Tracking *Toxoplasma gondii* in freshwater ecosystems: interaction with the invasive American mink (*Neovison vison)* in Spain

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Received: 24 February 2018 / Accepted: 9 May 2018

Abstract

Water-borne transmission may play an important role in the epidemiology of *Toxoplasma gondii*. Mammals closely related to freshwater ecosystems, such as the American mink (*Neovison vison*), are potentially valuable sentinels for *T. gondii*. To assess the importance of freshwater ecosystems in *T. gondii* epidemiology, sera of 678 American minks collected during the 2010 to 2015

Spanish national eradication campaigns were tested for the presence of *T. gondii* antibodies using the modified agglutination test (MAT, cut-off 1:25). A high prevalence of samples, 78.8% ($CI_{95\%}$: 75.5–81.8), were seropositive. In addition, a specific real-time PCR was performed in 120 brain samples and the parasite DNA was detected in 9.2% ($CI_{95\%}$: 5.2–15.7). Significant differences in seroprevalence were detected among bioregions, with the highest levels detected in coastal areas, and by age. The higher seroprevalence observed in older animals (80.0% adults versus 68.7% juveniles) confirms the importance of the horizontal transmission. These results indicate a widespread presence of *T. gondii* oocysts in freshwater ecosystems from Spain and further support the importance of water-borne transmission in the epidemiology of *T. gondii*.

Keywords

American mink (*Neovison vison*)
Seroprevalence
Toxoplasma gondii
Wildlife disease
Zoonosis

Section Editor: Larissa Howe

Introduction

Toxoplasma gondii is an intracellular protozoan parasite of worldwide distribution. Wild and domestic felines are the definitive hosts and excrete the environmentally resistant oocysts. Humans and potentially all warm-blooded species can become infected acting as intermediate hosts (Dubey 2010). Both the intermediate and the definitive host can acquire the infection via one of the following routes: (1) horizontally by oral ingestion of sporulated oocysts from the environment, (2) horizontally by ingestion of tissue cysts contained in the intermediate host, or (3) vertically by transplacental transmission of tachyzoites (Dubey 2010).

Water-borne transmission of *T. gondii* is a growing concern (Ahlers et al. 2015). Although traditionally considered a parasite of terrestrial habitats, recent reports

have identified *T. gondii* in seals, dolphins, and other marine and freshwater mammals, including the American mink (*Neovison vison*) (reviewed by Chadwick et al. 2013). Sporulated oocysts of *T. gondii* are resistant to environmental conditions and remain infective up to 54 months in freshwater at 4 °C (Dubey 1998) and for 24 months in seawater (Lindsay and Dubey 2009). Human settlements favor high felid densities that can excrete oocysts into sewage waters and contaminate aquatic ecosystems (Van~~w~~Wormer et al., 2013a). In fact, freshwater runoff has been considered as one of the main sources of *T. gondii* strains from land to coastal anthropogenic environments (Miller et al. 2002). The contamination of continental water with sporulated oocysts can be a threat for freshwater and marine mammals, as well as birds linked to aquatic ecosystems (Cabezón et al. 2004; Cabezón et al. 2016; Chadwick et al. 2013; Dubey et al. 2003; Jensen et al. 2010; Sulzner et al. 2012). Contamination of near-shore waters by *T. gondii* oocysts may negatively affect the survival of several sensitive marine mammals such as southern sea otters (*Enhydra lutris*) in the USA (Kreuder et al. 2003; Conrad et al. 2005) and the parasite represents the second most common infectious cause of death in dolphins from the Mediterranean Sea~~sea~~ (Domingo et al. 1992; Resendes et al. 2002). Contaminated run-off has also been ~~also~~ implicated in *T. gondii* outbreaks in humans (Jones and Dubey 2010).

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Mammals from a high trophic level in aquatic ecosystems can be good sentinels for the detection of pathogens (Bossart 2011; Moura et al. 2014). In this sense, serological and molecular studies on mammal species can be useful to provide a better understanding of the impact of human populations on aquatic ecosystems. The American mink (*Neovison vison*) is a semi-aquatic invasive species with a wide distribution throughout the North and Central Iberian Peninsula. This European-invasive mustelid ~~species~~ has opportunistic feeding habits, but is considered a predominantly freshwater carnivore. ~~This European-invasive mustelid~~ This species is a significant competitor that compromises the viability of populations of native species such as the European mink (*Mustela lutreola*) (Maran et al. 1998; Sidorovich et al. 1999), which is considered Critically Endangered by the International Union for Conservation of Nature (Maran et al. 2011). Eradication campaigns of American minks in Spain have been conducted in order to reduce their populations (MAGRAMA 2014) and samples are easily

available. Due to its close relation to freshwater ecosystems, we hypothesized that the American mink is an appropriate sentinel species for pathogens of great importance in public and animal health such as *T. gondii*.

The aim of the present study was to analyze the seroprevalence and infection of *T. gondii* in the invasive American mink, in order to assess the environmental contamination of freshwater ecosystems from Spain and to confirm the importance of water-borne transmission in the epidemiology of *T. gondii*.

Materials and methods

Animal samples

American minks were captured during the 2010 to 2015 Spanish national eradication campaigns. Captures were performed using baited boxtraps and animals were euthanized with an intramuscular injection of ketamine-medetomidine followed by an intracardiac injection of pentobarbital sodium (MAGRAMA 2014). All capture, data collection, and sampling procedures in the field, as well as euthanasia methods, were approved as part of the national eradication control programs for the American mink in Spain. Fieldwork permits were authorized by the relevant administrations from Generalitat de Catalunya, Junta de Castilla-y-León, La Rioja, and País Vasco.

Serum samples ($n = 678$) were obtained from four Autonomous Communities in the North of Spain (Castilla y León, La Rioja, Basque Country, and Catalonia) (Table 1). The regions correspond with the two main river basins (Ebro and Duero) in North Spain. Blood was collected by intracardiac puncture or from the thoracic cavity of euthanized animals. Blood was centrifuged to separate the serum and was stored at − 20 °C until the analysis was performed. Brain tissue was also collected in sterile tubes and stored at − 20 °C. The location of each captured animal was registered and each individual was examined to define sex and age stage (juvenile < 1 year and adult > 1 year) according to size, sexual maturity, and dental characteristics.

Table 1 The Table 1 contains mainly information of results. May be better placed it in the Results section.

Toxoplasma gondii seroprevalence (MAT ≥ 1:25) in American minks (*Neovison vison*) by the variables considered in this study. Statistically significant differences ~~when~~

~~different letters (a,b)~~ within earch categor~~ies~~ (p value < 0.05) are shown with different letters (a,b).

Category		Positive/examined (%, C Please note that a is already used to indicate significant differences. I have changed the symbol by *, but could be any other. I~~a~~*)
Autonomous community	Catalonia	38/46 (82.6, 69.3–90.9)
	Castilla y León	403/510 (79.0, 75.3–82.3)
	La Rioja	48/67 (71.6, 59.9–81.0)
	Basque Country	45/55 (81.8, 69.7–89.8)
Bioregion	2	510/653 (78.1, 74.8–81.1)[a]
	5	22/23 (95.7, 79.0–99.8)[b]
River basin	Duero	360/460 (78.3, 74.3–81.8)
	Ebro	119/161 (73.9, 66.6–80.1)
Sex	Male	289/370 (78.1, 73.6–82.0)
	Female	182/239 (76.2, 70.4–81.1)
Age stage	Juvenile	103/150 (68.7, 60.9–75.5)[a]
	Adult	385/481 (80.0, 76.2–83.4)[b]

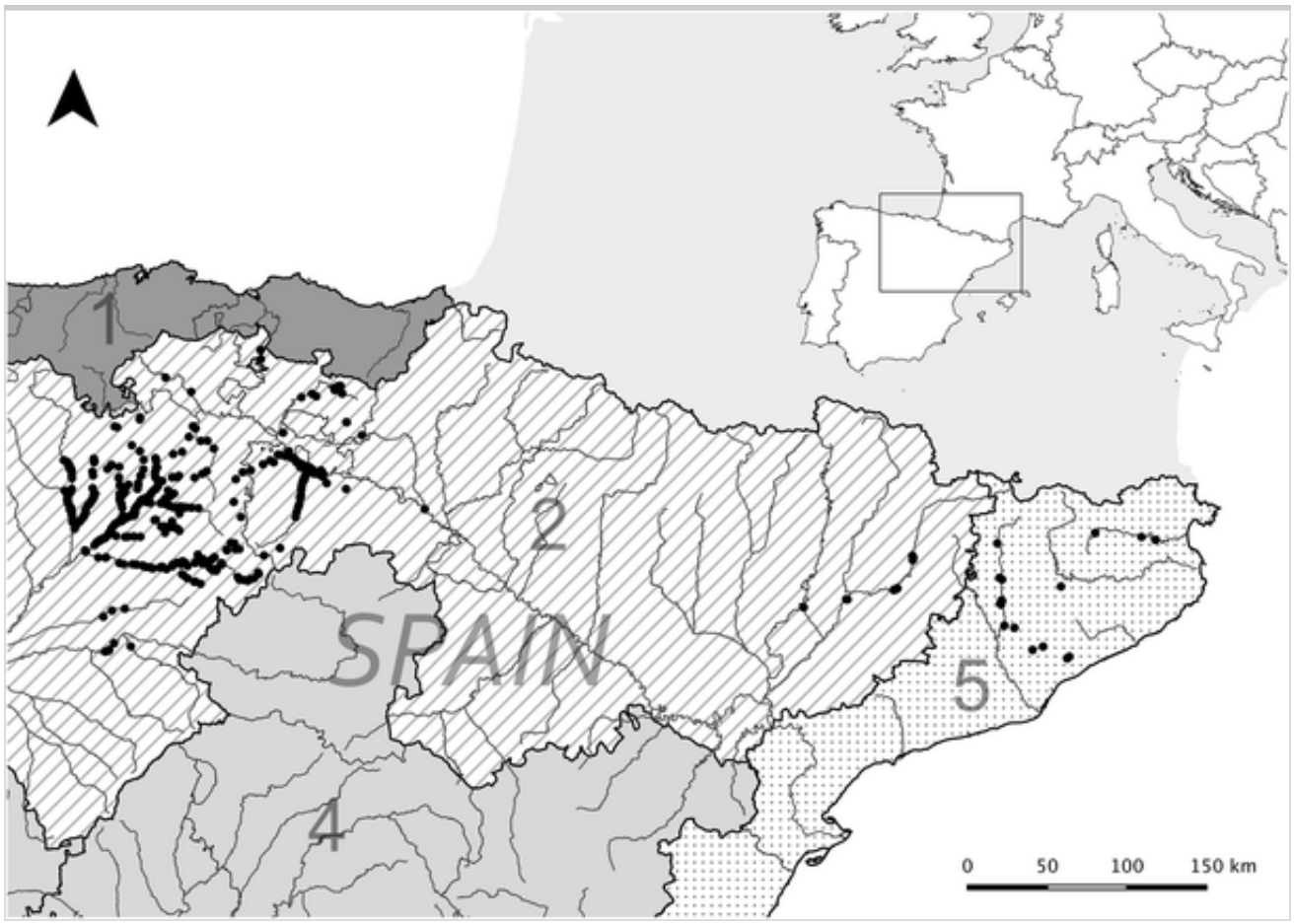
~~a~~* CI Please note that a is already used to indicate significant differences. I have changed the symbol by *, but could be any other. : 95% confidence interval

Study area

The great diversity of habitats and climates within Spain~~the Iberian Peninsula~~ can be simplified to five different bioregions (RASVE 2011). In order to consider the ecological features, each region was ascribed to its corresponding bioregion. Consequently, bioregion 2 (Northern plateau) includes León, Palencia, and Burgos provinces (Castilla y León); La Rioja (La Rioja); Álava province (Basque Country); and Lleida province (Catalonia). Likewise, bioregion 5 (Southeastern coast) includes Barcelona and Girona provinces (Catalonia) (Fig. 1).

Fig. 1

Map with the location of the serum samples (black spots) on the main rivers of Spain~~the Iberian Peninsula~~ (black lines). Bioregions 2 and 5 are shown in striped and spotted patterns, respectively. Bioregions not included in the study (1 and 4) are shown in different gray colors (QGIS 2.14 Essen 2016)



Serological analysis

Sera were tested by the modified agglutination test (MAT) to detect IgG antibodies against *T. gondii* at 1:25, 1:50, 1:100, and 1:500 dilutions (Dubey and Desmonts 1987). Positive and negative controls were also included in all tests. This technique has demonstrated a high sensitivity and specificity and has been previously evaluated in several wildlife species (Dubey et al. 2004), including mustelids (Sobrino et al. 2007). Titers of 1:25 or higher were considered positive and doubtful results were reexamined. Although hemolysis does not represent a problem (Dubey et al. 2003), hemolytic sera were filtered

using sterile 0.2-μm microfilters prior to the test.

DNA extraction and detection

The brain tissues of 120 American minks from which samples were available were mechanically homogenized, and DNA was extracted using the commercial kit NucleoSpin®Tissue (Macherey-Nagel) for genomic DNA following the manufacture's protocol. Real-time PCR (qPCR) specific for *T. gondii* was performed based on the 529-bp DNA fragment that is repeated 200–300 times in the *T. gondii* genome (Homan et al. 2000). The primers used were Toxo-SE (900 nM, 5′-AGGCGAGGGTGAGGATGA) and Toxo-AS (900 nM, 5′-TCGTCTCGTCTGGATCGCAT) and the probe Toxotaqman (300 nM, 5′-6FAM-CGACGAGAGTCGGAGAGGGAGAAGATGT--BHQ1). PCR reactions were performed in a 25-μl reaction mixture containing 2 μl of sample, 0.45 μl of each primer, 0.15 μl of probe, 12.5 μl of TaqMan®2× Universal PCR MasterMix (Applied Biosystem, Warrington, UK), and 9.45 μl of sterile water. Amplification was performed on Applied Biosystems® 7500 Fast Real-time PCR system by 2 min at 50 °C and 10 min at 95 °C initial incubation followed by 40 cycles of 15 s at 95 °C and 1 min at 61 °C. Positive and negative controls were included in each analysis.

Statistical analysis

Prevalence of antibodies against *T. gondii* was estimated from the ratio of positive to the total number of samples with the Wilson confidence intervals of 95%. Association between seropositivity and independent variables (Autonomous Community, bioregion, river basin, sex, and age) was assessed using a Pearson Chi-squared test or Fisher's exact test when observations/category were < 6. Year of sampling was excluded from statistic analysis due to large differences in the number of samples for each year. No statistical analysis was performed on PCR results due to the low number of positive samples observed. The differences were considered statistically significant when $P \leq 0.05$. Analyses were performed using the R Statistical Software 3.1.3 (R Development Core Team 3.1.3, 2015).

Results

Seroprevalence levels of *T. gondii* ~~in American mink~~ in each category of the

American minks are presented in Table 1 and locations of captured individuals are shown in Fig. 1. Overall seroprevalence was 78.8% (534/678, $CI_{95\%}$: 75.5–81.7), with titers of 1:25 in 11.2% of the samples, 1:50 in 7.3% of the samples, 1:100 in 32.6% of the samples, and 1:500 in 48.9% of the samples. No statistically significant differences in *T. gondii* seroprevalence were observed among Autonomous Communities, river basins, and sex of the animals. Statistically significant differences were found among bioregions (p value = 0.0399) with an odds ratio (OR) of 6.16 in bioregion 5 versus bioregion 2 and among age categories (p value = 0.0052) with an OR of 0.55 in adults versus juveniles.

T. gondii DNA was detected by qPCR in 11 of the 120 samples analyzed (9.2%, CI 5.2–15.7). Six of the PCR-positive samples corresponded to American minks captured in the province of Burgos (Castilla y León) located in the Duero river basin, while three positive samples were from La Rioja and two samples were from Alava (Basque Country), located in the Ebro river basin. Eight PCR-positive minks were also positive ~~to~~by the MAT with titers of ≥ 1:500 (four minks) and 1:100 (four minks) and one PCR-positive mink was negative by serologic analysis. In two animals, the serological status was unknown. Three PCR-positive animals were juveniles while eight PCR-positive samples were from adults. Two PCR-positive samples corresponded to females and nine PCR-positive samples were from males.

Discussion

The high seroprevalence and molecular detection of the parasite in the brain indicate a frequent exposure of American minks to *T. gondii*. No significant differences were observed among the four different Autonomous Communities sampled and the two main river basins in Spain, indicating that the parasite was widespread in freshwater ecosystems from Spain. Similar seroprevalences (70%) were described in a study of American minks from Chile (Sepúlveda et al. 2011). Despite most of *T. gondii* studies being focused on terrestrial hosts, these results suggest that the parasite can be commonly found in inland aquatic systems, as demonstrated also in marine coastal areas (Miller et al. 2008), and propose water courses as an important mechanism of oocysts spread.

Different ecological and climatic features of each bioregion or differences in

definitive host densities may explain the observed significant differences of seroprevalence among bioregions. Bioregion 5 mainly comprises the coastal areas of Catalonia, which has higher humidity and higher human density. Human settlements constitute artificial environments with a high density of domestic felids that excrete oocysts (Van~~w~~Wormer et al., ~~2013a~~b). The importance of human populations in *T. gondii* epidemiology is reinforced by the growing number of feral cat populations, the elevated number of oocysts excreted by each infected cat, the long-term viability of the oocysts, and the fact that a single oocyst can be infective for several species (reviewed by Torrey and Yolken 2013). Several studies have suggested a positive relationship between high *T. gondii* seroprevalences in suburban wildlife and elevated feral cat densities (Miller et al. 2002; Sepúlveda et al. 2011; Van~~w~~Wormer et al., ~~2013a~~b). To the authors' knowledge, no actual census of feral cat populations exists in the areas included in the present study. Future studies should be centered on determining factors that may influence the density of definitive hosts and the persistence and spread of oocysts, which may ~~to~~ explain the observed *T. gondii* variability in freshwater ecosystems.

Due to the feeding habits and the repetitive ingestion of tissue cysts in prey, carnivores and omnivores are more likely to be infected with *T. gondii* than herbivores (Hejlícek et al. 1997). The high seroprevalence in American minks was only comparable to the elevated seroprevalence of *T. gondii* previously reported in definitive hosts in Spain. Feral cats in Majorca, Balearic Islands, were highly seropositive (84.7%, $n = 59$), one of the highest levels reported worldwide in this species and the highest observed in Europe to date (Millán et al. 2009a). Similar results were observed in domestic cats in other areas of Spain (45 to 50% Gauss et al. 2003; Millán et al. 2009b, respectively) and in Iberian lynx (*Lynx pardinus*) (62.8%, $n = 29$) (García-Bocanegra et al. 2010) using the same serological technique. Several mustelids species, including Eurasian otters (*Lutra lutra*) in Spain (Sobrino et al. 2007), have also shown high seroprevalences. According to the high prevalences observed, mammals closely related to aquatic ecosystems may have a high risk of exposure to the parasite suggesting that water-borne transmission of oocysts may be of great importance for *T. gondii* epidemiology.

Juvenile animals were 50% less likely to be infected than adults. Given the lifelong persistence of *T. gondii* IgG antibodies in healthy individuals,

seroprevalence by MAT reflects the lifelong exposure to the parasite (Remington et al. 2004). Increased contact by age indicates that horizontal transmission was the main route of *T. gondii* infection for the American mink. Horizontal transmission can occur by consumption of environmental oocysts or by ingestion of cysts in tissues of prey. The diet of the American mink was not evaluated in the animals of the present study. However, previous work in the Iberian Peninsula showed that aquatic prey (fish and crayfish) represent 45.9–71.4% of the American mink's diet depending on the season (Melero et al. 2008). Ectothermic (cold-blooded) animals such as fish or crayfish reportedly do not develop tissue cysts of *T. gondii*, and consequently, their role in the epidemiology of the parasite is still unknown. However, some aquatic invertebrates such as bivalves or filter-feeding fish are able to remove sporulated *T. gondii* oocysts from seawater and retain their infectivity (Lindsay et al. 2004; Fayer et al. 2004; Massie et al. 2010). These filter-feeding aquatic animals could potentially act as mechanical vectors transporting oocysts and as a source of infection for freshwater and marine animals and for humans. In fact, the consumption of contaminated invertebrate prey was suspected as the vehicle for the exposure of southern sea otters (*Enhydra lutris nereis)* to *T. gondii* (Miller et al. 2008). Another route of exposure to the parasite would be the ingestion of sporulated oocysts present in water. Contamination of waterways could be facilitated by the hydrophilic nature and negative charge of *T. gondii* oocysts in freshwater, which could enhance transportation through water runoff (Shapiro et al. 2009, 2012). The high seroprevalence of *T. gondii* in a predominantly piscivorous freshwater mammal suggests widespread fecal contamination of freshwater ecosystems with oocysts. However, minks can also be opportunistic predators and occasionally take mammals and birds, and therefore become infected via ingestion of prey with tissue cysts of *T. gondii.* Therefore, confirmation of the specific route of exposure in minks is difficult.

The infection of *T. gondii* in American minks was also supported by the presence of DNA in tissues analyzed by real-time PCR. Most studies of *T. gondii* in Spain have been based on serology rather than molecular detection of the parasite. The fact that only 10% of the analyzed samples were PCR-positive compared to the high seroprevalence levels observed was probably due to the non-homogenous distribution of *T. gondii* tissue cysts and the small sample used for DNA detection. Burrells et al. (2013) also described similar results by

detection of *T. gondii* DNA in 20% (13/65) of brain tissues from American mink in the UK.

Due to the eradication programs in Spain and Europe, the American mink is potentially a useful sentinel species to track water-borne pathogens of importance in public and animal health, such as *T. gondii.* Although *T. gondii* infection was confirmed in this species by PCR, no mortality cases associated with *T. gondii* in mustelids have been confirmed in the Iberian Peninsula. Outbreaks and clinical cases have been observed in farmed American minks (Frank 2001; Śmielewska-Łoś and Turniak 2004) and even in free-feral minks (Jones et al. 2006). Surveillance of the invasive American mink for *T. gondii* is valuable because of conservation concerns for the European mink and the Eurasian otter, and also because of its suitability as a sentinel for freshwater contamination with oocysts. Anthropogenic activities and demographic changes are modifying the dispersion and emergence patterns of pathogens (Jones et al. 2008). Additionally, human coastal development can change populations of felids, increase *T. gondii* oocysts in water runoff, and disrupt disease dynamics, increasing the importance of a holistic approach to the study of this parasite (VanWormer et al. 2013a). Understanding the epidemiology of *T. gondii* in an ecosystem context is critical to human and animal health as this parasite represents a good example of the complexity in multi-host pathogen transmission. As populations of humans and their companion animals grow, the impacts of fecal contamination on public and wildlife health are likely to increase (Fayer et al. 2004; Chadwick et al. 2013). This study supports the growing body of evidence that freshwater ecosystems have an importante role on *T. gondii* transmission and its relevance on public helath should be further considered.

Conclusions

The high exposure of *T. gondii* detected in American minks indicates a widespread presence of the parasite in freshwater ecosystems in Spain. Horizontal transmission is the main route of infection for the American minks, strengthening the importance of water-borne transmission of *T. gondii* oocysts on its epidemiology. The highest seroprevalence was found in coastal regions, which are associated with areas of higher human density, and may reflect the influence of human settlements in the dynamics of the parasite. The invasive

American mink is a good sentinel species for *T. gondii* contamination in freshwater habitats.

Acknowledgements

The authors would like to thank the staff of the wildlife rehabilitation centres (WRC) of Burgos, especially to Ms. Nerea Falagán (Castilla-y-León), the WRC-Martioda (Basque Country) and WRC-La Fombera (La Rioja) for their help in the sample collection. Also, the authors would like to thank the people from of Castilla-y-León, La Rioja, Diputación Foral de Álava and Ministerio de Medio Ambiente governments, to the Asociación Visón Europeo, Tragsatec and to the Fundación Biodiversidad that have been directly or indirectly involved in the fieldwork. Most of the samples were collected in several Spanish territories within the National Program for control the American mink. This study was partly financied by FEDER and Spanish MINECO grant (AGL2012-39830-C02-01/02) and LIFE LUTREOLA SPAIN (LIFE 13 NAT/ES/001171). The authors are also very grateful for the technical contribution of Asunción Gómez from TRAGSATEC and Madis Pödra from Asociación Visón Europeo.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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