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The red fox (*Vulpes vulpes*) as a potential natural reservoir of human cryptosporidiosis by *Cryptosporidium hominis* in Northwest Spain

Running head: *Cryptosporidium hominis* infection in red foxes

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SUMMARY

Giardia duodenalis and *Cryptosporidium* spp. are ubiquitous intestinal protozoa that parasitize domestic and wild animals, as well as human beings. Due to their zoonotic potential, the objective of the present study was to determine the presence of these pathogens in the fox population (*Vulpes vulpes*) located in Northwest Spain. A total of 197 faecal samples from legally hunted foxes were collected in the autonomous region of Galicia. The presence of *G. duodenalis* and *Cryptosporidium* spp. was investigated by PCR-based methods amplifying the small subunit ribosomal RNA (*ssu* rRNA) gene of the parasites. Attempts to genotype obtained positive samples were subsequently conducted at the glutamate dehydrogenase (*gdh*) and β-giardin *(bg*) genes of *G. duodenalis*, and the 60 kDa glycoprotein (*gp60*) gene of *Cryptosporidium*. *Giardia duodenalis* and *Cryptosporidium* spp. were identified in 19 (9.6%) and 12 (6.1%) of the investigated samples, respectively. However, five *Cryptosporidium* species were detected at the *ssu* rRNA locus: *C. hominis* (33.4%, 4/12), *C. canis* (25.0%, 3/12), *C. parvum* (16.7%, 2/12), *C. ubiquitum* (8.3%, 1/12), and *C. suis* (8.3%, 1/12). An additional *Cryptosporidium*-positive sample was identified at the genus level only. Typing and subtyping of *Giardia*- and C*ryptosporidium*-positive samples was unsuccessful. The detection of *C. hominis* in wild foxes indicates the probable overlapping of sylvatic and domestic cycles of this parasite in rural settings. Besides, this finding raises the question of whether red foxes may act as natural reservoirs of *C. hominis*. The detection of *C. parvum* and *C. suis* is suggestive of active transmission events between farm and wild animals, opening up the possibility of transmission to human beings.

KEYWORDS: *Cryptosporidium hominis*; *Giardia*; Foxes; Genotyping; Prevalence; Sylvatic cycle; Spain

1. INTRODUCTION

Giardia (phylum Metamonada) and *Cryptosporidium* (phylum Apicomplexa) are worldwide intestinal parasites that infect a broad spectrum of vertebrates. Both are considered relevant pathogens in public and animal health and have a significant zoonotic impact because certain species are able to infect animals and human beings (Thompson, 2004; Xiao, 2010; Ryan & Cacciò, 2013; Ryan et al., 2016; Thompson & Ash, 2016). Infection typically occurs after ingestion of contaminated water or food. Not surprisingly, *Giardia* and *Cryptosporidium* are common causes of waterborne and foodborne outbreaks of diarrhoea globally (Chalmers et al., 2010; Efstratiou et al., 2017; Robertson, 2018).

Subclinical carriage of *Giardia* and *Cryptosporidium* is frequent (Reh et al., 2019), but both pathogens can cause a wide range of gastrointestinal-related conditions including chronic small bowel diarrhoea, vomiting, fever, and progressive weight loss. *Cryptosporidium* infection is a major cause of diarrhoea in immunocompromised adults and immunocompetent children, whereas *G. duodenalis* is the main intestinal parasite affecting people in developed countries (Cacciò & Chalmers, 2016; Mmbaga & Houpt, 2017). In Spain, according to data from the National Epidemiological Surveillance Network, human clinical cases of cryptosporidiosis and giardiosis have gradually increased since 2010, with children aged between 1 and 9 years being particularly at risk of these infections (NESN, 2016).

G. duodenalis is considered a multispecies complex with at least eight distinct assemblages (A-H) differing in host specificities and genetic content. There is extensive genetic sub-structuring within assemblages A and B, further divided within sub-assemblages AI-AIII and BIII-BIV, respectively (Feng & Xiao, 2011). Assemblages A and B infect a wide diversity of mammal species including humans and are therefore considered zoonotic. The remaining assemblages are likely to be host-specific and are only sporadically found infecting humans.

The genus *Cryptosporidium* encompasses thus far 38 recognized species (Feng et al., 2018). *C. hominis* primarily (but not exclusively) infects humans, whereas *C. parvum* is considered the most important *Cryptosporidium* zoonotic species, having as main reservoirs cattle and humans. Several other *Cryptosporidium* species from mammals and birds (e.g. *C. meleagridis*, *C. canis*, *C. felis*, and *C. ubiquitum*, among others) pose also zoonotic risk at varying degrees, causing animal contact-associated or waterborne and foodborne cryptosporidiosis in humans (Ryan et al., 2014; Efstratiou et al., 2017¸ Ryan et al., 2018). Importantly, the notion that C. *hominis* is a human-specific *Cryptosporidium* species has being increasingly challenged by numerous molecular epidemiological studies revealing that the actual host range of *C. hominis* is much wider than initially thought (Widmer et al., in press).

Although there is some controversy about the role played by production animals in transmission (e.g. O'Handley, 2007), genotyping of *Giardia*- and *Cryptosporidium*-positive samples is essential to ascertain the epidemiology of these pathogens and their public veterinary health relevance. Conventionally, livestock and companion animal species have been regarded as the most important sources of zoonotic human cryptosporidiosis cases (Ryan & Cacciò, 2013; Slapeta, 2013; Ryan et al., 2014). However, due to the steady but continuous human encroachment into wildlife habitats, free-living animals including foxes, raccoons, and wild boars are becoming an increasingly common sight on the urban and peri-urban areas of many European cities (Mackenstedt et al., 2015). Given this scenario, wild animals may play a more important role in the spreading of pathogens and as natural source of human and pet infections than previously anticipated (Thompson, 2013; Ryan et al., 2016; Zahedi et al., 2016).

In Spain, very few epidemiological surveys have attempted to investigate the occurrence of *G. duodenalis* and *Cryptosporidium* spp. infection in the red fox (*Vulpes vulpes*). These molecular-based studies revealed the presence of *C. canis*, *C. felis*, *C. parvum*, and *C. ubiquitum*

97 circulating in fox populations in the North and Central areas of the country (Mateo et al., 2017;
98 Navarro-i-Martinez et al., 2011). However, no data are currently available on the occurrence
99 and distribution of *G. duodenalis* and *Cryptosporidium* spp. in wild canids in Northwest Spain,
100 a region where these pathogens have been previously reported in wild and domestic animals,
101 humans, and even environmental (water) samples (Castro-Hermida et al., 2002, 2007, 2008,
102 2009, 2011; Castro-Hermida et al., 2006; Gómez-Couso et al., 2006; García-Presedo et al.,
103 2013; Gabín-García et al., 2017). Because red fox populations have significantly increased in
104 rural and peri-urban settings of this region (average density: 3.9–5.4 foxes/km^2) in recent years,
105 this epidemiological scenario may favour the transition from sylvatic to domestic transmission
106 cycles of these parasites (López Becerro, 2009). The aims of the present study were i) to
107 determine the presence and molecular diversity of zoonotic protozoa in faeces from foxes living
108 in Northwest Spain, ii) to conduct a preliminary assessment of the zoonotic potential risk that
109 fox populations pose in areas where sylvatic and domestic transmission cycles overlap, and iii)
110 to identify biological and environmental factors potentially associated to a higher risk of
111 infection.

112 ## 2. MATERIALS AND METHODS

113 ### 2.1 Ethical statement

114 This study was carried out in accordance with Spanish legislation guidelines (RD 8/2003) and
115 with the International Guiding Principles for Biomedical Research Involving Animals issued
116 by the Council for International Organization of Medical Sciences and the International Council
117 for Laboratory Animal Science (RD 53/2013).

118 ### 2.2 Study area, sampling and data collection

The carcases of 197 wild red foxes obtained in three out of the four provinces of the autonomous region of Galicia (NW Spain) between 2015 and 2019 were included in this study (Figure 1). The foxes had been legally shot during the official hunting season (from January to February) of each year. Faecal samples were collected from the rectum, transferred into sterile containers, and kept at 4 °C until further processing, usually within 72 h.

Information including specific coordinates of sampling sites, sample identification number, date, capture site, age, clinical status and sex were carefully recorded for each animal in an Excel spreadsheet. Clinical signs (change in the colour of mucous membranes, body and skin condition, lymphadenomegaly) were also assessed at the time of sampling. A body condition score (based on the thickness of the fat layer in the thoracic and abdominal cavities and the amount of visceral fat observed at necropsy) ranging from 1 to 5 was used, with a score of 1 being cachectic and 5 being overweight (Winstanley et al., 1998). Animal age was estimated according to several factors including body development (complete or not), external appearance, developmental stage of genitals (external or internal) and dentition (presence, development and teeth wear, periodontal disease). Three age groups were stablished: immature or juvenile (individuals <1 year-old), adults (reproductive individuals between 1–5 years-old), and old adults (individuals >5 years-old showing teeth wear and/or varying degree of periodontal disease or even loss of teeth).

2.3 Faecal sample processing

An aliquot (3–5 g) of each faecal sample was suspended into 20 mL volumes of 1× phosphate buffered saline (PBS) and thoroughly homogenized. The homogenate was then filtered through a sieve mesh (250 μm diameter) double gauze. The filtered suspension was divided into two 10 mL tubes and centrifuged at 500 × g for 10 min. After careful removal of the supernatant, the

remaining pellet was transferred to a clean 1.5 mL tube and stored at -20 ºC until DNA extraction was performed.

2.4 DNA extraction and purification

DNA was extracted from faecal samples using the QIAmp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was stored at 4 °C until PCR analyses. Elapsed time between sample processing and PCR testing was 1–6 months.

2.5 Molecular detection and characterisation of *G. duodenalis*

To detect *G. duodenalis*, a real-time PCR (qPCR) protocol was used to amplify a ~62-bp region of the small subunit ribosomal RNA (*ssu* rRNA) gene of the parasite (Verweij et al., 2003). The reaction mixture (25 µL) contained 3 µL of template DNA, 12.5 pmol of each primer Gd-80F (5′–GACGGCTCAGGACAACGGTT–3′) and Gd-127R (5′–TTGCCAGCGGTGTCCG–3′), 10 pmol of probe (6-carboxyfluorescein[FAM]–5′–CCCGCGGCGGTCCCTGCTAG–3′–blackhole quencher 1 [BHQ1]), and 1X TaqMan® Gene Expression Master Mix (Applied Biosystems, CA, USA). Negative and positive controls were included in all PCR runs. Amplification reactions were performed in a Corbett Rotor-Gene 6000 qPCR cycler (QIAGEN). Cycling conditions were: an initial hold step of 2 min at 55 ºC and 15 min at 95 ºC, followed by 45 cycles of 15 s at 95 ºC and 1 min at 60 ºC. For genotyping purposes, a semi-nested and a nested PCR protocols were used, respectively, to amplify partial fragments of the glutamate dehydrogenase (*gdh*; Read et al., 2004) and β-giardin (*bg*; Lalle et al., 2005) genes of *G. duodenalis*. Amplification reactions (25 µL) contained 0.4–0.5 µM of each primer, 2.5 units of MyTAQ™ DNA polymerase (Bioline GmbH, Luckenwalde, Germany), 5 µL of 5× MyTAQ™ Reaction Buffer containing 5 mM dNTPs and 15 mM $MgCl_2$, and 3–5 µL of

template DNA. Amplifications were conducted in a 2720 thermal cycler (Applied Biosystems). PCR products were resolved on 2% D5 agarose gels (Conda, Madrid, Spain) stained with Pronasafe nucleic acid staining solution (Conda).

2.6 Molecular detection and characterisation of *Cryptosporidium* spp.

Detection and identification of *Cryptosporidium* species was achieved using a nested PCR protocol to amplify a partial (~587 bp) fragment of the *ssu* rRNA gene of the parasite (Tiangtip & Jongwutiwes, 2002). The outer primers were CR-P1 (5′–CAGGGAGGTAGTGACAAGAA–3′) and CR-P2 (5′–TCAGCCTTGCGACCATACTC–3′) and the inner primers were CR-P3 (5′–ATTGGAGGGCAAGTCTGGTG–3′) and CPB-DIAGR (5′–TAAGGTGCTGAAGGAGTAAGG–3′). The reaction mix (50 µL) comprised 0.3 µM of each primer, 2.5 units of DNA polymerase, 10 µL of 5× Reaction Buffer, and 3 µL of template DNA. Cycling conditions for the primary and secondary PCR reactions included one cycle of 94 ºC for 3 min, followed by 35 cycles of 94 ºC for 40 s, 50 ºC for 40 s and 72 ºC for 1 min, and a final extension of 72 ºC for 10 min. DNA samples positive to *C. parvum*/*C. hominis* and *C. ubiquitum* at the *ssu*-PCR were subtyped at the 60-kDa glycoprotein (*gp60*) of the parasite using specific protocols (Feltus et al., 2006; Li et al., 2014). PCR reagents and equipment used were the same as described above for the *gdh*-PCR and the *bg*-PCR protocols.

2.7 Sequence analyses

Obtained PCR products were sequenced in both directions with the corresponding internal primer sets described above using Big Dye™ chemistries and an ABI 3730xl sequencer analyser (Applied Biosystems, Foster City, CA). Raw sequencing data in both forward and reverse directions were viewed using the Chromas Lite version 2.1 sequence analysis program. The BLAST tool was used to compare nucleotide sequences with appropriate

reference sequences retrieved from the National Center for Biotechnology Information (NCBI) database. Generated DNA consensus sequences were aligned to appropriate reference sequences using the MEGA 7 software to confirm species identity. Phylogenetic relationships among *Cryptosporidium* sequences identified in the present survey and known *Cryptosporidium* sequences retrieved from the NCBI public repository was done by the Neighbor-Joining (NJ) method using MEGA 7 (Tamura et al., 2013). Genetic distance was calculated with the Kimura 2-parameter model, and the rate variation among sites was modelled with a gamma distribution (shape parameter = 2).

2.8 Statistical analysis

Potential association between all the variables examined were investigated with the Chi-square test. We also explored whether proximity of infected foxes to river courses could be linked to an increased risk of environmental surface water contamination with *Cryptosporidium* oocysts and *Giardia* cysts, or, on the contrary, foxes may acquire these infections through consumption of water contaminated with faecal material from human or livestock origin. To do so, we first use the GIS software ArcGis Pro v.2.3.3 (ESRI, Redlands, CA) to assign each sampling site coordinate the real distance to the closest river course, taking into account spatial information derived from a 200 m resolution digital elevation model provided by the National Center for Geographic Information (CNIG). Secondly, T-student and Mann-Whitney U tests were used to assess correlation between foxes testing positive to *Cryptosporidium* and *Giardia* and distance from river courses. Analyses were conducted using SPSS Statistics package 17.0 (IBM, Chicago, IL, USA). Significance was set at $p < 0.05$.

3. RESULTS

A total of 197 faecal samples from individual red foxes were collected and included in the present survey. After examination, foxes were classified according to sex (107 males, 90 females) and estimated age (60 juveniles, 109 adults and 28 old adults). Body condition appraisal revealed that most (83%, 163/197) of the animals fell within scores 2 and 3. Overall, 40 animals were captured in the province of A Coruña, 94 in Lugo, 11 in Ourense, and 52 in Pontevedra (Figure 1 and Table 1).

Based on PCR methods, *G. duodenalis* and *Cryptosporidium* spp. were identified in 9.6% (19/197) and 6.1% (12/197) of the investigated faecal samples, respectively. *Giardia duodenalis* was equally present in male and female foxes of all age groups investigated. However, vixens were more likely ($P < 0.05$) to be infected by *Cryptosporidium* spp. than male foxes. Animals with a body condition score of 2 were more prone to have cryptosporidiosis, although this difference was not statistically significant. No foxes with a body condition of 5 (overweight) were found. The three foxes with a body condition score of 1 (cachectic) were found infected neither by *G. duodenalis* nor *Cryptosporidium* spp. None of these pathogens were identified infecting foxes captured in Ourense, although this finding may be associated to the relatively low number of animals sampled in that particular province (Table 1).

Samples that tested positive to *G. duodenalis* by qPCR had cycle threshold (Ct) values ranging from 33.0 to 43.4 (median: 39.1). Attempts to amplify these samples at the *gdh* and *bg* loci of the parasite failed repeatedly. BLAST sequence alignments of the *Cryptosporidium*-positive amplicons obtained at the *ssu*-PCR allowed the identification of five different *Cryptosporidium* species including *C. hominis* (33.4%, 4/12), *C. canis* (25.0%, 3/12), *C. parvum* (16.7%, 2/12), *C. ubiquitum* (8.3%, 1/12), and *C. suis* (8.3%, 1/12). An additional *Cryptosporidium*-positive sample was only confirmed at the genus level. The main

233 epidemiological and clinical features of the red foxes harbouring *Cryptosporidium* infections
234 are summarized in Table 2.

235 Table 3 shows the main molecular features of the 12 *Cryptosporidium ssu* rRNA
236 sequences generated in the present survey. Representative nucleotide sequences were deposited
237 in the GenBank database under accession numbers MK770260-MK770267 and MN996814-
238 MN996816. The two *C. parvum* sequences identified corresponded to known genetic variants
239 of the bovine genotype of the parasite, also known as *C. pestis* (GenBank accession number
240 AF108864) by some authors (Slapeta, 2006). Unexpectedly, a very high nucleotide diversity
241 was found among the four sequences assigned to *C. hominis*, including one known and three
242 novel genetic variants. The three sequences identified as *C. canis* are known to be circulating
243 in wild and domestic canids globally, but the *C. ubiquitum* and *C. suis* sequences corresponded
244 to genotypes not reported yet.

245 Attempts to determine the subtypes of the *C. hominis*, *C. parvum*, and *C. ubiquitum*
246 isolates at the *gp60* marker were unsuccessful. The phylogenetic tree for partial *ssu* rDNA
247 sequences including those generated in the present study and known and reference genotypes
248 of the parasite is shown in Fig. 2. The three novel *C. hominis* genotypes (GenBank accession
249 numbers MK770262-MK770264) formed a well-defined group together with other *C. hominis*
250 sequences previously obtained in wild mesocarnivore species and domestic dogs from Spain.
251 The novel *C. ubiquitum* sequence identified here (MK770267) clustered with reference
252 sequences belonging to this *Cryptosporidium* species but showed marked genetic differences
253 with the only fox sequence reported in Spain to date, belonging to an animal from the Basque
254 Country (Northern Spain). The novel *C. suis* sequence identified here (MN996816) clustered
255 together with reference sequence AF115377, but was also closely related (99.8% identify) to
256 *C. occultus* (MG699176).

Interestingly, fox faecal samples collected near main river courses were found significantly more infected with *G. duodenalis,* but not with *Cryptosporidium* spp., than those from more distant sites (Table 4).

4. DISCUSSION

Livestock and companion animal species have been long regarded as the main reservoir of protozoal diseases to humans (Feng & Xiao, 2011; Esch & Petersen, 2013; Ryan et al., 2014). However, wildlife are being increasingly recognised as an important source of emerging and/or re-emerging human pathogens, including the diarrhoea-causing protozoa *Giardia duodenalis* and *Cryptosporidium* spp. (Polley, 2005; Ryan et al., 2016). Data presented here fall within this frame of thinking, demonstrating that *G. duodenalis* and *Cryptosporidum* spp. are common findings in red foxes living in Northwest Spain, and that this host species can act as a suitable natural reservoir of species/genotypes potentially infective to human beings, including *C. hominis*.

The overall *G. duodenalis* prevalence found (9.6%) in the surveyed fox population was slightly higher than that (mean: 8%; range: 0–18%) previously reported also by PCR in other regions of the country (Mateo et al., 2017). In this very same area (Northwest Spain). *G. duodenalis* has been detected previously in 7% of otters (Méndez-Hermida et al., 2007), 5% of roe deer, and 1% of wild boars (Castro-Hermida et al., 2011b), but not in free-living foxes. In the European scenario, documented infection rates in foxes ranged from 2–5% in Norway and Croatia (Hamnes et al., 2007; Beck et al., 2011), and up to 19% in Poland (Stojecki et al., 2015). Our qPCR results revealed that all *Giardia*-positive samples delivered high (>33) Ct values, strongly suggesting that infected foxes harboured light parasite burdens. This fact can explain the failure to amplify *Giardia* DNA at the *gdh* and *bg* loci, as both markers are single-copy

genes with limited detection sensitivity. Unfortunately, this also means that we were unable to assess the *G. duodenalis* assemblages/sub-assemblages (and their zoonotic relevance) circulating in the fox population under study. This is a frequent problem encountered in many molecular epidemiological investigations focusing on wild mesocarnivore species including foxes (Mateo et al., 2017). Of note, zoonotic assemblages A and B have been identified in Croatian and Norwegian foxes (Hamnes et al., 2007; Beck et al., 2011). Other wild canids including wolves and raccoon dogs harboured infections with *G. duodenalis* assemblages A, C, and D in Croatia (Beck et al., 2011) and Romania (Adriana et al., 2016).

Cryptosporidium infection was found in 6.1% of foxes, a prevalence similar to the average rate (8%) previously reported at national level (Mateo et al., 2017). Additionally, the parasite has also been identified in one out of four foxes in Eastern Spain (Navarro-i-Martinez et al., 2010). In our study, female were significantly more infected by *Cryptosporidium* than male foxes. This could be related to stress-induced immune compromise during the reproductive season, since in the Iberian Peninsula matting usually occurs during the months of January and February (López Becerro et al., 2009), overlapping with the hunting and capture period of the foxes in the present study. This finding has not been described in previous studies, so further research would be necessary to unravel the true influence of sex on the prevalence of the infection by this parasite.

A striking finding was the confirmation of *C. hominis* as the most prevalent *Cryptosporidium* species circulating in the investigated fox population. Until recent, *C. hominis* was mainly thought to be specifically adapted to infect humans. However, an increasing number of investigations, including experimental infections in animal models and molecular epidemiological surveys in domestic and wildlife species, have demonstrated that *C. hominis* is able to successfully infect a broad range of hosts including cattle, sheep, horses, donkeys, pigs,

rodents, geese, deer, dingoes, hedgehogs, kangaroos, wallabies, and several species of non-human primates (e.g. Akiyoshi et al., 2002; Guk et al., 2004; Schiller et al., 2016; Zahedi et al., 2016; Feng et al., 2018; Chen et al., 2019). Only in Spain, *C. hominis* has been reported in a domestic dog from the Basque Country (Gil et al., 2017) and a free-living badger from Asturias (Mateo et al., 2017). These findings raise interesting questions about the host specificity and evolution of *C. hominis* (Widmer et al., in press). Even more intriguing was the fact that three out of the four *C. hominis* sequences generated at the *ssu* rRNA locus corresponded to genetic variants of the parasite not described previously, whereas the fourth one was identified as a genotype commonly seen in Spanish clinical patients (e.g. GenBank accession number KY499055) (de Lucio et al., 2016; Azcona-Gutiérrez et al., 2017). Such variety of distinct, novel sequences may be indicative of true *C. hominis* infections rather than accidental carriage (spurious infection) of ingested oocysts of the parasite. Unfortunately, our attempts to amplify these samples at the *gp60* marker did not yield readable sequences, so the subtypes of the parasite involved in these infection remains unknown. These findings provide preliminary molecular evidence supporting the existence of a peri-domestic transmission cycles of *C. hominis* maintained within humans and foxes. This does not preclude that a sylvatic transmission cycle of the parasite may also be occurring in this geographical area, as suggested by the three novel *C. hominis* sequences described above. The former scenario would be favoured by the increasing proximity of fox populations to human settlements in rural Galicia. These foxes may acquire the parasite by feeding from garbage and carrion remains of domestic animals, or from water or food contaminated with faeces of human origin (Navarro-i-Martinez et al., 2011). This epidemiological situation fits well with the rural land tenure structure in Galicia, characterized by the presence of small family farms, holdings and parcels and a resulting landscape in the form of a complex mosaic, unlike the rest of Spain (Crecente et al.,

2002). The extent and exact meaning of these results should be corroborated in further typing and subtyping molecular surveys investigating simultaneously *Cryptosporidium*-positive faecal specimens from human and animal (including wildlife) origin, and also from environmental (soil, water) samples in this geographical area. Whatever the case, it seems clear that foxes carrying and disseminating *C. hominis* oocysts should be considered as a potential source of environmental contamination including surface waters intended for human consumption (Gómez-Bautista et al., 2000; Navarro-i-Martínez et al., 2011).

The finding of zoonotic *C. parvum* is also relevant, as this *Cryptosporidium* species is a major diarrhoea-causing agent in livestock (primarily calves) causing substantial economic losses. *Cryptosporidium parvum* has been frequently reported in humans, domestic ruminants, wildlife, and surface waters from Galicia (Castro-Hermida et al., 2011; García-Presedo et al., 2013; Abal-Fabeiro et al., 2014), so the finding of this species in free-living foxes was somehow expected, pointing out to the existence of transmission events between sylvatic and domestic (involving livestock species and humans) cycles of the parasite (Navarro-i-Martínez et al., 2003). Similar conclusions can be drawn for *C. suis*, a *Cryptosporidium* species adapted to infect swine that has been previously described in farmed pigs in north-eastern Spain (Suárez-Luengas et al., 2007). This is, to the best of our knowledge, the first description of *C. suis* in red foxes. Interestingly, *C. suis* has also been described in five of 209 wild boars in Galicia (García-Presedo et al., 2013). These facts support the hypothesis of cross-transmission of *Cryptosporidium* spp. between domestic and free-living animal species.

Zoonotic *C. ubiquitum* has been regarded as a pathogen emerging in humans (Li et al., 2014), although no human infections by this *Cryptosporidium* species have been documented in Spain yet. The parasite is known to have a broad host spectrum including ruminants, rodents and primates. Few studies have reported the presence of *C. ubiquitum* in different geographical

areas (Zahedi et al., 2016) including Spain (Mateo et al., 2017). Finally, *C. canis* is primarily found infecting domestic and wild (including foxes) canids (Mateo et al., 2017; Zahedi et al., 2016). Because of its narrower host preferences, human infections by *C. canis* are rarely described, mainly in children and immunocompromised individuals. Therefore, this *Cryptosporidium* species is considered of low zoonotic potential.

Finally, we also found that fox faecal samples collected near main water streams were more likely to harbour *G. duodenalis* cysts than those recovered from more distant sites. This finding may pose a significant (but still not fully evaluated) public health threat, as foxes carrying *G. duodenalis* (and *Cryptosporidium*) infections can contribute to the environmental burden of infective (oo)cysts and contaminate surface waters intended for human consumption or recreation. This does not preclude that foxes may also acquire these infections, at least partially, through drinking water contaminated with human or livestock faecal material. In this regard we should keep in mind that Galician surface water bodies have been shown to be heavily polluted with viable *G. duodenalis* cysts (range: 1–400 per litre) belonging to sub-assemblages AI and AII and assemblage E, and *Cryptosporidium* oocysts (range: 1–1,200 per litre) assigned to *C. hominis*, *C. parvum* and *C. andersoni* (Castro-Hermida et al., 2009, 2010). More research is clearly needed to ascertain the frequency, directionality, and extent of these events.

CONCLUSIONS

In addition to previously known *C. canis*, *C. parvum* and *C. ubiquitum*, this is the first description of *C. hominis* and *C. suis* infections from foxes globally, and the first report of *G. duodenalis* infection in free-living fox populations from Northwest Spain. Molecular data presented here, although preliminary and in need of confirmation, may indicate that *C. hominis* can be naturally infecting wild red foxes, and that this host may be a significant reservoir of

Cryptosporidium in humans and domestic animals. Given this scenario, the increasing urbanization of fox habitats favoured by their scavenging behaviour and the accessibility of anthroponotic food may pose a greater public veterinary health risk than previously anticipated.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

REFERENCES

Abal-Fabeiro, J.L., Maside, X., Llovo, J., Bello, X., Torres, M., Treviño, M., Moldes, L., Muñoz, A., Carracedo, A., & Bartolomé, C. (2014). High-throughput genotyping assay for the large-scale genetic characterization of *Cryptosporidium* parasites from human and bovine samples. *Parasitology*, *141*(4), 491–500. https://doi.org/10.1017/S0031182013001807

Adriana, G., Zsuzsa, K., Mirabela Oana, D., Mircea, G.C., & Viorica, M. (2016). *Giardia duodenalis* genotypes in domestic and wild animals from Romania identified by PCR-

RFLP targeting the gdh gene. *Veterinary Parasitology*, *217*, 71–75. https://doi.org/10.1016/j.vetpar.2015.10.017

Akiyoshi, D.E., Feng, X., Buckholt, M.A., Widmer, G., & Tzipori, S. (2002). Genetic analysis of a *Cryptosporidium parvum* human genotype 1 isolate passaged through different host species. *Infection and Immunity*, *70*, 5670–5675. https://doi.org/10.1128/iai.70.10.5670-5675.2002

Azcona-Gutiérrez, J.M., de Lucio, A., Hernández-de-Mingo, M., García-García, C., Soria-Blanco, L.M., Morales, L., Aguilera, M., Fuentes, I., & Carmena, D. (2017). Molecular diversity and frequency of the diarrheagenic enteric protozoan *Giardia duodenalis* and *Cryptosporidium* spp. in a hospital setting in Northern Spain. *PLoS One*, 1*2*(6), e0178575. https://doi.org/10.1371/journal.pone.0178575

Beck, R., Sprong, H., Lucinger, S., Pozio, E., & Cacciò, S.M. (2011). A large survey of Croatian wild mammals for *Giardia duodenali*s reveals a low prevalence and limited zoonotic potential. Vector Borne Zoonotic Dis. Larchmt. N 11, 1049–1055. https://doi.org/10.1089/vbz.2010.0113

Cacciò, S.M., & Chalmers, R.M. (2016). Human cryptosporidiosis in Europe. *Clinical Microbiology and Infection*, *22*, 471–480. https://doi.org/10.1016/j.cmi.2016.04.021

Castro-Hermida, J.A., González-Losada, Y.A., & Ares-Mazás, E. (2002). Prevalence of and risk factors involved in the spread of neonatal bovine cryptosporidiosis in Galicia (NW Spain). *Veterinary Parasitology*, *106*, 1–10. https://doi.org/10.1016/S0304-4017(02)00036-5

Castro-Hermida, JA., AlmeidA, A., González-Warleta, M., Da Costa, J.M.C., & Mezo, M. (2006a). Prevalence and preliminary genetic analysis of *Giardia* isolated from adult sheep in Galicia (northwest Spain). *Journal of Eukaryotic Microbiology*, *53 Suppl 1*,

S172–173. https://doi.org/10.1111/j.1550-7408.2006.00220.x

Castro-Hermida, J. A., Carro-Corral, C., González-Warleta, M., & Mezo, M. (2006b). Prevalence and intensity of infection of *Cryptosporidium* spp. and *Giardia duodenalis* in dairy cattle in Galicia (NW Spain). *Journal of Veterinary Medicine, Series B*, *53*, 244–246. https://doi.org/10.1111/j.1439-0450.2006.00946.x

Castro-Hermida, J.A., Almeida, A., González-Warleta, M., Correia da Costa, J.M., Rumbo-Lorenzo, C., & Mezo, M. (2007). Occurrence of *Cryptosporidium parvum* and *Giardia duodenalis* in healthy adult domestic ruminants. *Parasitology Research*, *101*, 1443–1448. https://doi.org/10.1007/s00436-007-0624-6

Castro-Hermida, J.A., García-Presedo, I., Almeida, A., González-Warleta, M., Correia Da Costa, J.M., & Mezo, M. (2008). Contribution of treated wastewater to the contamination of recreational river areas with *Cryptosporidium* spp. and *Giardia duodenalis*. *Water Research*, *42*, 3528–3538. https://doi.org/10.1016/j.watres.2008.05.001

Castro-Hermida, J.A., García-Presedo, I., Almeida, A., González-Warleta, M., Da Costa, J.M.C., & Mezo, M. (2009). Detection of *Cryptosporidium* spp. and *Giardia duodenalis* in surface water: a health risk for humans and animals. *Water Research*, *43*, 4133–4142. https://doi.org/10.1016/j.watres.2009.06.020

Castro-Hermida, J.A., García-Presedo, I., Almeida, A., González-Warleta, M., Correia Da Costa, J.M., & Mezo, M. (2011a). *Cryptosporidium* spp. and *Giardia duodenalis* in two areas of Galicia (NW Spain). *Science of the Total Environment*, *409*, 2451–2459. https://doi.org/10.1016/j.scitotenv.2011.03.010

Castro-Hermida, J.A., García-Presedo, I., González-Warleta, M., Mezo, M. (2010) *Cryptosporidium* and *Giardia* detection in water bodies of Galicia, Spain. *Water*

Research, *44*, 5887–5896. https://doi.org/10.1016/j.watres.2010.07.010

Castro-Hermida, J.A., García-Presedo, I., González-Warleta, M., & Mezo, M. (2011b). Prevalence of *Cryptosporidium* and *Giardia* in roe deer (*Capreolus capreolus*) and wild boars (*Sus scrofa*) in Galicia (NW, Spain). *Veterinary Parasitology*, *179*, 216-219. https://doi.org/10.1016/j.vetpar.2011.02.023

Chalmers, R.M., Robinson, G., Elwin, K., Hadfield, S.J., Thomas, E., Watkins, J., Casemore, D., & Kay, D. (2010). Detection of *Cryptosporidium* species and sources of contamination with *Cryptosporidium hominis* during a waterborne outbreak in north west Wales. *Journal of Water & Health*, *8*, 311–325. https://doi.org/10.2166/wh.2009.185

Chen, L., Hu, S., Jiang, W., Zhao, J., Li, N., Guo, Y., Liao, C., Han, Q., Feng, Y., & Xiao, L. (2019). *Cryptosporidium parvum* and *Cryptosporidium hominis* subtypes in crab-eating macaques. *Parasites and Vectors*, *12*, 350. https://doi.org/10.1186/s13071-019-3604-7

Crecente, R., Alvarez, C., & Fra, U. (2002). Economic, social and environmental impact of land consolidation in Galicia. *Land Use Policy*, *19*, 135–147. https://doi.org/10.1016/S0264-8377(02)00006-6

de Lucio, A., Merino, F.J., Martínez-Ruiz, R., Bailo, B., Aguilera, M., Fuentes, I., & Carmena, D. (2016). Molecular genotyping and sub-genotyping of *Cryptosporidium* spp. isolates from symptomatic individuals attending two major public hospitals in Madrid, Spain. Infection, Genetics and Evolution, 37, :49-56. https://doi.org/10.1016/j.meegid.2015.10.026

Efstratiou, A., Ongerth, J.E., & Karanis, P. (2017). Waterborne transmission of protozoan parasites: Review of worldwide outbreaks - An update 2011-2016. *Water Research*, *114*, 14–22. https://doi.org/10.1016/j.watres.2017.01.036

468 Esch, K.J., & Petersen, C.A. (2013). Transmission and epidemiology of zoonotic protozoal
469 diseases of companion animals. *Clinical Microbiology Reviews*, *26*, 58–85.
470 https://doi.org/10.1128/CMR.00067-12
471 Feltus, D.C., Giddings, C.W., Schneck, B.L., Monson, T., Warshauer, D., & McEvoy, J.M.
472 (2006). Evidence supporting zoonotic transmission of *Cryptosporidium* spp. in
473 Wisconsin. *Journal of Clinical Microbiology*, *44*, 4303–4308.
474 https://doi.org/10.1128/JCM.01067-06
475 Feng, Y., & Xiao, L. (2011). Zoonotic potential and molecular epidemiology of *Giardia* species
476 and giardiasis. *Clinical Microbiology Reviews*, *24*, 110–40.
477 https://doi.org/10.1128/CMR.00033-10
478 Feng, Y., Ryan, U.M., & Xiao, L. (2018). Genetic diversity and population structure of
479 *Cryptosporidium*. *Trends in Parasitology*, *34*, 997–1011.
480 https://doi.org/10.1016/j.pt.2018.07.009
481 Gabín-García, L.B., Bartolomé, C., Abal-Fabeiro, J.L., Méndez, S., Llovo, J., & Maside, X.
482 (2017). Strong genetic structure revealed by multilocus patterns of variation in *Giardia*
483 *duodenalis* isolates of patients from Galicia (NW-Iberian Peninsula). *Infection, Genetics*
484 *and Evolution*, *48*, 131–141. https://doi.org/10.1016/j.meegid.2016.12.014
485 García-Presedo, I., Pedraza-Díaz, S., González-Warleta, M., Mezo, M., Gómez-Bautista, M.,
486 Ortega-Mora, L.M., & Castro-Hermida, J.A. (2013). Presence of *Cryptosporidium*
487 *scrofarum*, *C. suis* and *C. parvum* subtypes IIaA16G2R1 and IIaA13G1R1 in Eurasian
488 wild boars (*Sus scrofa*). *Veterinary Parasitology*, *196*, 497–502.
489 https://doi.org/10.1016/j.vetpar.2013.04.017
490 García-Presedo, I., Pedraza-Díaz, S., González-Warleta, M., Mezo, M., Gómez-Bautista, M.,
491 Ortega-Mora, L.M., Castro-Hermida, J.A. (2013). The first report of *Cryptosporidium*

bovis, *C. ryanae* and *Giardia duodenalis* sub-assemblage A-II in roe deer (*Capreolus capreolus*) in Spain. *Veterinary Parasitology*, *197*, 658–664. https://doi.org/10.1016/j.vetpar.2013.07.002

Gil, H., Cano, L., de Lucio, A., Bailo, B., Mingo, M.H., Cardona, G.A., Fernández-Basterra, J.A., Aramburu-Aguirre, J., López-Molina, N., & Carmena, D. (2017). Detection and molecular diversity of *Giardia duodenalis* and *Cryptosporidium* spp. in sheltered dogs and cats in Northern Spain. *Infection, Genetics and Evolution*, *50*, 62–69. https://doi.org/10.1016/j.meegid.2017.02.013

Gomez-Bautista, M., Ortega-Mora, L.M., Tabares, E., Lopez-Rodas, V., & Costas, E. (2000). Detection of infectious *Cryptosporidium parvum* oocysts in mussels (*Mytilus galloprovincialis*) and cockles (*Cerastoderma edule*). *Applied and Environmental Microbiology*, *66*, 1866–1870. https://doi.org/10.1128/aem.66.5.1866-1870.2000

Gómez-Couso, H., Mendez-Hermida, F., Ares-Mazas, E. (2006). First report of *Cryptosporidium parvum* 'ferret' genotype in American mink (*Mustela vison* Shreber 1777). *Parasitology Research*, *100*, 877–879. https://doi.org/10.1007/s00436-006-0338-1

Guk, S.M., Yong, T.S., Park, S.J., Park, J.H., & Chai, J.Y. (2004). Genotype and animal infectivity of a human isolate of *Cryptosporidium parvum* in the Republic of Korea. *Korean Jornal of Parasitology*, *42*, 85–89. https://doi.org/10.3347/kjp.2004.42.2.85

Hamnes, I.S., Gjerde, B.K., Forberg, T., & Robertson, L.J. (2007). Occurrence of *Giardia* and *Cryptosporidium* in Norwegian red foxes (*Vulpes vulpes*). *Veterinary Parasitology*, *143*, 347–353. https://doi.org/10.1016/j.vetpar.2006.08.032

Lalle, M., Pozio, E., Capelli, G., Bruschi, F., Crotti, D., & Cacciò, S.M. (2005). Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia*

duodenalis and identification of potentially zoonotic subgenotypes. *International Journal for Parasitology*, *35*, 207–213. https://doi.org/10.1016/j.ijpara.2004.10.022

Li N, Xiao L, Alderisio K, Elwin K, Cebelinski E, Chalmers R, Santin M, Fayer R, Kvac M, Ryan U, Sak B, Stanko M, Guo Y, Wang L, Zhang L, Cai J, Roellig D, & Feng Y. (2014). Subtyping *Cryptosporidium ubiquitum*, a zoonotic pathogen emerging in humans. *Emerging Infectious Diseases*, *20*, :217–224. https://doi.org/10.3201/eid2002.121797

López Beceiro, A.M., Riguera Rey, L., Espino López, L., & González Machado M.A. (2009) O Raposo en Galicia. Gráficas Sogal (ed.). Universidad de Santiago de Compostela. Conselleria de Medio Ambiente e Desenvolvemento Sostible. Xunta de Galicia. pp 70.

Mateo, M., de Mingo, M.H., de Lucio, A., Morales, L., Balseiro, A., Espí, A., Barral, M., Lima Barbero, J.F., Habela, M.Á., Fernández-García, J.L., Bernal, R.C., Köster, P.C., Cardona, G.A., & Carmena, D. (2017). Occurrence and molecular genotyping of *Giardia duodenalis* and *Cryptosporidium* spp. in wild mesocarnivores in Spain. *Veterinary Parasitology*, *235*, 86–93. https://doi.org/10.1016/j.vetpar.2017.01.016

Mackenstedt, U., Jenkins, D., & Romig, T. (2015). The role of wildlife in the transmission of parasitic zoonoses in peri-urban and urban areas. *International Journal for Parasitology: Parasites and Wildlife*, *4*, 71–79. https://doi.org/10.1016/j.ijppaw.2015.01.006

Méndez-Hermida, F., Gómez-Couso, H., Romero-Suances, R., & Ares-Mazás, E. (2007). *Cryptosporidium* and *Giardia* in wild otters (*Lutra lutra*). *Veterinary Parasitology*, *144*, 153–156. https://doi.org/10.1016/j.vetpar.2006.09.029

Mmbaga, B.T., & Houpt, E.R. (2017). *Cryptosporidium* and *Giardia* infections in children: A Review. *Pediatric Clinics of North America*, *64*, 837–850.

https://doi.org/10.1016/j.pcl.2017.03.014

Navarro-i-Martinez, L., Bornay-Llinares, F.J., Rueda, C., del Aguila, C., da Silva, A.J., Oleaga, A., Ramajo, V., Fenoy, S., & Pieniazek, N.J. (2003). Molecular characterization of *Cryptosporidium* sp. from animals in Spain. *Journal of Eukaryotic Microbiology*, *50*, Suppl, 553–554.

Navarro-i-Martinez, L. (2010) Detección y Caracterización Molecular de *Cryptosporidium* spp. Aislados de Humanos y Animales [Doctoral thesis]. Sant Joan D'Alacant: Universidad Miguel Hernández. p. 197.

Navarro-i-Martinez, L., del Águila, C., & Bornay-Llinares, F.J. (2011). *Cryptosporidium*: a genus in revision. The situation in Spain. *Enfermedades Infecciosas y Microbiología Clínica*, *29*, 135–143. https://doi.org/10.1016/j.eimc.2010.12.002

NESN (National Network of Epidemiological Surveillance). Red Nacional de Vigilancia Epidemiológica. Instituto de Salud Carlos III. Ministerio de Ciencia, Innovación y Universidades.Resultados de la Vigilancia Epidemiológica de las enfermedades transmisibles. Informe anual. Año 2016. p. 146.

O'Handley, R.M. (2007). *Cryptosporidium parvum* infection in cattle: are current perceptions accurate? *Trends in Parasitology*, *23*, 477–480. https://doi.org/10.1016/j.pt.2007.08.005

Polley, L. (2005). Navigating parasite webs and parasite flow: emerging and re-emerging parasitic zoonoses of wildlife origin. Int J Parasitol. 35(11-12):1279-94. https://doi.org/

Read, C.M., Monis, P.T., & Thompson, R.C. (2004). Discrimination of all genotypes *of Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. *Infection, Genetics and Evolution*, 4, 125–130. https://doi.org/10.1016/j.ijpara.2005.07.003

Reh, L., Muadica, A.S., Köster, P.C., Balasegaram, S., Verlander, N.Q., Chércoles, E.R., &

564 Carmena, D. (2019). Substantial prevalence of enteroparasites *Cryptosporidium* spp.,
565 *Giardia duodenalis* and *Blastocystis* sp. in asymptomatic schoolchildren in Madrid,
566 Spain, November 2017 to June 2018. *Euro Surveillance*, *24*.
567 https://doi.org/10.2807/1560-7917.ES.2019.24.43.1900241.
568 Robertson, L.J. (2018). Parasites in food: From a neglected position to an emerging issue.
569 *Advances in Food and Nutrition Research*, *86*, 71–113.
570 https://doi.org/10.1016/bs.afnr.2018.04.003
571 Ryan, U., Fayer, R., & Xiao, L. (2014). *Cryptosporidium* species in humans and animals:
572 current understanding and research needs. *Parasitology*, *141*, 1667–1685.
573 https://doi.org/10.1017/S0031182014001085
574 Ryan, U., & Cacciò, S.M. (2013). Zoonotic potential of *Giardia*. *International Journal for*
575 *Parasitology*, *43*, 943–956. https://doi.org/10.1016/j.ijpara.2013.06.001
576 Ryan, U., Zahedi, A., & Paparini, A. (2016). *Cryptosporidium* in humans and animals-a one
577 health approach to prophylaxis. *Parasite Immunology*, *38*, 535–547.
578 https://doi.org/10.1111/pim.12350
579 Ryan, U., Hijjawi, & N., Xiao, L. (2018). Foodborne cryptosporidiosis. *International Journal*
580 *for Parasitology*, *48*, 1–12. https://doi.org/10.1016/j.ijpara.2017.09.004
581 Schiller, S.E., Webster, K.N., & Power, M. (2016). Detection of *Cryptosporidium hominis* and
582 novel *Cryptosporidium* bat genotypes in wild and captive Pteropus hosts in Australia.
583 *Infection, Genetics and Evolution*, *44*, 254–260.
584 https://doi.org/10.1016/j.meegid.2016.07.002
585 Šlapeta, J. (2006). *Cryptosporidium* species found in cattle: a proposal for a new species. *Trends*
586 *in Parasitology*, *22*, 469–474. https://doi.org/10.1016/j.pt.2006.08.005

587 Šlapeta, J. (2013). Cryptosporidiosis and *Cryptosporidium* species in animals and humans: a
588 thirty colour rainbow? *International Journal for Parasitology*, *43*, 957–970.
589 https://doi.org/10.1016/j.ijpara.2013.07.005
590 Stojecki, K., Sroka, J., Caccio, S.M., Cencek, T., Dutkiewicz, J., & Kusyk, P. (2015).
591 Prevalence and molecular typing of *Giardia duodenalis* in wildlife from eastern Poland.
592 *Folia Parasitologica (Praha)*, *30*, 62. https://doi.org/10.14411/fp.2015.042
593 Suárez-Luengas, L., Clavel, A., Quílez, J., Goñi-Cepero, M.P., Torres, E., Sánchez-Acedo, C.,
594 & del Cacho, E. (2007). Molecular characterization of *Cryptosporidium* isolates from
595 pigs in Zaragoza (northeastern Spain). Veterinary Parasitology, 148, 231–235.
596 https://doi.org/10.1016/j.vetpar.2007.06.022
597 Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: molecular
598 evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*, 2725–
599 2729. https://doi.org/10.1093/molbev/mst197
600 Thompson, R.C.A. (2004). The zoonotic significance and molecular epidemiology of *Giardia*
601 and giardiasis. *Veterinary Parasitology*, *126*, 15–35.
602 https://doi.org/10.1016/j.vetpar.2004.09.008
603 Thompson, R.C. (2013). Parasite zoonoses and wildlife: One Health, spillover and human
604 activity. *International Journal for Parasitology*, *43*, 1079–1088. https://doi.org/
605 Thompson, R.C.A., & Ash, A. (2016). Molecular epidemiology of *Giardia* and
606 *Cryptosporidium* infections. *Infection, Genetics and Evolution*, *40*, 315–323.
607 https://doi.org/10.1016/j.ijpara.2013.06.007
608 Tiangtip, R., & Jongwutiwes, S. (2002). Molecular analysis of *Cryptosporidium* species
609 isolated from HIV-infected patients in Thailand. *Tropical Medicine and International*
610 *Health*, *7*, 357–364. https://doi.org/10.1046/j.1365-3156.2002.00855.x

Verweij, J.J., Schinkel, J., Laeijendecker, D., van Rooyen, M.A., van Lieshout, L., & Polderman, A.M. (2003). Real-time PCR for the detection of *Giardia lamblia*. *Molecular and Cellular Probes*, *17*, 223–225. https://doi.org/10.1016/S0890-8508(03)00057-4

Widmer, G., Köster, P.C., & Carmena, D. 2020. *Cryptosporidium hominis* infections in non-human animal species: Revisiting the concept of host specificity. *International Journal for Parasitology*, in press.

Winstanley, R.K., Saunders, G., Buttermer, & W. Indices for predicting total body fat in red foxes from Australia. 1998. J*ournal of Wildlife Management*, *62*, 1307–1312. https://doi.org/10.2307/3801995

Xiao, L. (2010). Molecular epidemiology of cryptosporidiosis: an update. *Experimental Parasitology*, *124*, 80–89. https://doi.org/10.1016/j.exppara.2009.03.018

Zahedi, A., Paparini, A., Jian, F., Robertson, I., & Ryan, U. (2016). Public health significance of zoonotic *Cryptosporidium* species in wildlife: Critical insights into better drinking water management. *International Journal for Parasitology: Parasites and Wildlife*, *5*, 88–109. https://doi.org/10.1016/j.ijppaw.2015.12.001

FIGURE CAPTIONS

Figure 1. Map of the autonomous region of Galicia (Northwest Spain) showing the geographical location where wild red foxes were sampled. Green and orange filled circles/quadrants represent *Cryptosporidium*- and *Giardia*-positive results by PCR assays, respectively. Yellow filled triangles represent samples that tested negative to both pathogens.

Figure 2. Phylogenetic tree depicting evolutionary relationships among *Cryptosporidium* sequences at the *ssu* rRNA gene. The analysis was inferred using the Neighbor-Joining method. Bootstrap values lower than 75% were not displayed. Filled triangles represent sequences generated in the present study. Empty triangles indicate sequences from red foxes previously reported in Spain and other countries, used for comparison purposes. Filled circles represent reference sequences retrieved from the GenBank database. *Cryptosporidium fragile* was used as outgroup taxa.