeye et al., 2011
	Czech Republic	13	ELISA	23.1	Lorencova et al., 2015
		143	IFAT	17.0	Bartova et al., 2007
		3	SF	100	Hejlíček et al., 1997
	France	4	MAT	25.0	Aubert et al., 2010
	Poland	167	ELISA	10.0	Moskwa et al., 2018
	Spain	372	MAT	37.1	**Present study**
		62	MAT	19.0	Almeria et al., 2021
		452	MAT	29.7	Barroso et al., 2020
		294	MAT	15.6	Almeria et al., 2018
		79	MAT	24.0	Gauss et al., 2006

TABLE 3 (Continued)

Species	Country	No. tested	Serological test[a]	% Positive	Reference
Mouflon	Czech Republic	41	ELISA	24.4	Lorencova et al., 2015
		105	IFAT	9.0	Bartova et al., 2007
		20	SF	10.0	Hejlíček et al., 1997
	France	143	MAT	14.7	Gotteland et al., 2014
		31	MAT	16.0	Aubert et al., 2010
	Germany	138	MAT	22.5	Heddergott, Steinbach, et al., 2018; Heddergott, Osten-Sacken, et al., 2018
	Spain	209	MAT	11.5	**Present study**
		64	MAT	3.1	Almeria et al., 2021
		216	MAT	5.6	Almeria et al., 2018
		27	MAT	14.8	Gauss et al., 2006
Southern chamois	France	101	MAT	16.8	Gotteland et al., 2014
	Spain	186	MAT	13.9	**Present study**
		149	IFAT	4.0	San Miguel et al., 2016
		10	MAT	20.0	Gauss et al., 2006

[a]DA, Direct Agglutination; ELISA, Enzyme-Linked Immunosorbent Assay; IFAT, Indirect Fluorescent Antibody Test; LAT, Latex Agglutination Test; MAT, Modified Agglutination Test; SF, Sabin–Feldman dye test.

Italy and Poland (Table 3). Even though accurate comparisons cannot be made given the differences in the number of animals tested, the populations sampled and/or the different serological methods used, the seroprevalence observed in roe deer in the present study was high in every BR where this species was sampled. Higher seroprevalence of *T. gondii* infection in roe deer compared to other wild ruminant species has also been observed in other European countries, suggesting that consumption of roe deer raw or undercooked meat may be an important source for human infection (Fanelli et al., 2021; Gotteland et al., 2014; Vikoren et al., 2004).

Fallow deer is the third most important wild ruminant game species in Spain (MAP A, 2021). Information about the seroprevalence of *T. gondii* in this species in Europe is still scarce and, in some studies, the number of samples analysed was too low to accurately estimate seroprevalence (Table 3). Data have been only reported from Belgium, the Czech Republic, France, Poland and Spain (Table 3). The seroprevalence of *T. gondii* detected in fallow deer in our study (37.1%) is the highest reported in Europe to date, except for a study in the Czech Republic (100% of the three fallow deer tested) (Hejlíček et al., 1997). In the present study, fallow deer showed the highest seroprevalence levels compared with the other wild ruminant species analysed in four of the five BRs, being the third in BR4. The higher seroprevalence of *T. gondii* in fallow deer compared with other wild ruminant species, with exception of roe deer, is consistent with previous serological studies in Spain (Almería et al., 2018, 2021; Barroso et al., 2020; Gauss et al., 2006). A molecular study in South-West Spain reported the highest prevalence (48% of 21 fallow deer) of *T. gondii* DNA in this species compared with other wild ruminants analysed (Calero-Bernal et al., 2015). Further research is required to determine the

factors involved in the high susceptibility to *T. gondii* exposure in fallow deer.

Our results highlight a widespread exposure to *T. gondii* in wild ruminant populations in Spain. However, the spatial distribution of this parasite was not homogenous, with significantly higher prevalence of *T. gondii* antibodies in BR1. This finding is in accordance with the geographical differences observed in previous studies of wild ruminants in different areas/regions of Spain. Gauss et al., (2006) observed significantly higher *T. gondii* seroprevalence in red deer from wetter areas of north-eastern Spain than those from central and southern areas, while Gamarra et al., (2008) found that roe deer from the northern coastal habitats had higher seropositivity values than those sampled in central Spain. A north-south gradient of *T. gondii* seropositivity was also reported by Jokelainen et al., (2010) in Finland. The geographical differences in seroprevalence observed in the present study could be related to the type of habitat, presence, abundance of domestic/feral or wild felids and environmental (stochastic) factors that influenced the persistence of viable oocysts in each region. BR1, the BR with significantly higher seroprevalence of *T. gondii* in wild ruminants compared to the rest of BRs in Spain, is characterized by mild temperatures and high average annual rainfall, which provides high humidity. These climatic conditions are optimal for survival and sporulation of oocysts in soil, food and water contaminated with domestic or wild feline faeces, as sources of infection for wild ruminants (Dubey, 2010; Dubey & Beattie, 1988). Unfortunately, there is almost no information on *T. gondii* in felids in BR1 (Sobrino et al., 2007), and no large-scale studies have been carried out to assess *T. gondii* distribution in these species in this area of the country. Because most of the sampling sites of the present study were located far from urban areas, feral and wild felines that

share habitat with wild ruminants could play a more relevant role than domestic cats in the epidemiology of *T. gondii* in these Iberian ecosystems. Seropositivity to *T. gondii* in feral cats [free-living cats with generally little or no direct human interaction or dependency (Sparkes et al., 2013)] has been shown to be high in mainland Spain, ranging between 12.3% and 52% (Millán et al., 2009; Villanueva-Saz et al., 2021). Although some studies have shown that seroprevalence to *T. gondii* in the endangered Iberian lynx (*Lynx pardinus*) is high in Spain, ranging between 44.0% and 81.5% (Roelke et al., 2008; Sobrino et al., 2007), its spatial distribution in south and central Spain could not have affected the high seroprevalence observed in wild ruminants in northern Spain. Thus, other wild felids could be more involved in the transmission of *T. gondii* to wild ruminant populations in those scenarios. Although studies in European wild-cats (*Felis silvestris silvestris*) are scarce in Spain (Candela et al., 2019; Sobrino et al., 2007), reported seroprevalences (≥50.0%) indicate that this species could be important in the epidemiology of *T. gondii*, particularly in BR1, the Spanish region with the highest distribution of this wild felid species (MITECO, 2020). Further studies are needed to determine the importance of feral cat and European wildcat populations in the epidemiology of *T. gondii* in the North of Spain.

Spain is one of the countries in Europe with the largest number of wild ruminants hunted per year (Garrido et al., 2019), with around 145,000 red deer, 143,000 roe deer, 18,000 fallow deer, 11,000 mouflon, 6,700 Iberian wild goats, 1,400 Southern chamois and 600 Barbary sheep hunted every year (MAP A, 2021). Butchers, Veterinarians and/or hunters could be at risk of *T. gondii* infection during evisceration and handling of harvested wild ungulates (Dubey et al., 2020; EFSA, 2007). It should be noted that cervids (red, roe and fallow deer) are the main wild ruminant species destined for human consumption in Spain and, in many parts of Spain, hunters frequently prepare home-made products derived from meat of these large game species, leading to a risk of food-borne transmission of *T. gondii*. Large game consumers might also be exposed to the parasite when eating raw or undercooked meat or meat products derived from these species (Ross et al., 2001). Based on the number of seropositive cervids found in the present study, around 1,900, 460 and 660 tons of meat and derived products from red deer, roe deer and fallow deer, respectively, could be contaminated by *T. gondii* cysts. Meat from wild ruminants, in particular wild cervids, could therefore be an important source of *T. gondii* infection in humans, not only in Spain, but also throughout Europe, given that 90% of the meat and products from large game in Spain are exported to other European countries (Fundacion Artemisan, 2017).

The highest antibody titres (≥ 1:100) were observed in Iberian wild goat (56.6%; 15/27), roe deer (27.7%; 39/141) and red deer (26.1%; 24/92). The high antibody titres detected in Iberian wild goat could indicate recent or recurrent infections in this species (Almería et al., 2021), which could be of animal health concern. The low overall seropositivity found in this wild caprine (7.8%) suggests a limited circulation of *T. gondii* in their populations. It is worth noting that the main wild ruminant species both hunted and destined for human consumption in Spain (red deer and roe deer) showed high antibody

titres against *T. gondii*. This finding could have significant implications for public health since the rate of isolating viable parasites has been shown to be positively associated with MAT titres in wild ruminants (Dubey et al., 2020, 2021).

Our study had some limitations. First, the temporal distribution of the sampling, from 1999 and 2020, was not homogenous. Second, information on sex and age was not available for every animal analysed which could explain the lack of a significant association between these individual factors with *T. gondii* seropositivity in the present study. Previous studies observed a significant correlation with those variables in wild ruminant species in Spain (Almería et al., 2018; Barroso et al., 2020). In addition, a third limitation was that it was not possible to reach enough sample size for certain species within each BRs (Figure 1); therefore, the seropositivity obtained at species level at each BR should be carefully interpreted. On the other hand, the overall sample size for each species allowed us to adequately establish the species seroprevalence at national level as discussed above.

In summary, our results suggest that wild ruminants can potentially be considered good indicators of environmental contamination by *T. gondii* oocysts. The overall seroprevalence found in the present study, as well as the variations of seropositivity at BR level, indicates widespread but not homogeneous distribution of *T. gondii* in wild ruminant populations in Spain, which can be of animal and public health concern. Undercooked game meat should not be consumed by humans or fed to cats. Proper cooking or freezing large game meat will greatly reduce the risk of *T. gondii* infection (Dubey et al., 2021). Precautions should also be taken when handling or eviscerating carcasses of harvested wild ruminants. In addition, trap–neuter–release programmes could be implemented as control tool of the feral cat populations. Further studies are warranted to elucidate the *T. gondii* infection levels in meat and derived products from these exposed large game species, particularly cervids, and the risk of transmission of this food-borne zoonotic disease.

CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

ETHICAL APPROVAL

All the animals were legally hunted under Spanish and EU legislation by hunters with appropriate permits during the hunting

season (October to February) or culled within population control programmes of game reserves. This study did not involve purposeful killing of animals, and the blood samples were not collected specifically for this study. Protocols, amendments and other resources were performed according to the guidelines approved by each Autonomous government following the R.D.1201/2005 of the Ministry of Presidency of Spain. No ethical approval was deemed necessary. The collection of blood samples was performed for routine procedures in compliance with the Ethical Principles in Animal Research before the design of this study.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available on request from the authors.

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