Seroepidemiology of *Toxoplasma gondii* in wild ruminants in Spain

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Original Article

**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%...
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 (Feková et al., 2020). Importantly, clinical toxoplasmosis and even *T. gondii* outbreaks have been reported after consumption of 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.

### 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.
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%) of 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 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

| Variable | Category | No. positives/overall | 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 |

*Missing values omitted.
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; CI95%: 34.5–44.7) in roe deer, 37.1% (138/372; CI95%: 32.2–42.0) in fallow deer, 16.6% (92/553; CI95%: 13.5–19.7) in red deer, 14.0% (26/186; CI95%: 9.0–18.9) in Southern chamois, 11.5% (24/209; CI95%: 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; CI95%: 0.0–16.1) in Barbary sheep.

Geographically, seroprevalence values ranged between 45.6% (171/375; CI95%: 40.6–50.6) in BR1 and 13.0% (60/461; CI95%: 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; CI95%: 12.1–17.2) during the second study period (2013–2015) and fell to 11.4% (65/568; CI95%: 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 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,
| Species            | Country       | No. tested | Serological test | % 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             |
|                    | 24            | ELISA      | 20.8             |            | Lorencova et al., 2015             |
|                    | 377           | IFAT       | 45.0             |            | Bartova et al., 2007               |
|                    | 303           | SF         | 15.0             |            | Hejilieček et al., 1997            |
|                    | 24            | MAT        | 4.0              |            | Aubert et al., 2010                |
|                    | 47            | ELISA      | 6.4              |            | Bier et al., 2020                  |
|                    | 60            | IFAT       | 22.0             |            | Rocchigiani et al., 2016           |
|                    | 81            | ELISA      | 39.5             |            | Formenti et al., 2015              |
|                    | 348           | LAT        | 6.6              |            | Halova et al., 2013                |
|                    | 571           | DA         | 7.7              |            | Vikoren et al., 2004               |
|                    | 99            | SF         | 12.0             |            | Kapperud, 1978                     |
|                    | 552           | ELISA      | 24.1             |            | Witkowski et al., 2015             |
|                    | 14            | MAT        | 21.4             |            | Waap et al., 2016                  |
|                    | 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              |            | González-Barrio et al., 2015       |
|                    | 441           | MAT        | 15.6             |            | Gauss et al., 2006                 |
| (Continues)        |               |            |                  |            |                                    |
### TABLE 3 (Continued)

| Species     | Country         | No. tested | Serological test | % 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       | Heddergott, Steinbach, et al., 2018; Heddergott, Osten-Sacken, et al., 2018 |
|             |                 | 295        | MAT              | 29.0       |                                               |
|             | 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       | Sevilla 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       | Lorenco 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                           |

(Continues)
TABLE 3

| Species            | Country         | No. tested | Serological testa | % 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               |

*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 homogeneous, 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, prevalence, 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% (Milián 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.

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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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