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Human and environmental factors driving *Toxoplasma gondii* prevalence in wild boar (*Sus scrofa*)

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ABSTRACT

As one of the most relevant foodborne diseases, it is essential to know the factors related to the transmission, persistence and prevalence of *Toxoplasma gondii* infection. Eurasian wild boar (*Sus scrofa*) might play a relevant role in *T.gondii's* life cycle. This species is the most consumed big game animal in Spain and may act as a source of infection if the meat is eaten raw or undercooked or due to cross-contaminations. Additionally, wild boar can act as an excellent bioindicator of *T.gondii* circulation in the ecosystem, because its natural behaviour leads to exposure to oocysts from the soil when rooting and tissular bradyzoites when scavenging. A total of 1003 wild boar were sampled from 2010 to 2017 in Mediterranean Spain. Blood samples were tested with an indirect ELISA test giving a total of 14.1% (95% confidence interval 12.0–16.4%) positive results. The prevalence was not homogeneous in neither the animals nor the sampled districts. Significant differences were found regarding age, climatic conditions and human space occupancy. Human population aggregation, assessed by Demangeon's index, was identified as an influential factor in *T.gondii* infection risk. This multiple approach allows us to evaluate local risks for human and environmental contamination.

1. Introduction

Toxoplasma gondii is an important tissue cyst-forming coccidia of great medical and veterinary importance, with worldwide distribution (Antolová et al., 2007). Domestic and wild felids are the only definitive hosts. After primary infection, cats produce and release millions of oocysts in their faeces (Dubey, 2009). The definitive and intermediate hosts may be infected by the ingestion of water or food contaminated with oocysts, by the ingestion of tissue cysts in infected animal tissues or congenitally (Dubey and Beattie, 1988).

Between a quarter and a third of the human population is infected with *T. gondii* (Larkin, 2007; Kijlstra and Jongert, 2008). It is the most common parasitic disease in the European Union affecting between 50 and 80% of the population (EFSA, 2012). Although most infections are asymptomatic, the social and economic impact of *T.gondii* infection is estimated to be comparable to those of other major foodborne diseases (Kijlstra and Jongert, 2008). Infections can have serious consequences by causing abortion, foetal abnormality and perinatal death in pregnant women and in other mammals (Hill et al., 2008).

Severe clinical infection is considered rare in pigs (Dubey, 2009). However, congenital infection, with significant foetal malformations, has been described in Eurasian wild boar (*S. scrofa*) (Calero-Bernal et al., 2013). Wild boar are in contact with *T. gondii* from their local environment, and therefore could act as indicators for understanding the geographical variations of the parasite (Beral et al., 2012). Additionally, the species plays an important role as one of the most relevant scavengers in the Mediterranean ecosystem (Sánchez-Zapata et al., 2010; Mateo-Tomás et al., 2015; Vicente et al., 2016), exposing themselves to

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an increased infection risk due to this trophic behaviour.

Wild boar meat is usually consumed by hunters or distributed locally (GfK, 2017. When the meat is eaten undercooked it could act as a source of infection for people (Choi et al., 1997). Moreover, a lack of hygiene during the manipulation and culinary preparation can cause cross-contaminations, becoming an additional origin of human contagion (Hughes et al., 2000).

Wild boar is widespread in mainland Spain and due to their abundance, is one of the most popular big game species (Gortazar et al., 2000). In the autonomous region of the Valencia Community (Eastern Spain) it is the hegemonic big game target (86% of all big game species) (Torres et al., 2019). The annual hunting harvest has doubled from the 2000/01 season (11,406) to 2017/18 (26,832) (Torres et al., 2019), while the number of hunters has decreased by 55% (from 81,892 to 36,829 in the same period) (Generalitat Valenciana, 2019) suggesting an exponential population growth.

This region is experiencing accelerated habitat changes mainly due to the increasing development of coastal and some inland areas, the abandonment of traditional farming activities and the expansion of modern intensive agricultural methods (Serra et al., 2008). The wild boar is able to increase rapidly in those fragmented landscapes (Rutten et al., 2019) in which the combination of forest regeneration, croplands, anthropogenic food resources and water availability throughout the year constitutes an attractive habitat (Fernández-Llario et al., 2003; Castillo-Contreras et al., 2018). Spatial and temporal variations in parasite prevalence are expected in the context of recent habitat change and range expansion (Lizana et al., 2021).

The goal of this study is to determine the seroprevalence of *T. gondii* in wild boar (*S. scrofa*) in the recently colonized Mediterranean ecosystem of Eastern Spain and to assess the influence of factors such as age, sex, climate, game management, wild boar abundance and human influence on the territory.

2. Materials and methods

2.1. Study area

The Valencian Community is an autonomous region located in the East of the Iberian Peninsula (Fig. 1). It is administratively divided into (North to South) 3 provinces (Castellon, Valencia and Alicante) with 31 districts and 542 municipalities.

2.1.1. Climate

Weather conditions of the area tend to be mild, especially on the coast, but present different variants of the Mediterranean climate. According to the classification of Köppen-Geiger (Kottek et al., 2006)

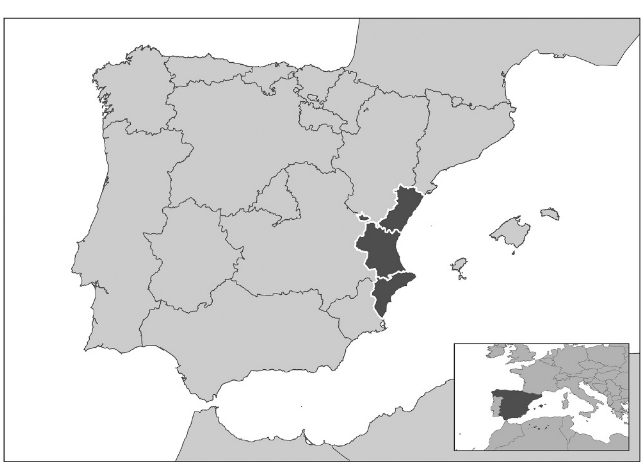

Fig. 1. Location of the Valencian Community in the Iberian Peninsula.

(Fig. 2), the Typical Mediterranean climate (Csa), is extended along the northern and central coastland and centre of the Community. Winters are not very cold, and summers are long, dry and hot, with maximums around 30 °C. The Continental Mediterranean climate (Csa - Bsk), is a transition between the Continental climate and the Typical Mediterranean. It is located in the northern and central inlands. Winters have frequent snowfalls, and summers are very warm and dry with temperatures reaching 40 °C. The Dry Mediterranean climate (Bsh - Bsk), occurs at the southern end of the territory, where the temperatures are very warm in summer (maximums above 30 °C) with mild winters around 10 °C (Fig. 2). This area has the lowest rainfalls of the whole region, less than 350 mm per year, normally concentrated in just a few days (De Luís et al., 2000; González-Hidalgo et al., 2003). Data processing was done clustering the districts according to the bioclimatic area they mainly belong to.

2.1.2. Wild boar density

Information about the geographic origin of the samples and management systems (fenced game estates and free populations) was obtained in order to compare different scenarios and *T.gondii* prevalence. The potential influence of wild boar density in free populations was evaluated classifying the study area in four categories, based on hunting bags. Category 1 with 0.35–1 wild boar/km2; Category 2 from 1.01 to 2 wild boar/km2; Category 3, 2.08–3 wild boar/km2 and Category 4 with >3 wild boar/km2 (Torres et al., 2019).

2.1.3. Human population

In order to evaluate the potential impact of *T.gondii* presence related with human occupation, the territory was classified following two indexes:

Human density: as the number of people in a given area. Districts were categorized as Rural (<100 inhabitants/km^2), Intermediate (100–499 inh/km^2) and Urban (>500 inh/km^2) (EU, 2018) (See Table 2).

Demangeon's index: This parameter describes the human aggregation and spreading in a given administrative unit (municipality). It is expressed with the formula: $k = (E^*N) / T$.

E corresponds to the total human population in the municipal territory, except for the inhabitants living in the main nucleus. N is the number of human settlements (urban areas, hamlets and isolated houses) except for the main nucleus. T is the total population of the administrative unit.

Following this classification, municipalities can be categorized as Maximum Concentration (Demangeon 0.00 to 0.09) inherent to small villages, with few inhabitants living outside the main town. Normal Concentration (0.10 to 0.99) corresponds to medium towns, with hamlets and isolated buildings dispersed around the municipal area. Normal Dispersion (rate 1.00–9.99) comprises of bigger settlements such as big towns and even medium to big cities surrounded by extensive commuter belts of residential homes (Petrizan-Lombaña, 2010). Finally, Maximum Dispersion municipalities (rate > 10) are where most of the territory has been occupied. Only a few locations in the south of our study area show this value. There are villages with a long residential tradition, where most of the human population live spread out from the main foundation nucleus.

2.2. Sample collection

A total of 1003 wild boar were sampled from the 2010 to 2017 hunting seasons. The game events providing the samples took place in 26 of the 31 districts in which the region is divided. References of the animals (geographic origin, date, sex, weight and age) were taken. Age estimation was done by dental eruption patterns (Boitani and Mattei, 1992; Oroian et al., 2010; Boitani et al., 2014) and coat colour (Rosell et al., 2001). Animals were categorized in piglets (until 6 months old, striped coat pattern, < 20 kg), juveniles (7 to 18 m.o., between 20 and

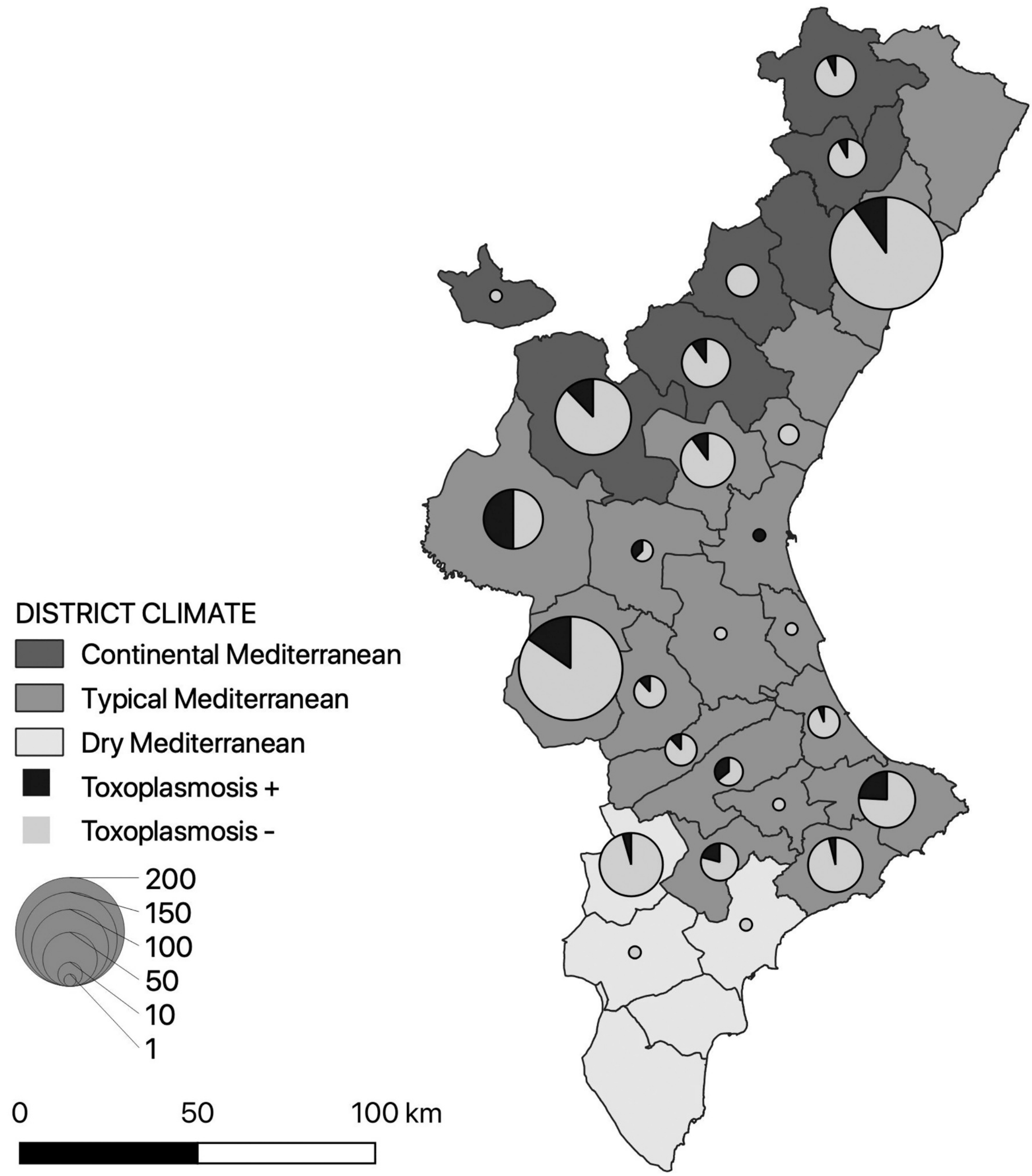

Fig. 2. *T.gondii* prevalence in sampled wild boars by district. Districts were clustered according with the bioclimatic area they mainly belong to. Variants of the Mediterranean climate are represented. (Colour clave: Dark Grey = Continental Mediterranean / Csa-Bsk; Grey = Typical Mediterranean / Csa; Pale Grey = Dry Mediterranean / Bsh-Bsk).

40 kg) and adults (> 18 m.o., > 40 kg).

Blood samples were collected from the thoracic cavity, heart (Gauss et al., 2006) or from the cavernous sinus of the dura mater (Arenas-Montes et al., 2013) and placed in 14 mL plastic tubes. The samples were centrifuged (3500 rpm for 10 min (Sroka et al., 2008)) and the resulting serum was stored in aliquots at −20 °C (Gauss et al., 2005; Beral et al., 2012) until analysis.

2.3. Laboratory analyses

Antibodies against *T. gondii* were detected using the commercial indirect ELISA test ID Screen® Toxoplasmosis Indirect Multi-species (IDvet, Grabels, France) following the manufacturer's instructions. This kit has been used to detect *T.gondii* infection in wild boar by previous studies (Wallander et al., 2015; Laforet et al., 2019; Crotta et al., 2021).

2.4. Data analysis

The prevalence and the confidence intervals (95% CI) were calculated with Quantitative Parasitology (QPweb) (Reiczigel et al., 2019). The associations among qualitative and categorical variables were analyzed by Pearson's Chi square function in SPSS 19.0 software for Windows.

Several models of logistic regression were prepared with the forward stepwise (conditional) method, in order to detect the factors involved in the *T.gondii* contagion risk.

Variables with a probability (p) value ≤0.05 were considered statistically significant.

3. Results

The total apparent prevalence rate of *T.gondii* was 14.1% (141/1003; 95% CI 12.0–16.4%). A total of 15.3% (70/458) females were affected, compared to 13.4% (67/500) males ($p = 0.418$), gender was not registered in 45 specimens (8.9% positives; 4/45). Concerning age, 11 out of 78 piglets were found positive (14.1%; CI 7.3–23.8%), compared to 22 out of 263 juveniles (8.4%; CI 5.3–12.4%) and 17% of adults (101/593; CI 14.1–20.3%). This is a significant difference ($p = 0.007$) stratifying by age. In 69 individuals the age was not registered, and 7 of them tested positive (10.1%; CI 4.2–19.8%).

Seroprevalence in fenced estates was not significantly higher (18.4%; CI 10.5–29%; 14/76) than in free living populations (13.7%; CI 11.6–16.1%; 127/927) ($p = 0.255$). By contrast, densities of free wild boars, classified in four categories considering hunting bags, detected significant differences between them ($p < 0.001$).

Concerning the climatic conditions, the prevalence found in the Continental Mediterranean area (Csa-Bsk) was 9.5% (CI 6.4–14.7%; 20/211), decreasing to 4.2% (CI 0.4–10.7%; 3/71) in the Dry Mediterranean (Bsh-Bsk). By contrast, seropositivity rose to 16.4% (CI 13.7–19.2%; 118/721) in Typical Mediterranean climate areas (Csa), a statistically significant result ($p = 0.002$) compared with the other bioclimates.

Regarding human densities, Rural districts showed a prevalence of 15.7% (CI 13–19.2%; 86/548), while Intermediate ones were 11.9% seropositive (7.9–14.2%; 54/453). In Urban districts prevalence reached 50%, but the low number of samples (only 2 wild boar samples were taken from urban areas, because the full colonisation of those areas has not yet happened) did not allow a statistical evaluation (CI 1.3–98.7%). The comparison between the results of rural and intermediate districts gave a marginal-significant result ($p = 0.087$).

The relation between the prevalence of *T.gondii* infection and the human occupancy in the territory was further studied with Demangeon's index, to determine the effect of human spatial dispersion. We found a significant relation of *T.gondii* prevalence and Demangeon's index ($p = 0.025$).

A logistic regression model was used to study the relations between seropositivity and climate, wild boar density, age, human density and Demangeon's index (see Table 1).

The model allowed us to demonstrate the action of the Typical Mediterranean climate (Csa) as a risk factor in comparison with the other bioclimates, which act as protective variables, essentially the Dry Mediterranean (OR = 0.125; CI 0.026–0.589). The same results are achieved with adult age, acting as a supplementary risk factor (OR 2.387; CI 1.019–5.592) compared with younger groups, especially piglets.

Concerning wild boar density, the lowest density areas (named as Category 1) resulted in a lower prevalence than expected, while categories 2 and 4 had higher ratios than expected.

Referring to Demangeon's index, it was found that Normal Dispersion municipalities showed a higher seropositivity than expected. This category acts as a risk factor, compared with areas of a higher human aggregation, with a protective effect. The protection is even more significant in the Maximum Concentration municipalities (OR 0.453; CI 0.269–0.762) than in the Normal Concentration ones (OR 0.679; CI 0.416–1.108). Maximum Dispersion municipalities were not evaluated, only a few locations in the south of our study area show this value and no wild boar from this area were sampled, due to low wild boar densities in this region.

Table 1
Logistic regression model, designed to study the relations between seropositivity and human density, wild boar density, age, climate and Demangeon's index.

			CI 95%	
Variables	Sig.	OR	Inferior	Superior
URBAN_DENSITY (Reference)	0.022			
INTERMEDIATE_DENSITY	0.051	0.027	0.001	1.018
RURAL_DENSITY	0.103	0.049	0.001	1.843
WILD_BOAR/KM2_CAT (4) (Reference)	0.000			
WILD_BOAR /KM2_CAT(1)	0.002	0.303	0.144	0.640
WILD_BOAR/KM2_CAT(2)	0.733	0.860	0.361	2.047
WILD_BOAR /KM2_CAT(3)	0.064	0.435	0.180	1.050
AGE_UNKNOWN (Reference)	0.005			
AGE_ADULT	0.045	2.387	1.019	5.592
AGE_JUVENIL	0.897	1.064	0.414	2.732
AGE_PIGLET	0.382	1.606	0.555	4.646
BIOCLIMATE_Csa (Reference)	0.000			
BIOCLIMATE_Bsh-Bsk	0.009	0.125	0.026	0.589
BIOCLIMATE_Csa-Bsk	0.000	0.311	0.172	0.559
DEMANGEON_Norm_Dispersion (Reference)	0.011			
DEMANGEON_Max_Concentration	0.003	0.453	0.269	0.762
DEMANGEON_Norm_Concentration	0.122	0.679	0.416	1.108
Constant	0.265	8.668		

Table 2
Sampling sites. Cont. Med (Continental Mediterranean / Csa-Bsk), Typ. Med (Typical Mediterranean / Csa) and Dry Med (Dry Mediterranean / Bsh-Bsk). Seropositivity for each bioclimatic area was 9.5% (20/211) in Continental Mediterranean, 16.4% (118/721) in Typical Mediterranean and 4.2% (3/71) in Dry Mediterranean. Attending human density, districts were classified as Rural (<100 inh/km^2), Intermediate (100–499 inh/km^2) and Urban (>500 inh/km^2) (EU 2018).

Sampling site (North to South)	Climatic area	Human density	No. examined	No. positive	% positives
Castellon Province					
Els Ports	Cont. Med	R	29	2	6.89
Baix Maestrat	Typ. Med	R	0	0	–
Alt Maestrat	Cont. Med	R	25	2	8.00
Alcalaten	Cont. Med	R	0	0	–
Plana Alta	Typ. Med	I	215	21	9.76
Alt Millars	Cont. Med	R	18	0	0.00
Plana Baixa	Typ. Med	I	0	0	–
Alt Palancia	Cont. Med	R	40	4	10.00
Valencia Province					
Camp de Morvedre	Typ. Med	I	7	0	0.00
Rincon de Ademuz	Cont. Med	R	1	0	0.00
Serrans	Cont. Med	R	98	12	12.24
Camp de Turia	Typ. Med	I	50	5	10.00
Horta	Typ. Med	U	1	1	100.00
Requena-Utiel	Typ. Med	R	60	30	50.00
Foia de Bunyol	Typ. Med	R	8	3	37.50
Ribera Alta	Typ. Med	I	1	0	0.00
Ribera Baixa	Typ. Med	I	1	0	0.00
Valle de Ayora	Typ. Med	R	183	28	15.30
Canal de Navarres	Typ. Med	R	17	2	11.76
Safor	Typ. Med	I	17	1	5.88
Costera	Typ. Med	I	17	2	11.76
Vall Albaida	Typ. Med	I	14	5	35.71
Alicante Province					
Marina Alta	Typ. Med	I	54	13	24.07
Comtat	Typ. Med	R	1	0	0.00
Marina Baixa	Typ. Med	I	51	2	3.92
Alcoia	Typ. Med	I	24	5	20.83
Alt Vinalopo	Dry Med	R	68	3	4.41
Alacanti	Dry Med	U	1	0	0.00
Vinalopo Mitja	Dry Med	I	2	0	0.00
Baix Vinalopo	Dry Med	U	0	0	–
Vega Baja	Dry Med	I	0	0	–

4. Discussion

We studied the exposure of wild boar to *T.gondii* in a region with important gradients in climate, human population distribution and wild boar density. In comparison with previous studies carried out in Europe using ELISA as a diagnostic technique, the final result of 14.1% positives is higher than those found in Switzerland (6.7%) (Berger-Schoch et al., 2011) or the Slovak Republic (8.1%) (Antolová et al., 2007), but lower than in the Czech Republic (26.2%) (Bártová et al., 2006), continental Denmark (27.7%) (Laforet et al., 2019), Latvia (33.2%) (Deksne and Kirjušina, 2013), Northern Italy (49.5%) (Crotta et al., 2021) or Sweden (50%) (Wallander et al., 2015).

Regarding individual factors (gender and age), non-statistically significant differences among sexes ($p = 0.418$) were detected in this research, which is consistent with previous studies (Laforet et al., 2019) where gender has been revealed as a factor with no importance in disease prevalence. However, significant differences ($p = 0.007$) were found stratifying by age groups. Those results also coincide with other research, where increasing relations among age and infection have been found in both domestic and wild animals (Richomme et al., 2010; Katzer et al., 2011; Rostami et al., 2017), indicating the relevance of environmental exposure to oocysts in comparison with prenatal infection (Malmsten et al., 2011).

In contrast to other parts of the country, such as the southwestern quadrant, fenced game estates, with high wild boar densities, are not a common way to manage populations for hunting purposes in Valencia (Acevedo et al., 2006; Giménez-Anaya et al., 2020). Only 76 individuals came from these places, in comparison with 927 free roaming wild boars. Consistent with previous studies (Gauss et al., 2005), seroprevalence in fenced estates was higher than in free-living populations, but differences were not statistically significant in our results.

The study of wild boar density in relation to *T.gondii* prevalence showed significant differences between categories. Coinciding with previous reports (Gauss et al., 2005), a higher prevalence was found in more densely populated areas, showing rates of positivity higher than expected in category 4 (>3 wild boar/km^2). Surprisingly, category 2 (1.01 to 2 wild boar/km^2) showed infection rates higher than expected too. Category 1 acted as a protective factor, with prevalence rates lower than expected.

Weather conditions have been revealed as a capital factor for oocyst survival (Meerburg and Kijlstra, 2009). Consequently, positivity rates showed variations between the three bioclimatic territories, with lower prevalence in the most extreme climatic regions from our study area. Low levels of annual rainfall have been related to a reduction of oocyst survival and, coincidently to our results in the Dry Mediterranean climate (Bsh-Bsk), arid and sub-arid regions have the lowest rates of infection in definitive and intermediate hosts (Vollaire et al., 2005; Meerburg and Kijlstra, 2009; Afonso et al., 2010).

The categorization of Districts according to human density (inhabitants/km^2) provided no relevant information about *T.gondii* presence.

Despite being present in inland districts and coastal mountains in our study area, European wild cat (*Felis silvestris*) populations are scarcely known and considered rare (Barona, 2012), therefore classified as near threatened on a country level (López-Martín et al., 2007). Thus, it can be assumed the key role of free-ranging domestic cats (*Felis catus*), both stray cats and pets allowed go outdoors, acting as the most relevant definitive hosts of *T.gondii*. In Mediterranean environments of the Iberian Peninsula, domestic cats are highly dependent on human settlements. Free-ranging cats do not venture far from inhabited houses (Ferreira, 2010; Gil-Sánchez et al., 2015; Belda et al., 2017). Because there are no data about feral and free-ranging domestic cats in Spain, the study of human occupancy on the territory, as a driver of cat presence, could conditionate the levels of environmental *T.gondii* (Gilot-Fromont et al., 2012).

Due to cat's dependence on human populations, Demangeon's index can be used as an indirect way to study cat's spreading around the territory, in their epidemiologic role of oocyst producers. The lowest levels of seroprevalence were associated with Maximum Concentration municipalities. As a result of high human aggregation, cats live in the core area and most of the territory remains free of felines, thus becoming free of infective oocysts. Intermediate levels were found in Normal Concentration villages, as a result of a greater proportion of the land being accessible to free ranging cats. Normal Dispersion municipalities, with a greater spread of the human population have, because of the greatest area with cat presence, the highest prevalence. This effect is confirmed by the logistic regression model, evidencing Normal Dispersion as a risk factor, in comparison to the protective effect (cat contention) of Maximum and Normal concentration. The fragmented areas formed by the mosaic of detached houses and progressively abandoned croplands create attractive environments for wild boar, who find refuge and anthropogenic food resources (Castillo-Contreras et al., 2018). This kind of environment increases the interaction between domestic or stray cats and wildlife (Hill et al., 2008). This result is coherent with previous studies, which have related higher levels of *T.gondii* infection in wild boars in proximity to inhabited areas (Almería et al., 2021).

5. Conclusions

The rate of infection points to wild boar as a potential transmitter of *T.gondii* tissue cysts in game meat consumers. Their generalist feeding behaviour makes wild boar a good bioindicator of *T.gondii* presence, being in contact with soil oocysts because of rooting and with tissular bradyzoites when scavenging (Almería et al., 2021). Wild boar offal and butchery remains, when not properly disposed, can also act as a source of contamination for the environment and new hosts, such as free-ranging cats, able to complete *T.gondii*'s life cycle (spreading new oocysts) or other scavenger species, including the wild boar itself (Tenter et al., 2000; Vicente et al., 2011; Coelho et al., 2014; Mateo-Tomás et al., 2015).

Climatic conditions have been revealed as key factors in *T.gondii* prevalence. Higher rates of infection are found in mild climate areas, where oocysts find suitable conditions for survival.

The principal new discovery of this research is providing evidence that human aggregation acts as an influential factor in *T.gondii* infection prevalence, and the usefulness of the Demangeon's index as a tool to study the spread of *T.gondii* in the environment. This index allows us to deduce the potential influence of domestic cats (as the most relevant *T. gondii* definitive host in absence of other felids) in their local environment.

Previous studies have shown the importance of a regional approach to *T.gondii* investigation (Richomme et al., 2010; Beral et al., 2012). Local conditions (environment, microclimate, wild boar populations and human management) have relevant effects in prevalence rates. Those subtle factors become unclear in large-scale surveys.

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Ethics approval

All animal sampling took place post-mortem. The wildlife samples were obtained from hunter-harvested individuals that were hunted independently from our research. According to EU and National legislation (2010/63/UE Directive and Spanish Royal Decree 53/2013) and to the University of Castilla—La Mancha guidelines, no permission or consent is required to conduct the research reported herein.

When consulted on this matter, the Bioethics Commission of the Cardenal Herrera University, through report 20/012, decided that no special authorization was necessary, given that the samples were taken postmortem.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Additional data and materials are available under reviewers' request.

Code availability

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interest or personal relationships that could have influenced in any way the work reported in this paper.

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