



# Molecular epidemiology of parasitic protozoa and *Ehrlichia canis* in wildlife in Madrid (central Spain)

Angel Criado-Fornelio<sup>1</sup> · T. Martín-Pérez<sup>1</sup> · C. Verdú-Expósito<sup>1</sup> · S. A. Reinoso-Ortiz<sup>1</sup> · J. Pérez-Serrano<sup>1</sup>

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## Abstract

Wildlife species are involved in the transmission of diverse pathogens. This study aimed to monitor raccoons (*Procyon lotor*), American minks (*Neovison vison*), and red foxes (*Vulpes vulpes*) as potential reservoirs in central Spain. Specifically, 200 spleen and fecal samples (from 194 raccoons, 3 minks, and 3 foxes) were analyzed molecularly by PCR/qPCR and sequencing for the presence of piroplasmids, *Hepatozoon* spp., *Toxoplasma gondii*, and *Ehrlichia canis* infections in the Community of Madrid (Spain). Biological samples were obtained in the years 2014, 2015, and 2016. No pathogen DNA was found in fecal samples. In contrast, analysis of raccoon spleen samples revealed that *Toxoplasma* was the most prevalent pathogen (prevalence  $3.6 \pm 2.6\%$ ), followed by *Hepatozoon canis* and *E. canis* (each with a prevalence of  $2.57 \pm 2.2\%$ ). *Hepatozoon canis* was also diagnosed in all three of the analyzed foxes. Analysis of yearly prevalence showed that tick-borne pathogens were less frequent in raccoon in 2015, a dry and warm year compared both to 2014 and 2016. These data suggest that fecal PCR assays are unsuitable for detection of DNA of non-erythrocytic pathogens. Furthermore, they demonstrate that the raccoon (an invasive species often living in proximity to domestic areas) and the red fox are putative reservoirs for pathogenic organisms in the Community of Madrid.

**Keywords** Wildlife · Parasitic protozoa · *Ehrlichia canis* · Epidemiology · PCR · qPCR

## Introduction

The raccoon (*Procyon lotor*) and the American mink (*Neovison vison*) are small- to medium-sized carnivores living mainly in North America, although the geographical range of procyonids reaches Central America. Non-native populations of both carnivores have been established worldwide, including Southern Europe (Canova and Rossi 2008). Populations of wild raccoons and American minks have been reported in central Spain (Barona and García-Román 2005; García et al. 2012; Melero and Palazón 2017). On the other hand, red foxes (*Vulpes vulpes*) show a wide distribution in the northern

hemisphere, including Spain (López-Martín 2010). These three species are not highly specialized, thus thrive in diverse habitats (including peri-urban areas), making them significant reservoirs of infection for both domestic animals and humans.

The molecular epidemiology of tissue and hematic protozoa in raccoons and minks has been studied in many geographic locations. Thus, piroplasmids have been found in raccoons in the USA (Telford and Forrester 1991; Birkenheuer et al. 2006; Clark et al. 2012; Hersh et al. 2012), and two different *Babesia* species have been detected in raccoons and American minks in Japan (Kawabuchi et al. 2005; Hirata et al. 2013). Red foxes are known to frequently harbor both piroplasmids and *Hepatozoon* (Criado-Fornelio et al. 2003a; Torina et al. 2013; Ebani et al. 2017; Koneval et al. 2017). *Hepatozoon* spp. have also been diagnosed in raccoons in the USA (Clark et al. 1973; Schaffer et al. 1978; Panciera et al. 2001; Allen et al. 2011). In a previous survey, no piroplasmid or *Hepatozoon* infections were found in American minks in Spain; however, this result is not very informative as only two animals were analyzed (Barandika et al. 2016).

*Toxoplasma* infection seems to be present in small carnivores worldwide (Sepúlveda et al. 2011; Heddergott et al. 2017; Martino et al. 2017). The molecular epidemiology of

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✉ Angel Criado-Fornelio  
angel.criado@uah.es

<sup>1</sup> Departamento de Biomedicina y Biotecnología, Laboratorio de Parasitología, Grupo ECOMYP, Facultad de Farmacia, Universidad de Alcalá, 28805 Alcalá de Henares, Madrid, Spain

toxoplasmosis in raccoons, minks, and foxes has been studied in Europe, which has shown that prevalence varies greatly depending on location (Verin et al. 2013; Suteu et al. 2014; Ferroglio et al. 2014; Calero-Bernal et al. 2015).

Anaplasmataceae infections have been found in raccoons, minks, and foxes. Molecular studies of procyonids from the USA and Japan showed a low number of samples positive to bacterial DNA (< 6%) for *Ehrlichia canis* and *Anaplasma phagocytophilum* in raccoons (Dugan et al. 2005; Inokuma et al. 2007; Yabsley et al. 2008; Sashika et al. 2011). A different situation occurs in red foxes in Italy and Spain, as *Ehrlichia canis* DNA was detected in more than 16% of the sampled animals (Millán et al. 2016; Ebani et al. 2017). To our knowledge, no molecular studies of Anaplasmataceae in American minks have been reported to date. Furthermore, no studies of these bacteria in the raccoon, American mink, or red fox in Spain have been conducted. However, *E. canis* and *Anaplasma platys* are known to be present in Madrid based on evidence from infected dogs (Sainz et al. 1998, 1999; Aguirre et al. 2004). Therefore, it is unknown if the Anaplasmataceae infecting dogs in central Spain may be transmitted (through ticks) to raccoons or minks there, as the susceptibility of these exotic animals to the local strains of these bacteria has not yet been studied. Additionally, it is important to point out that *A. phagocytophilum* and *Neorickettsia risticii* have not been detected in central Spain (Amusatogui et al. 2008; Sainz et al. 2015).

A PCR technique using fecal DNA has been used as a non-invasive method to analyze the presence of some hemoparasites (such as *Plasmodium* or piroplasmids) in wildlife (Liu et al. 2010; Hornok et al. 2015). Some authors have suggested the feasibility of detecting other non-erythrocytic hematic parasites by fecal PCR (see, e.g., Criado-Fornelio 2012). However, no attempts to perform such diagnosis have been reported to date, despite it being a better alternative to current procedures that involve the collection of blood or spleen samples in wildlife (Torina et al. 2013).

The presence of parasitic protozoa and bacteria in raccoons and minks invading Europe, and specifically Spain, has been scarcely studied, even though the subject is highly relevant due to its sanitary implications for both animals and humans. To fill this gap in pathogen epidemiology, a molecular survey (based on PCR/quantitative PCR (qPCR) and sequencing of *Toxoplasma* B1 or ribosomal genes) for piroplasmids, *Hepatozoon*, *Toxoplasma*, and *E. canis* has been carried out in wild raccoons, minks, and red foxes from the Community of Madrid (central Spain). Furthermore, given that fecal PCR has been suggested as an alternative method for hematic (non-erythrocytic) pathogen detection in carnivores, molecular diagnosis assays for protozoa and bacteria were performed using both spleen and fecal samples for comparative purposes.

## Materials and methods

### Biological samples

During the survey period (January 2014 to December 2016), spleen and fecal samples (the latter consisting of a fragment of the end of the colon containing formed feces) were collected from 194 raccoons (*Procyon lotor*), 3 American minks (*Neovison vison*), and 3 red foxes (*Vulpes vulpes*). Animals were captured by trappers (under authorization of the Regional Government of Madrid, with the following yearly references: 10/236833.9/13, issued on 11/25/2013; 10/039635.9/15, issued on 03/04/2015; and 10/047369.9/16, issued on 03/09/2016) and transported to the Wildlife Recovery Center of the Regional Government of Madrid in Tres Cantos (Madrid, Spain), where veterinarians removed the organs required for this study. Biological samples were frozen in sterile plastic containers at  $-20\text{ }^{\circ}\text{C}$  and subsequently sent on cold packs to the Parasitology Laboratory at the Universidad de Alcalá.

### DNA extraction

DNA was extracted from 0.15 g of either spleen or feces. For tissue and fecal DNA extractions, respectively, the Ultra Clean® Tissue & Cells DNA Isolation Kit and the Power Fecal® DNA Isolation kit (MO BIO, Carlsbad, CA, USA) were used. Molecular diagnosis was performed using three different samples: spleen DNA, undiluted fecal DNA, and fecal DNA diluted to 1/25 with sterile distilled water. Diluted fecal DNA was used to facilitate PCR amplification in case inhibitors were present in undiluted fecal samples after purification.

### PCR and qPCR assays for different pathogens and DNA sequencing

The presence of piroplasmid DNA was diagnosed by standard PCR targeting the 5' end of the small subunit ribosomal RNA gene using the primer pair BT1-F (GGTTGATCCTGCCA GTAGT) and BT1-R (GCCTGCTGCCTTCCTTA), as previously described (Criado-Fornelio et al. 2003b). DNA of *Babesia vulpes*-infected foxes, which were already available in our laboratory, were used as positive controls.

qPCR with SYBR green, targeting the 5' end of the small subunit ribosomal RNA gene, was used to detect the presence of *Hepatozoon* sp. DNA, as previously described by Criado-Fornelio et al. (2007). The primers HEP-1 (CGCGCAA TTACCCAATT) and HEP-2 (CAGACCGGTTACTT TYAGCAG) were used in amplification reactions. Positive control samples (of foxes positive for *H. canis*) were already available in our laboratory (Criado-Fornelio et al. 2006).

The presence of *Toxoplasma* sp. DNA in biological samples was diagnosed by amplification of a fragment of the B1 gene using qPCR with SYBR green and primers previously described by Costa et al. (2000): TOX-1 (GGAGGACT GGCAACCTGGTGTCTCG) and TOX-2 (TTGTTTCA CCCGGACCGTTTAGCAG). No FRET probes were included in the assay. *Toxoplasma gondii* (strain RH-type I) DNA, available in our laboratory from a previous study (Criado-Fornelio et al. 2009), was used as the positive control.

*Ehrlichia canis* DNA was amplified by a qPCR assay with SYBR green using the newly designed primers ERC-1 (AAGCCTAACACATGCAAG) and ERC-2 (TGGCTATT CCGTACTACT). These primers amplify a 117-bp fragment of the 5' end of the *E. canis* 16S small subunit ribosomal RNA gene. The thermal cycling profile for this qPCR assay included an initial denaturation step at 95 °C for 2 min and then 40 cycles, each comprising denaturation at 95 °C for 10 s, annealing at 60 °C for 5 s, and extension at 72 °C for 6 s. The temperature transition rates were programmed at 20 °C/s. After completion, a melting curve was recorded by decreasing the temperature to 35 °C at a rate of 20 °C/s, holding at 35 °C for 30 s, and then slowly increasing the temperature to 90 °C at a rate of 0.2 °C/s. DNA obtained from the blood of 10 animals with canine ehrlichiosis (as determined by microscopy and immunological procedures) were used as positive controls to validate the technique; 10 dogs positive to other pathogens [*Babesia canis* (3), *B. vulpes* (3), and *H. canis* (4)] and 50 healthy dogs were used as negative controls. In addition, amplifications using 0.2 ng of DNA from *Mycobacterium tuberculosis* (H37Rv), *Escherichia coli* (CECT-100), *Pseudomonas aeruginosa* (CECT-108), *Clostridium perfringens* (CECT-4110), or *Brucella abortus* (292) were used to check for specificity as previously described (Criado-Fornelio et al. 2003b). Standard curves were prepared by using serial dilutions of plasmid DNA including the targeted fragment of the 16S small subunit ribosomal RNA gene of *E. canis*. This fragment was cloned using the NZY-A PCR cloning kit (NZY, Lisbon, Portugal), following the manufacturer's instructions. Plasmid DNA was purified with the Mini Plasmid Prep kit (MO BIO).

To detect cross-contamination artifacts during DNA amplification, all PCR assays included a negative control (sterile water). In addition, a positive control consisting of DNA from infected animals was used as well to ensure the reproducibility of the technique. Template DNA (2 µl) was added at a final concentration of 5 ng/µl. All qPCR assays were performed in a Lightcycler 1.5 instrument (Roche diagnostics GmbH, Mannheim, Germany). The SYBR Premix Ex Taq II kit (Takara-Bio, Shiga, Japan) was used for amplification. Procedures for DNA electrophoresis and band purification from agarose gels were as previously described (Criado-Fornelio et al. 2003b). In all cases, if one of the analyzed samples had positive amplification, another PCR reaction

was performed. The DNA products from both reactions were sequenced using an ABI 3130 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA). For each *E. canis*-positive sample, 6 clones were sequenced to assess, to some extent, if multiple species were detected with this qPCR assay. Sequences were processed with the BioEdit 5.0.9 sequence editor (Hall 1999). A BLASTn search was performed to confirm the sequence identities of pathogens and determine the most similar sequence match available in GenBank.

## Climate data

Climate data were compiled (along with parasitization rates) to infer the potential influence of climate on pathogen temporal distribution in raccoons in Spain (Table 2). Average annual temperature and rainfall data from 2014 to 2016 were obtained from records available from the Spanish Meteorological Service, Agencia Española de Meteorología (AEMET 2018). Monthly climatic records for five weather stations in the Community of Madrid (namely Retiro, Barajas, Torrejón, Getafe, and Cuatro Vientos) were used in the calculations. In addition, the temperature and rainfall categories for Madrid were determined using the annual climatic records for Spain that were graphically represented in color-coded maps (AEMET 2018).

## Statistical analysis

The absolute error for prevalence in the raccoon was calculated with the *Win Episcopy* 2.0 software package (Thrusfield et al. 2001), assuming a raccoon population of 20,000 animals and setting a confidence level of 95%. The statistical significance of the results on the melting curve analysis was determined using the Student *t* test. A Kruskal-Wallis test was performed to test for homogeneity of the comparison among animals positive for tick-borne pathogens in raccoons by year (Table 2). These analyses were performed using the *GraphPad Prism 5*® software package (GraphPad Software, San Diego, CA, USA). Statistical significance was defined at  $P < 0.05$ .

## Results

Fecal samples were negative for all pathogens. In contrast, PCR and sequencing in raccoon spleen samples (Table 1) showed seven animals positive for *Toxoplasma* DNA and five animals positive for each *H. canis* and *E. canis* DNA. The three pathogens found in the raccoon all showed less than 4% prevalence and did not appear in combined infections (Table 1). *Hepatozoon canis* DNA was found in the three red foxes studied. The three American minks analyzed were negative for all pathogens tested.

**Table 1** Results of the molecular survey of 200 spleen samples of three wildlife species from the Community of Madrid

Host type (number of samples) and pathogens diagnosed	% positive $\pm$ absolute error (number positives/total analyzed)	Closest GenBank entry/% identity (BLASTn)
<i>Procyon lotor</i> (194)		
<i>Hepatozoon canis</i>	2.57 $\pm$ 2.2 (5/194)	MG 758124 <sup>a</sup> and MG 018464 <sup>a</sup> /99%
<i>Toxoplasma gondii</i>	3.60 $\pm$ 2.6 (7/194)	AF 179871/100%
<i>Ehrlichia canis</i>	2.57 $\pm$ 2.2 (5/194)	CP 025749/100%
<i>Vulpes vulpes</i> (3)		
<i>Hepatozoon canis</i>	100% (3/3)	AF 179871/100%
<i>Neovison vison</i> (3)		
No pathogens found	0%	NA

<sup>a</sup> Four different sequences were obtained for *H. canis* in raccoon: two of these were closest in % identity with one GenBank entry (MG758124), while the other two were closest to another GenBank entry (MG018464). Therefore, both of these GenBank sequences represent the closest match of the four obtained sequences

The results of the BLASTn comparison of the sequences obtained in the survey are shown in Table 1. Four different *Hepatozoon* small ribosomal subunit sequences were obtained from the five positive raccoon samples. A BLASTn search showed that these procyonid isolates were 99% identical to *H. canis* from Australian ticks (showing one to three base differences in the 235-bp fragment). These sequences were deposited into GenBank with the following accession numbers MG897468, MG897469, MG897470, and MG897471 (the last sequence was found in two raccoons). The *H. canis* sequence from the red fox samples was 100% identical to an isolate from the Spanish fox. The partial sequence of the *Toxoplasma* B1 gene infecting procyonids was 100% identical to other GenBank entries for that gene. Likewise, all of the putative *E. canis* 16S rRNA gene sequences obtained from the qPCR assay of the raccoon samples (either by direct or plasmid sequencing) were verified as *E. canis*. However, *Toxoplasma* and *E. canis* sequences were shorter than 200 bp and therefore could not be submitted to GenBank per their requirements.

The qPCR assay with the newly designed *E. canis* primers showed good specificity and sensitivity (100%). All dogs positive for *Ehrlichia* DNA were correctly identified by qPCR (as demonstrated by sequencing), and no false-positive amplification occurred in any of the control reactions using DNA from dogs infected with other common pathogens. Furthermore, no amplification was obtained in assays performed with control bacterial DNA or DNA from healthy dogs. The sensitivity of the assay is such that concentrations as low as 0.1 fg of target DNA can be detected. The standard curve obtained in different assays showed a linear response at a wide range of DNA concentrations, and standard curve parameters [correlation coefficient ( $r$ ) = -0.99; slope = -3.32] indicated that the amplification reaction was well optimized (see supplementary material for more details about the *E. canis* qPCR assay). Melting curve analysis showed that the *E. canis*-specific product had a melting temperature ( $T_m$ ) of

80.97  $\pm$  0.12 °C; differences in  $T_m$  among different *E. canis* isolates were non-significant. Presence of primer-dimer artifacts was not common; when present, they had lower melting temperatures than specific products, allowing easy identification of positive samples.

The data concerning monthly temperatures and rainfall in the Community of Madrid are shown as supplementary material. Annual average temperature and rainfall were calculated based on these variables, and the results obtained are shown in Table 2, in combination with the annual prevalence data for the pathogens studied in raccoon (see the “Discussion” section).

## Discussion

Native populations of procyonids show relatively high frequencies of presence of *Babesia microti* DNA in the USA (Clark et al. 2012; Hersh et al. 2012). Piroplasmid DNA has been found in raccoons from Japan (Kawabuchi et al. 2005; Hirata et al. 2013). Molecular analysis of two American minks in northern Spain found no piroplasmid infections (Barandika et al. 2016). Therefore, these previous findings and those from this study suggest that small carnivores (such as raccoon) are not susceptible to non-autochthonous piroplasmids. Although *B. vulpes* is common in red fox in central Spain (Criado-Fornelio et al. 2006; Criado-Fornelio 2012), it seems unable to parasitize the procyonids living there. The local tick vector for *B. vulpes* in central Spain is likely not prone to parasitizing raccoons, or, alternatively, it may be absent in the areas where these animals thrive. A recent study suggests that *Dermacentor reticulatus* is a possible vector of *B. vulpes* (Hodzic et al. 2017). Another species of this genus (*D. marginatus*) is present in Madrid (Cordero del Campillo et al. 1994), but it is unknown if it is a vector for the protozoa. Further studies on ticks infesting raccoons in the study area are needed to address this hypothesis.

**Table 2** Summary of the molecular diagnosis of parasites and *E. canis* in 194 raccoon spleen samples according to year and annual climate data (temperature and rainfall—AEMET 2018) in the Community of Madrid for the period 2014–2016

Variable	Years		
	2014	2015	2016
Number of raccoon samples studied	31	71	92
Average annual temperature (°C)—temperature category <sup>a</sup>	15.7—very to extremely warm	16.22—very to extremely warm	15.63—warm to very warm
Average annual rainfall (mm)—rainfall category <sup>a</sup>	406—rainy to very rainy	272.5—very dry	467.5—rainy to very rainy
<i>Hepatozoon</i> -positive animals	5	0	0
Piroplasmid-positive animals	0	0	0
<i>Toxoplasma</i> -positive animals	1	2	4
<i>E. canis</i> -positive animals	3	0	2

<sup>a</sup> Categories are based on AEMET annual climatic maps for Spain and consist of up to seven temperature categories ranging from “extremely cold” to “extremely warm” and up to seven rainfall categories (ranging from “extremely dry” to “extremely rainy”). In most cases, two different categories were found for the community of Madrid: one for a particular area of Madrid and another for the rest of the Community. Therefore, the temperature and rainfall categories typically fall between two categories. AEMET categories were delineated by comparing the average values of temperature and rainfall for each year with the averages of a defined reference period (from 1971 to 2000). Each step (up or down) of the map scale represents an increase/decrease of 20% of the average value compared to the reference period

The present study is the first to record *H. canis* infection of non-native carnivores (raccoons). Indeed, a *Hepatozoon* species (probably *H. procyonis*) has been shown to be present in procyonids in the USA as demonstrated by molecular methods (Allen et al. 2011). It is unclear if the Spanish hepatozoons are imported or local as *H. canis* is relatively common in red foxes and dogs in central Spain (Criado-Fornelio et al. 2003a; Criado-Fornelio et al. 2006; Giménez et al. 2009; Criado-Fornelio 2012; this study).

Serological studies have shown that raccoons are frequently seropositive to *Toxoplasma*, with highly variable prevalence ranging from 9.9 to 65% (Sato et al. 2011; Rainwater et al. 2017). Sepúlveda et al. (2011) also reported high toxoplasmosis prevalence (70%) in American minks. Molecular diagnosis of *Toxoplasma* infection has been far less used, probably due to the low prevalence found by these methods. Only animals in the acute disease stage (or those with immunosuppression) are likely to be diagnosed by PCR-based assays. However, a few molecular studies of the red fox in different parts of Europe have also shown highly variable toxoplasmosis levels. Verin et al. (2013) and Suteu et al. (2014) observed a relatively low prevalence of *Toxoplasma* (< 6%), which is consistent with the data presented here. Ferroglio et al. (2014) found a moderate frequency (20.21%), whereas Calero-Bernal et al. (2015) reported high prevalence in southern Spain (51.2%). In terms of the qPCR assay, although Costa et al. (2000) used FRET probes to confirm *Toxoplasma* infection with the TOX-1 and TOX-2 primers, our results demonstrate that these primers are also suitable for molecular detection of this protozoa in qPCR assays with SYBR green (combined with sequencing), as no false positives were detected in our diagnostic tests.

Generally speaking, epidemiological studies of carnivore ehrlichiosis have been based mainly on immunological evidence. Dugan et al. (2005) found that 21.7% of the animals they sampled in the USA were seropositive for *E. canis*, yet molecular analysis of the same pathogen indicated that only 1 raccoon out of 60 animals was positive (prevalence of 1.66%), which is in agreement with the findings presented here. Inokuma et al. (2007) found a low prevalence of *E. canis* (0.5%) in a sample of 187 raccoons in Japan based on serological tests. Consistent with this, Sashika et al. (2011), using PCR-based assays, did not detect *E. canis* in a sample of 699 animals in the same country. Likewise, Yabsley et al. (2008) suggested that raccoons are unlikely reservoirs of *E. canis* and *E. ewingii* in the USA, likely harboring *E. chaffeensis* instead. According to our data, a different situation appears to occur in Spain, where *E. canis* DNA was detected in raccoons, albeit with low prevalence. The fact that many raccoons in Spain are located in peri-domestic habitats (Barona and García-Román 2005; García et al. 2012) makes them potential reservoir for both ticks and dogs. Dogs show a low *E. canis* infection rate in Madrid (5–6.5%, according to immunological tests; Sainz et al. 1998; Miró et al. 2013) that is comparable to the rate found here for raccoons. These data suggest that in central Spain, *E. canis* may be transmitted by ticks between domestic animals (dogs) and wildlife (mainly carnivores) and vice versa. In terms of the molecular analysis, the *E. canis* qPCR assay reported in this work performed well, detecting *E. canis* DNA in positive control samples and yielding no amplification in negative control samples. Furthermore, a limited number of positive raccoons were detected and sequencing demonstrated that direct amplification or cloning led to the

identification of *E. canis* in these samples. The method seems reliable as far as amplified DNA samples are sequenced to confirm the pathogen identity.

Host, pathogens, and vectors are influenced by environmental factors that regulate their biological densities. Adverse climatic conditions affect survival and, moreover, decrease contact rates, thus influencing the circulation of vector-borne pathogens in ecosystems (Estrada-Peña and de la Fuente 2017). In an attempt to evaluate the putative influence of environmental factors on the temporal distribution of tick-borne pathogens in the raccoon analyzed in this study, their prevalence was compared to annual climatic conditions (temperature and rainfall) of the Community of Madrid during the period of study (Table 2). Based on these data, 2015 was a very warm and dry year, suggesting that tick populations were smaller in that year. Consistent with this hypothesis, tick-borne pathogens were not detected in any of the animals sampled that year; however, they were in both 2014 and 2016, which were both more humid than the average. A statistical analysis performed with the nonparametric Kruskal-Wallis test clearly indicated that the yearly distribution of positive animals (for *Hepatozoon* and *Ehrlichia*) shows no homogeneity ( $P < 0.0001$ ). This observation may partially explain the great variability in parasite prevalence observed by different researchers. Variations in climatic and biological factors affecting either the host, the pathogen, or its vectors dramatically affect prevalence, particularly for vector-borne pathogens (Estrada-Peña et al. 2013). Furthermore, variations in sensitivity and specificity among the different diagnostic techniques may contribute to the observed differences in prevalence.

Molecular detection methods have provided new insights for studies of pathogens in wildlife (Thompson et al. 2010). Fecal material is abundant and easy to collect. Moreover, it can be obtained without disturbing the study animals (Wasser et al. 2004). This type of experimental approach is best suited for temperate countries, as exposure to humidity, warm temperatures, rain, and sunlight is more limited; hence, DNA degradation in feces is less extensive than in tropical countries. This method worked well for detecting intraerythrocytic parasite DNA, such as *Plasmodium* in gorillas (Liu et al. 2010) or *Babesia canis* in bats (Hornok et al. 2015). In our study, the fecal material was removed directly from the digestive tract and quickly processed, so that the DNA samples analyzed were as intact as possible for PCR amplification compared to those obtained in the field. However, *Hepatozoon*, *Toxoplasma*, and *E. canis* are mainly found within white blood cells, which comprise a small fraction of the cells found in blood. Therefore, the likelihood of amplifying parasite DNA in fecal material is much smaller, which likely accounts for the lack of positive amplifications in our samples. The presence of piroplasmid DNA should have been detectable by fecal PCR; however, none was found in any of the fecal or spleen samples analyzed.

Although the frequency of *H. canis*, *Toxoplasma*, and *E. canis* infection in raccoons is low, the finding of pathogen DNA in an invasive host species widens the spectrum of putative mammalian carriers of parasites and Anaplasmataceae in Spain. Moreover, as our data suggests, climate may affect the distribution of pathogens. Additional pathogen monitoring studies are necessary to investigate the current transmission patterns among wildlife, domestic animals, and humans. In this sense, molecular epidemiology may help in the decision-making process to ensure the adequate control measures are implemented to limit the potential sanitary impact of invasive species.

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## Compliance with ethical standards

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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