Received: 12 May 2021 | Revised: 11 June 2021 | Accepted: 23 June 2021

DOI: 10.1111/tbed.14206

Transboundary and Emerging Diseases

WILEY

RAPID COMMUNICATION

Molecular survey of *Besnoitia* spp. (Apicomplexa) in faeces from European wild mesocarnivores in Spain

David González-Barrio[1,2,3,4] | Pamela C. Köster[2] | Miguel A. Habela[5] | Manuel Martín-Pérez[5] | Jose L. Fernández-García[5] | Ana Balseiro[6,7] | Marta Barral[8] | Fernando Nájera[9] | Ana M. Figueiredo[10,11] | Maria Jesus Palacios[9] | Marta Mateo[12] | David Carmena[2] | Gema Álvarez-García[1] | Rafael Calero-Bernal[1]

[1] SALUVET, Department of Animal Health, Faculty of Veterinary, Complutense University of Madrid, Madrid, Spain

[2] Parasitology Reference and Research Laboratory, Spanish National Centre for Microbiology, Health Institute Carlos III, Majadahonda, Madrid, Spain

[3] Viral Hepatitis Reference and Research Laboratory, Spanish National Centre for Microbiology, Health Institute Carlos III, Majadahonda, Madrid, Spain

[4] SaBio Working Group, Institute for Game and Wildlife Research IREC (CSIC-UCLM-JCCM), Ciudad Real, Spain

[5] Department of Animal Health, Veterinary Sciences Faculty, Extremadura University, Cáceres, Spain

[6] Animal Health Department, Veterinary School, University of León, León, Spain

[7] Departamento de Sanidad Animal, Instituto de Ganadería de Montaña (CSIC-Universidad de León), Finca Marzanas, Grulleros, León, Spain

[8] NEIKER-Basque Institute for Agricultural Research and Development, Derio, Bizkaia, Spain

[9] Directorate-General for Environment, Regional Government of Extremadura, Mérida, Badajoz, Spain

[10] Department of Biology & CESAM, University of Aveiro, Campus Universitário de Santiago, Aveiro, Portugal

[11] Department of Bioscience & CEES, University of Oslo, Oslo, Norway

[12] Veterinary Faculty, Alfonso X El Sabio University, Avenida Universidad 1, Villanueva de la Cañada, Madrid, Spain

Correspondence
David González-Barrio, SALUVET, Department of Animal Health, Faculty of Veterinary, Complutense University of Madrid, Av. Puerta de Hierro s/n, 28040, Madrid, Spain.
Email: davigo20@ucm.es, dgonzalezbarrio@gmail.com
Rafael Calero-Bernal, SALUVET, Department of Animal Health, Faculty of Veterinary, Complutense University of Madrid, Av. Puerta de Hierro s/n, 28040, Madrid, Spain.
Email: r.calero@ucm.es

Funding information
Health Institute Carlos III (ISCIII); Ministry of Economy and Competitiveness, Grant/Award Number: PI16CIII/00024; Complutense University of Madrid, Grant/Award Number: CT65/16; Spanish Ministry of Economy and Competitiveness, Grant/Award Number: AGL-2016-75202-R; Community of Madrid; University of Aveiro

Abstract

Numerous studies have unsuccessfully tried to unravel the definitive host of the coccidian parasite *Besnoitia besnoiti*. Cattle infections by *B. besnoiti* cause a chronic and debilitating condition called bovine besnoitiosis that has emerged in Europe during the last two decades, mainly due to limitations in its control associated with the absence of vaccines and therapeutical tools. Although the exact transmission pathways of *B. besnoiti* is currently unknown, it is assumed that the parasite might have an indirect life cycle with a carnivore as definitive host. Current lack of studies in wildlife might underestimate the importance of free-living species in the epidemiology of *B. besnoiti*. Thus, the aim of the present study is to assess the presence of *Besnoitia* spp. in free-ranging mesocarnivores in Spain. DNA was searched by PCR on faeces collected from wild carnivores as a first approach to determine which species could be considered as potential definitive host candidates in further research. For this purpose, a total of 352 faecal samples from 12 free-living wild carnivore species belonging to the Canidae, Felidae, Herpestidae, Mustelidae, Procyonidae and Viverridae families were collected in seven Spanish regions. PCR testing showed that *Besnoitia* spp. DNA was present in four faecal samples from red foxes collected in western Spain, an area with the greatest density of extensively reared cattle and associated with high incidence of bovine besnoitiosis in

the country. To date, this is the first report of a *B. besnoiti*-like sequence (99.57% homology) from carnivore faeces in a worldwide context. Red foxes might contribute to the epidemiology of *B. besnoiti*, although further studies, mostly based on bioassay, would be needed to elucidate the accuracy and extent of these interesting findings.

KEYWORDS
Apicomplexa, *Besnoitia besnoiti*, biological cycle, carnivore, Spain, wildlife

1 | INTRODUCTION

Besnoitia besnoiti (Apicomplexa) is the aetiological agent of a chronic and debilitating disease of cattle called bovine besnoitiosis characterized by a low body score, non-specific systemic clinical signs and skin lesions. Reproductive failure is a major concern as males may develop infertility, sterility or even may die during the acute phase of the infection (González-Barrio et al., 2020). Bovine besnoitiosis causes considerable economic losses in many countries in Africa, the Middle East and Europe. In these regions, the disease has been increasingly spreading over the last two decades (European Food Safety Authority, 2010; Álvarez-García et al., 2013), mainly due to control drawbacks including the absence of vaccines and therapeutical tools, also cattle testing prior to movement may be insufficient and contribute to the spread of the disease. Several surveys confirmed the increased prevalence and geographical expansion of this disease in cattle, in areas of Western and Northern Europe (Álvarez-García, 2016). A few studies carried out in Spain reported high seroprevalence rates in beef cattle herds, ranging from 36 to 87% in Urbasa Andía and the Pyrenees mountain ranges in North Spain (Álvarez-García et al., 2014; Cortes et al., 2014; Gutiérrez-Expósito et al., 2014).

Despite the fact that its biological cycle is not fully known, it is assumed that *B. besnoiti* might have an indirect life cycle with a carnivore as definitive host able to shed oocysts after ingestion of tissues harbouring mature cysts (Cortes et al., 2014; Jacquiet et al., 2010). Although domestic cattle act as intermediate hosts of the parasite, specific antibodies against *B. besnoiti* were also described in roe deer (*Capreolus capreolus*) (Arnal et al., 2017) and red deer (*Cervus elaphus*) in North Easter Spain, regions where bovine besnoitiosis is endemic, showing that these wild ruminant species are also intermediate hosts (Gutiérrez-Expósito et al., 2013, 2016). Despite this fact, their role in the epidemiology of bovine besnoitiosis seems to be of scarce importance (Gutiérrez-Expósito et al., 2016). In contrast, specific antibodies were not detected in small ruminants (Gutiérrez-Expósito et al., 2017). Similarly, a serosurvey on *Besnoitia* spp. in wild carnivore species from Spain provided no evidence to support that wild carnivores are implicated in the epidemiology of *B. besnoiti* within the geographical regions covered by the analysis (Millán et al., 2012).

Domestic and wild felines have been suggested as definitive hosts for *B. besnoiti*; however, experimental infections in several felid species failed to confirm their suitability as potential definitive hosts (Basso et al., 2011; Diesing et al., 1988). The putative role of a sylvatic life cycle, involving other Carnivora species, in the epidemiology of the disease has not been fully elucidated (see Table 1).

The aim of the present survey is to assess the presence of *Besnoitia* spp. DNA in faeces from wild mesocarnivores in Spain as a first step to determine which species might be considered as potential definitive host candidates in further investigations.

2 | MATERIAL AND METHODS

2.1 | Sample collection

A total of 352 faecal samples from 12 free-living carnivore species belonging to the Canidae (n = 193), Felidae (n = 15), Herpestidae (n = 1), Mustelidae (n = 131), Procyonidae (n = 1) and Viverridae (n = 11) families were collected in seven Spanish regions between December 2013 to October 2017 (Table 2 and Figure 1). The sampling was mainly focused on areas with higher densities of extensively reared cattle and where positive cases of bovine besnoitiosis had been previously reported (Nieto-Rodríguez et al., 2016), as is the case of Central and Western Spain (Figure 1). Samples were obtained from road- and hunter-killed animals, from accidentally found carcasses, camera-trap surveys or animals entering rescue shelters (see Calero-Bernal et al., 2020). Faeces were collected directly from the rectum of each animal and placed in individual plastic cups with records of the date, location and species, and frozen within 12 h after collection.

2.2 | DNA extraction and molecular detection of *Besnoitia* spp

Genomic DNA was isolated from about 200 mg of faecal material by using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except that samples mixed with ASL lysis buffer were incubated for 10 min at 95°C for improving oocyst wall rupture. Purified DNA samples (200 μl) were stored at −20°C until downstream PCR-based diagnostic and subtyping analyses were conducted. A water extraction control was routinely included in each sample batch processed for DNA extraction. The products of the DNA extraction process were tested for the specific detection of *Besnoitia* spp. by *ITS-1* rDNA PCR (Cortes et al., 2007). The

TABLE 1 Summary of the available studies reporting investigations to elucidate possible definitive host of *Besnoitia besnoiti*

Order	Family	Common name	Scientific name	*n*	Country	Experimental/ natural	Method	Result	Reference
Carnivora	Canidae	Black-backed jackal	*Canis mesomelas*	2	South Africa	Experimental	Coprology	Negative	Diesing et al. (1988)
				39	Namibia	Natural	Serology	Negative	Seltmann et al. (2020)
		Cape fox	*Vulpes ehama*	1	South Africa	Experimental	Coprology	Negative	Diesing et al., 1988
		Bat-eared fox	*Otocyon megalotis*	4	Namibia	Natural	Serology	Negative	Seltmann et al., 2020
		Domestic dog	*Canis familiaris*	1	Israel	Experimental	Coprology	Negative	Diesing et al., 1988
				9	n.a.	Experimental	Coprology/ serology and PCR	Negative	Basso et al., 2011
				28	Uganda	Natural	Coprology	Negative	Rommel, 1975
		Golden jackal	*Canis aureus*	189	Israel	Natural	Serology	Negative	Mazuz et al., 2018
		Red fox	*Vulpes vulpes*	41	Spain	Natural	Serology	Negative	Millán et al., 2012
				25	Portugal	Natural	Serology	Negative	Waap et al., 2016
				75	Israel	Natural	Serology	Negative	Mazuz et al., 2018
				n.a.	France	Natural	Serology	Negative	Berhault & L'Hostis, 2008
		Wolf	*Canis lupus*	16	Spain	Natural	Serology	Negative	Millán et al., 2012
		African wild dog	*Lycaon pictus*	7	Namibia	Natural	Serology	Negative	Seltmann et al., 2020
		Honey badger	*Mellivora capensis*	10	Namibia	Natural	Serology	Negative	Seltmann et al., 2020
	Hyaenidae	Brown hyena	*Hyaena brunnea*	13	Namibia	Natural	Serology	Negative	Seltmann et al., 2020
		Spotted hyena	*Crocuta crocuta*	11	Namibia	Natural	Serology	Negative	Seltmann et al., 2020
	Felidae	Cheetah	*Acynonyx jubatus*	2	South Africa	Experimental	Coprology	Negative	Diesing et al., 1988
				250	Namibia	Natural	Serology	Negative	Seltmann et al., 2020
				2	Namibia	Natural	Coprology/ serology and PCR	Negative[a]	Schares et al., 2021
		Caracal	*Caracal caracal*	2	Israel	Experimental	Coprology	Negative	Diesing et al., 1988
				15	Namibia	Natural	Serology	Negative	Seltmann et al., 2020
		Domestic cat	*Felis silvestris catus*	2	Israel	Experimental	Coprology	Negative	Diesing et al., 1988
				8	n.a.	Experimental	Coprology/ serology and PCR	Negative	Basso et al., 2011
				n.a.	Kazakhstan	Experimental	Coprology	Negative	Peteshev et al., 1974
		European wildcat	*Felis silvestris silvestris*	28	Spain	Natural	Serology	Negative	Millán et al., 2012
				6	Portugal	Natural	Serology	Negative	Waap et al., 2016
		Wild cat	*Felis silvestris libyca*	n.a.	Kazakhstan	Experimental	Coprology	Negative	Peteshev et al., 1974
		Feral cat	*Felis silvestris catus*	43	Spain	Natural	Serology	Negative	Millán et al., 2012
				79	Portugal	Natural	Serology	Negative	Waap et al., 2016
		Iberian lynx	*Lynx pardinus*	3	Spain	Natural	Serology	Negative	Millán et al., 2012
		Jungle cat	*Felis chaus*	2	Israel	Experimental	Coprology	Negative	Diesing et al., 1988
		Lion	*Panthera leo*	1	South Africa	Experimental	Coprology	Negative	Diesing et al., 1988
				59	Namibia	Natural	Serology	Positive	Seltmann et al., 2020
		Leopard	*Panthera pardus*	3	South Africa	Experimental	Coprology	Negative	Diesing et al., 1988
				58	Namibia	Natural	Serology	Negative	Seltmann et al., 2020
		Serval	*Leptailurus serval*	1	Uganda	Natural	Coprology	Negative	Rommel, 1975

(Continues)

TABLE 1 (Continued)

Order	Family	Common name	Scientific name	*n*	Country	Experimental/ natural	Method	Result	Reference
	Herpestidae	Banded mongoose	*Mungos mungo*	2	South Africa	Experimental	Coprology	Negative	Diesing et al., 1988
		Egyptian mongoose	*Herpestes ichneumon*	5	Spain	Natural	Serology	Negative	Millán et al., 2012
				34	Portugal	Natural	Serology	Negative	Waap et al., 2016
		Marsh mongoose	*Herpestes palludinosus*	2	Israel	Experimental	Coprology	Negative	Diesing et al., 1988
	Mustelidae	American mink	*Neovison vison*	1	Spain	Natural	Serology	Negative	Millán et al., 2012
		Eurasian badger	*Meles meles*	12	Spain	Natural	Serology	Negative	Millán et al., 2012
				6	Portugal	Natural	Serology	Negative	Waap et al., 2016
				n.a.	France	Natural	Serology	Negative	Berhault & L'Hostis, 2008
		Eurasian otter	*Lutra lutra*	4	Spain	Natural	Serology	Negative	Millán et al., 2012
				2	Portugal	Natural	Serology	Negative	Waap et al., 2016
				n.a.	France	Natural	Serology	Negative	Berhault & L'Hostis, 2008
		European polecat	*Mustela putorius*	2	Spain	Natural	Serology	Negative	Millán et al., 2012
				3	Portugal	Natural	Serology	Negative	Waap et al., 2016
		Least weasel	*Mustela nivalis*	1	Spain	Natural	Serology	Negative	Millán et al., 2012
		Pine marten	*Martes martes*	21	Spain	Natural	Serology	Negative	Millán et al., 2012
		Small-spotted genet	*Genetta genetta*	1	Israel	Experimental	Coprology	Negative	Diesing et al., 1988
		Stone marten	*Martes foina*	8	Spain	Natural	Serology	Negative	Millán et al., 2012
				6	Portugal	Natural	Serology	Negative	Waap et al., 2016
	Procyonidae	Northern raccoon	*Procyon lotor*	1	Spain	Natural	Serology	Negative	Millán et al., 2012
		South American coati	*Nasua nasua*	1	Spain	Natural	Serology	Negative	Millán et al., 2012
	Viverridae	Common genet	*Genetta genetta*	18	Spain	Natural	Serology	Negative	Millán et al., 2012
Chiroptera	Vespertilionidae	Western barbastelle	*Barbastella barbastellus*	6	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Schreiber's bent-winged Bat	*Miniopterus schreibersii*	1	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Alcathoe whiskered Bat	*Myotis alcathoe*	23	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Bechstein's Myotis	*Myotis bechsteini*	21	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Brandt's Myotis	*Myotis brandtii*	6	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Lesser Mouse-eared Myotis	*Myotis blythii*	5	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Pond Myotis	*Myotis dasycneme*	4	Hungary and Netherlands	Natural	PCR	Positive	Hornok et al., 2015a
		Daubenton's Myotis	*Myotis daubentonii*	49	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Geoffroy's bat	*Myotis emarginatus*	6	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a

(Continues)

TABLE 1 (Continued)

Order	Family	Common name	Scientific name	*n*	Country	Experimental/ natural	Method	Result	Reference
		Lesser noctule	*Nyctalus leisleri*	9	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Greater mouse-eared bat	*Myotis myotis*	8	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Natterer's bat	*Myotis nattereri*	3	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Noctule	*Nyctalus noctula*	21	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Nathusius' pipistrelle	*Pipistrellus nathusii*	3	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Common pipistrelle	*Pipistrellus pipistrellus*	14	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Soprano pipistrelle	*Pipistrellus pygmaeus*	1	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Brown big-eared bat	*Plecotus auritus*	1	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Greater horseshoe bat	*Rhinolophus ferrumequinum*	3	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
		Lesser horseshoe bat	*Rhinolophus hipposideros*	2	Hungary and Netherlands	Natural	PCR	Negative	Hornok et al., 2015a
Squamata	Colubridae	Cat snake	*Telescopus dhara*	2	Israel	Experimental	Coprology	Negative	Diesing et al., 1988
		European whip snake	*Coluber jugularis*	3	Israel	Experimental	Coprology	Negative	Diesing et al., 1988
		Ravergier's whip snake	*Coluber ravergieri*	2	Israel	Experimental	Coprology	Negative	Diesing et al., 1988
	Lamprophiidae	Montpellier snake	*Malpolon monspessulanus*	3	Israel	Experimental	Coprology	Negative	Diesing et al., 1988
	Viperidae	Palestinian viper	*Vipera palaestinae*	8	Israel	Experimental	Coprology	Negative	Diesing et al., 1988
		Puff adder	*Bitis arietans*	2	Israel	Experimental	Coprology	Negative	Diesing et al., 1988
Accipitriformes	Accipitridae	White-backed	*Gyps africanus*	2	South Africa	Experimental	Coprology	Negative	Diesing et al., 1988
		vulture		11	Uganda	Natural	Coprology	Negative	Rommel, 1975
Ciconiiformes	Ciconiidae	Marabou	*Leptoptilos crumenifer*	4	Uganda	Natural	Coprology	Negative	Rommel, 1975

[a]The authors suggest that a so far unknown *Besnoitia* species exists in Namibian wildlife, which is closely related to *B. darlingi*, *B. neotomofelis*, *B. oryctofelisi*, *B. akodoni* or *B. jellisoni*.
n.a.: not available.

forward primer ITS1F (50-TGACATTTAATAACAATCAACCCTT-30) and the reverse primer ITS1R (50-GGTTTGTATTAACCAATCCGTGA-30) were added at a concentration of 10 μM, and the rest of reagents were incorporated in the mixture (final volume: 25 μl), as indicated by Frey et al. (2013). The amplified products were visualized after electrophoresis on a 1.5% agarose gel containing 0.1 μl/ml GelRed™ Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA). DNA extraction and PCR were performed in separate laboratories under biosafety level II conditions (BIO II A Cabinet, TELSTAR, Madrid, Spain) to avoid cross contamination. The positive control was DNA extracted from *in vitro* cultured tachyzoites of *B. besnoiti*, and PCR grade water was used as the negative control.

2.3 | Sequence and phylogenetic analyses

In order to confirm the specificity of the amplicons yielded, positive PCR products were subjected to direct sequencing at the Center for Genomic Technologies of the Complutense University of Madrid (Spain). Briefly, amplicons were sequenced in both directions with the same internal primer pair used for PCR amplification, Big Dye™ chemistries and an ABI 3730xl sequencer analyzer (Applied Biosystems, Foster City, CA, USA). Raw sequencing data in both forward and reverse directions were manually edited by Bioedit Software, and BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare resulting nucleotide sequences with sequences retrieved

TABLE 2 Wild carnivore species examined, region, number of samples tested and number of samples in which DNA from *Besnoitia* spp. has been detected in Spain

Family	Species	Scientific name	Region	N° samples	DNA positive samples
Canidae	Iberian wolf	*Canis lupus signatus*	Castilla-La Mancha	1	0
			País Vasco	5	0
	Red fox	***Vulpes vulpes***	Andalucía	1	0
			Castilla-La Mancha	19	0
			Castilla y León	125	2
			Extremadura	21	2
			País Vasco	21	0
Felidae	European wild cat	*Felix silvestris*	Castilla y León	3	0
			Extremadura	1	0
			País Vasco	1	0
	Iberian lynx	*Lynx pardinus*	Castilla-La Mancha	2	0
			Extremadura	8	0
Herpestidae	Egyptian mongoose	*Herpestes ichneumon*	Castilla y León	1	0
Mustelidae	American mink	*Neovison vison*	Extremadura	25	0
	Beech marten	*Martes foina*	Castilla-La Mancha	5	0
			Castilla y León	14	0
			Extremadura	1	0
			País Vasco	2	0
	Eurasian otter	*Lutra lutra*	Castilla-La Mancha	1	0
			Castilla y León	2	0
			Extremadura	1	0
	European badger	*Meles meles*	Asturias	68	0
			Castilla-La Mancha	1	0
			Castilla y León	7	0
	European polecat	*Mustela putorius*	Castilla-La Mancha	2	0
			Extremadura	2	0
Procyonidae	Raccoon dog	*Procyon lotor*	Madrid	1	0
Viverridae	Common genet	*Genetta genetta*	Castilla-La Mancha	1	0
			Castilla y León	5	0
			Extremadura	3	0
			País Vasco	2	0
Total				352	4

In bold, the species and number of faeces samples in which DNA from *Besnoitia* spp. has been found and confirmed by sequencing.

from the National Center for Biotechnology Information (NCBI) database.

The evolutionary relationships among the identified *Besnoitia* sp.-positive samples were inferred by a phylogenetic analysis using the Neighbor-Joining method (Saitou & Nei, 1987) in MEGA 6. The evolutionary distances were computed using the Kimura 2-parameter method and modelled with a gamma distribution. The reliability of the phylogenetic analyses at each branch node was estimated by the bootstrap method using 1000 replications. Representative sequences of different *Besnoitia* spp. isolates were retrieved from the NCBI database and included in the phylogenetic analysis for reference and comparative purposes. Representative sequences obtained in the present study were deposited in GenBank under the accession numbers MW035607 to MW035610.

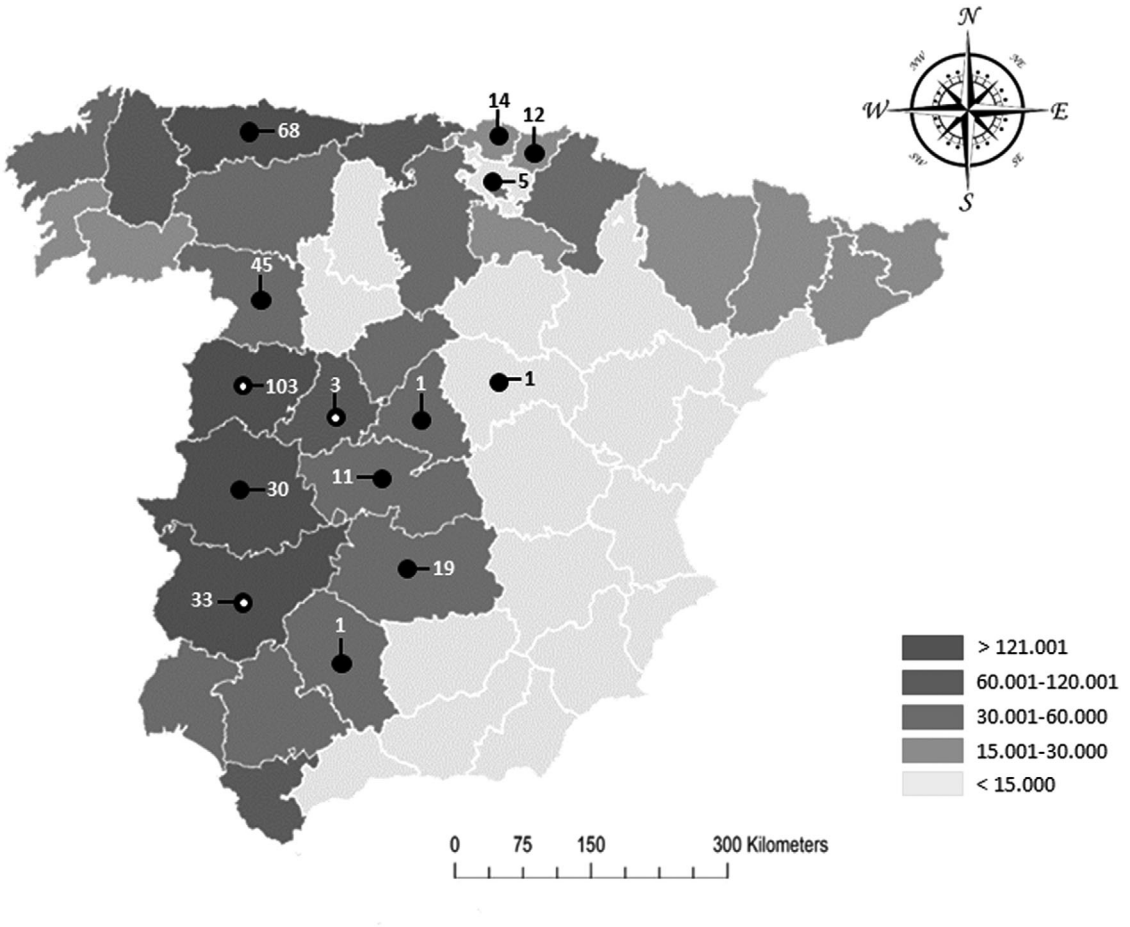

FIGURE 1 Spatial distribution of sample collection. Each black dot represents the province of sampling. The current geographic distribution and census of beef cattle raised under extensive husbandry conditions for each province is represented by greyscale. The number of faecal samples per province is shown. The white dot indicates that *Besnoitia* spp. DNA has been found

3 | RESULTS AND DISCUSSION

PCR and sequencing results showed that *B. besnoiti*-like DNA was present in four faecal samples (1.13%) analyzed from 352 wild carnivores. Those positive samples corresponded to four red foxes from Castilla y León and Extremadura in western Spain. To date, this is the first finding of a *B. besnoiti*-like sequence from a carnivore in Europe, and from any carnivore species in a worldwide context.

To the best of our knowledge, this is the first large-scale molecular survey for *Besnoitia* spp. DNA in free-living carnivores in Europe. The survey benefits from the inclusion of 12 different species of free-living carnivores and a national coverage, paying special attention to regions where bovine besnoitiosis is present (except in North West Spain) in conjunction with greater densities of extensive cattle production.

Serological, molecular and parasitological techniques have been used in an attempt to elucidate the role of several animal species as potential definitive host of *B. besnoiti*, but they failed to find it in wild and domestic carnivores, in addition to mammals, reptiles and birds (see Table 1). Several studies on felines, including the domestic cat (*Felis catus*), have attempted to clarify its role as a definitive host (see Table 1). Rommel (1975) and Peteshev et al. (1974) reported inconclusive results to confirm domestic cats as definitive hosts of *B. besnoiti* in experimental studies. Despite detecting oocysts in the faeces, authors could not achieve further characterization for fully confirmation of their identity as *B. besnoiti*. Other authors did not find *B. besnoiti* oocysts in the faeces of *per os* challenged cats over a 3 to 20 weeks observation period (Basso et al., 2011; Diesing et al., 1988). Several serological studies have been also carried out to detect antibodies against *B. besnoiti* in felines (Table 1). Millán et al. (2012) found antibodies by indirect fluorescent antibody test in eight feral cats (*Felis silvestris catus*). However, no individual tested positive by Western blot assay. These animals originated from areas where no cases of bovine besnoitiosis had been detected until year 2010. The results suggested their unlikely implication in the parasite transmission. In a recent study in Namibian wildlife, antibodies have been detected in two lions, *Panthera leo* (Seltmann et al., 2020). On the other hand, two studies have managed to detect by molecular techniques *Besnoitia* spp. DNA in faeces from pond bat (*Myotis dasycneme*) in the Netherlands (Hornok et al., 2015a) and in faecal matter from cheetahs (*Acinonyx jubatus*) in Namibia (Schares

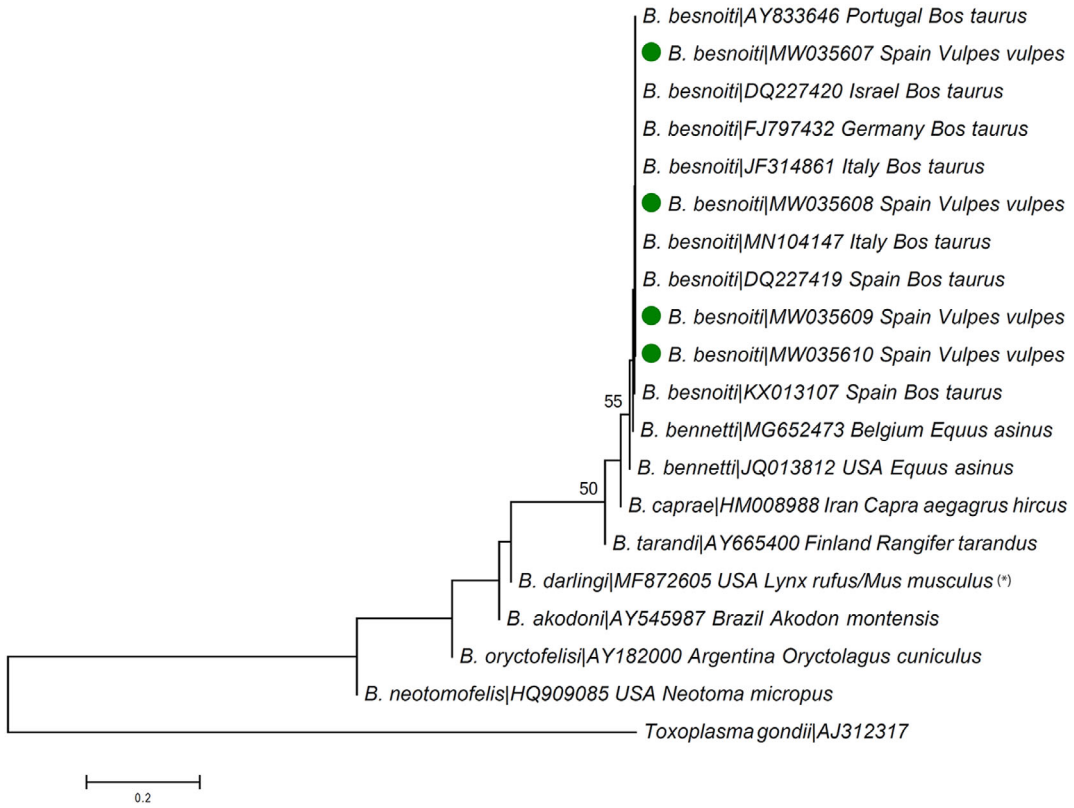

FIGURE 2 Phylogenetic relationships among *Besnoitia* spp. sequences identified in this study and known *Besnoitia* spp. isolates, as inferred by a neighbour-joining analysis of *ITS-1* ribosomal RNA gene partial sequences, based on genetic distances calculated by the Kimura two-parameter model. Nucleotide sequences determined in this study are identified with dark green filled circles. Bootstrap values lower than 50% are not displayed. *Toxoplasma gondii* was used as outgroup taxa

et al., 2021); in the first report, authors hypothesized that *B. besnoiti*-like sequences might have originated from French cattle via bloodsucking dipterans (*Stomoxys calcitrans*, *Tabanus* spp.). In this regard, bats frequently use cattle stables for roosting, where they can prey on the mechanical vectors of *B. besnoiti*. In addition, the later study (Schares et al., 2021) suggests that a so far unknown *Besnoitia* species closely related to *B. darlingi*, *B. neotomofelis*, *B. oryctofelisi*, *B. akodoni* or *B. jellisoni* is cycling in Namibian wildlife.

In the present survey, *B. besnoiti*-like DNA has been demonstrated in four individual faecal samples from red foxes from Ávila, Badajoz and Salamanca provinces (Table 2) in western Spain. All four fox-derived *Besnoitia* spp. sequences were equivalent to positions 527–737 of reference sequence KX013107 (a bovine isolate of the parasite previously reported in Spain), differing from it by a single di-nucleotide site (a G/C double peak) at position 706. An additional ambiguous position (an A/G double peak) was also detected at position 711 of reference sequence KX013107 in one (GenBank accession number MW035609) of the four generated sequences. The topology of the produced phylogenetic tree clearly clustered all these sequences with other *Besnoitia* species in large mammals (*B. bennetti*, *B. caprae* and *B. tarandi*) but particularly with *B. besnoiti*, from European countries (Belgium, Finland, Italy, Germany, Portugal and Spain), Israel and Iran. The *ITS-1* rDNA sequence of *B. besnoiti*-like from red foxes suggest a closer relationship to *B. besnoiti*, which infects cattle in the Old World. In a separate phylogenetic cluster and with large evolutionary divergence, other species of *Besnoitia* genus (*B. neotomofelis*, *B. oryctofelisi*, *B. akodoni* and *B. darlingi*) infecting small mammals from Argentina, Brazil and USA (Figure 2) are placed. These results are in agreement with those described by Olias et al. (2011), in which *ITS-1* region shows the most informative nucleotide variances and phylogenetically clearly splits small mammalian from large mammalian *Besnoitia* species. Of note, all foxes with *Besnotia* sp. PCR-positive faecal samples were caught within Western Spain (Figure 1), where the highest number of bovine besnoitiosis clinical cases were found in a previous survey (Nieto-Rodríguez et al., 2016).

This is the first molecular evidence of the occurrence of *B. besnoiti*-like DNA in a European mesocarnivore gut. The red fox is present in a wide range of habitats in the Iberian Peninsula (Macdonald & Reynolds, 2004) with densities of 0.7–2.5 foxes/km^2, depending on environmental conditions (Sarmento et al., 2009). In addition, this wild canid is a highly adaptable omnivorous mammal distributed across all continents on the northern hemisphere. Numerous studies on the red fox diet show it as a generalist predator, feeding mainly on preys which are abundant and easily accessible. Red foxes feed most frequently on small mammals as rodents and wild rabbits, but utilize also other food items such as carrion, birds, reptiles, amphibians, invertebrates, fruit and vegetables (Díaz-Ruiz et al., 2013).

The prevalence rate found in red fox (2.1%; four DNA samples out of a total of 187 samples) is in agreement with worldwide reported data

for the detection of oocysts or DNA of the closely related Toxoplasmatinae parasite *Toxoplasma gondii* by Felidae (Hatam-Nahavandi et al., 2021). Nonetheless, the different incidence of bovine besnoitiosis on the European continent cannot be fully explained by animal trade and mechanical transmission together with the dissemination of oocysts by red foxes (abundant and widespread in Europe), and other factors such as the involvement of vectors may be facilitating transmission (Hornok et al., 2015b).

Taking into account that in our study the faecal samples were collected from regions where beef cattle are usually raised in extensive production systems (Figure 1) and bovine besnoitiosis is widespread (Nieto-Rodríguez et al., 2016), there are three possible explanations for such interesting findings; (i) our first hypothesis is that red foxes may have a role in the transmission of the parasite as definitive host: the red fox is considered to be one of the most widespread generalist vertebrate predators in the world (Macdonald & Reynolds, 2004). Therefore, predation on small mammals as rodents and wild rabbit, intermediate hosts of *B. darlingi*, *B. neotomofelis* and *B. oryctofelisi*, respectively, suggests that it could make us think that red fox might have a role as a definitive host in other species of *Besnoitia*. However, the sequences found in fox faeces are genetically related to *B. besnoiti* and not so closely related to small mammalian *Besnoitia* spp. In addition, other small mammalian prey of the fox may be unknown intermediate hosts of *B. besnoiti*, and the fox may act as a definitive host for *B. besnoiti*; (ii) our second hypothesis is that there has been consumption of carrion infected with *B. besnoiti*, and foxes are acting as passive carriers without developing the infection; (iii) and the third and last hypothesis is that the red fox could act as carrier, after the accidental ingestion of the hypothetical *B. besnoitia* oocysts from the contaminated soil (and foodstuff) and we would find parasite DNA in the fox faeces.

Although we have found *Besnoitia* spp. DNA in red fox faeces and subsequently confirmed it by Sanger sequencing, the present survey has several limitations. First, no serological analysis has been performed on these species, sampling was carried out in most cases on road- and hunter-killed animals, from accidentally found carcasses, and camera-trap surveys. Thus, fresh, good quality blood samples were unavailable for serological testing, in addition to the difficulty of finding validated techniques in wildlife for detecting this parasite (González-Barrio & Ruiz-Fons, 2019). Second, no additional parasitological techniques (e.g., floatation, isolation and/or concentration of oocysts) were used to enhance oocysts detection due to the retrospective nature of this study and the insufficient amount of remaining faecal material for performing complementary techniques. Finally, identification of *Besnoitia* spp. was accomplished on a single locus. Low quantity and quality of genomic DNA from faeces prevented us of conducting multilocus microsatellite analyses. However, on the other hand, foxes were the only species among the 12 studies, where the presence of *B. besnoiti*-like DNA was identified. With this survey, we hypothesized that red foxes might have a role in the epidemiology of bovine besnoitiosis; we suspect that as in the case of *Neospora caninum* in domestic dogs, the number of oocysts shed by the definitive host might be low limiting parasite dissemination, since the red fox is the world's most widely distributed carnivore and bovine besnoitiosis is not a disease that is present worldwide. On the other hand, the main transmission route described so far has been the vector borne transmission evidenced by natural and experimental assays but more dependent on climatic factors and vector ecology; this transmission coupled with the natural mating identified as a major risk factor (Gazzonis et al., 2017) are the main suspects for the spread of bovine besnoitiosis in Europe. Our finding together with these two possible routes of transmission ensure that the life cycle of *B. besnoitia* remains a mystery.

To conclude, Sanger sequencing analysis of four of the 187 faecal samples revealed the presence of *B. besnoiti*-like DNA in red fox (*Vulpes vulpes*) faeces in Spain. Further epidemiological and experimental studies with a similar approach may help in the search for the definitive host of this parasite. In addition, future studies are needed to identify its natural intermediate host in small mammalian prey of foxes.

ACKNOWLEDGEMENTS

This study was funded by the Health Institute Carlos III (ISCIII), Ministry of Economy and Competitiveness (Spain), under project PI16CIII/00024. Additional funding was obtained from the Complutense University of Madrid (grant CT65/16), Spanish Ministry of Economy and Competitiveness (AGL-2016-75202-R), Community of Madrid (PLATESA P2018/ BAA-4370), University of Aveiro (Department of Biology) and FCT/MEC for the financial support to CESAM (UID/AMB/50017/2019) through national funds co-financed by the FEDER (PT2020 Partnership Agreement). G. Álvarez-García and R. Calero-Bernal are part of the TOXOSOURCES consortium, supported by funding from the European Union's Horizon 2020 Research and Innovation programme under grant agreement No 773830: One Health European Joint Programme. D. González-Barrio was funded by the Spanish Ministry of Science through a Juan de la Cierva postdoctoral fellowship (FJCI-2016-27875) and currently is funded by the Spanish Ministry of Science through Sara Borrell postdoctoral fellowship (CD19CIII/00011).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

No ethical approval was required as this is a paper in which samples were obtained from road- and hunter-killed animals, from accidentally found carcasses, camera-trap surveys, or animals entering rescue shelter.

ORCID

David González-Barrio https://orcid.org/0000-0001-5083-8854
David Carmena https://orcid.org/0000-0002-4015-8553
Rafael Calero-Bernal https://orcid.org/0000-0003-2323-0135

REFERENCES

Arnal, M. C., Gutiérrez-Expósito, D., Martínez-Durán, D., Regidor-Cerrillo, J., Revilla, M., Fernández de Luco, D., Jiménez-Meléndez A., Ortega-Mora L. M., & Álvarez-García, G. (2017). Systemic Besnoitiosis in a juvenile roe deer (*Capreolus capreolus*). *Transboundary and Emerging Diseases*, *64*, e8-e14. https://doi.org/10.1111/tbed.12514

Álvarez-García, G., Frey, C. F., Ortega-Mora, L. M. O., & Schares, G. (2013). A century of bovine besnoitiosis: An unknown disease re-emerging in Europe. *Trends in Parasitology*, *29*, 407–415. https://doi.org/10.1016/j.pt.2013.06.002

Álvarez-García, G., García-Lunar, P., Gutiérrez-Expósito, D., Shkap, V., & Ortega-Mora, L. M. (2014). Dynamics of *Besnoitia besnoiti* infection in cattle. *Parasitology*, *141*(11), 1419–1435. https://doi.org/10.1017/S0031182014000729

Álvarez-García, G. (2016) From the mainland to Ireland - bovine besnoitiosis and its spread in Europe. *Veterinary Record*, *178*(24), 605–607. https://doi.org/10.1136/vr.i3175

Basso, W., Schares, G., Gollnick, N. S., Rütten, M., & Deplazes, P. (2011). Exploring the life cycle of *Besnoitia besnoiti*-Experimental infection of putative definitive and intermediate host species. *Veterinary Parasitology*, *178*, 223–234. https://doi.org/10.1016/j.vetpar.2011.01.027

Berhault, G., & L'Hostis, M. (2008). La besnoitiose à Besnoitia besnoiti: Étude de reservoirs sauvages potentiels dans un foyer en Maine et Loire. *Th Vet Med Nantes*.

Calero-Bernal, R., Santín, M., Maloney, J. G., Martín-Pérez, M., Habela, M. A., Fernández-García, J. L., Figueiredo, A., Nájera, F., Palacios, M. J., Mateo, M., Balseiro, A., Barral, M., Lima-Barberoi, J. F., Köster, P. C., & Carmena, D. (2020). *Blastocystis* sp. subtype diversity in wild carnivore species from Spain. *Journal of Eukaryotic Microbiology*, *67*, 273–278. https://doi.org/10.1111/jeu.12772

Cortes, H. C., Reis, Y., Gottstein, B., Hemphill, A., Leitao, A., & Muller, N. (2007). Application of conventional and real-time fluorescent ITS1 rDNA PCR for detection of *Besnoitia besnoiti* infections in bovine skin biopsies. *Veterinary Parasitology*, *146*, 352–356. https://doi.org/10.1016/j.vetpar.2007.03.003

Cortes, H., Leitão, A., Gottstein, B., & Hemphill, A. (2014). A review on bovine besnoitiosis: A disease with economic impact in herd health management, caused by Besnoitia besnoiti (Franco and Borges,). *Parasitology*, *141*(11), 1406–1417. https://doi.org/10.1017/S0031182014000262

Díaz-Ruiz, F., Delibes-Mateos, M., García-Moreno, J. L., María López-Martín, J., Ferreira, C., & Ferreras, P. (2013). Biogeographical patterns in the diet of an opportunistic predator: The red fox *Vulpes vulpes* in the Iberian Peninsula. *Mammal Review*, *43*, 59–70. https://doi.org/10.1111/j.1365-2907.2011.00206.x

Diesing, L., Heydorn, A. O., Matuschka, F. R., Bauer, C., Pipano, E., de Waal, D. T., & Potgieter, F. T. (1988). *Besnoitia besnoiti*: Studies on the definitive host and experimental infections in cattle. *Parasitology Research*, *75*, 114–117. https://doi.org/10.1007/BF00932710

European Food Safety Authority. (2010) Bovine besnoitiosis: An emerging disease in Europe. *EFSA Journal*, *8*, 1499.

Frey, C. F., Gutiérrez-Expósito, D., Ortega-Mora, L. M., Benavides, J., Marcén, J. M., Castillo, J. A., Casasús, I., Sanz, A., García-Lunar, P., Esteban-Gil, A., & Álvarez-García, G. (2013). Chronic bovine besnoitiosis: Intra-organ parasite distribution, parasite loads and parasite-associated lesions in subclinical cases. *Veterinary Parasitology*, *197*(1-2), 95–103. https://doi.org/10.1016/j.vetpar.2013.04.023

Gazzonis, A. L., Alvarez-Garcia, G., Maggioni, A., Zanzani, S. A., Olivieri, E., Compiani, R., Sironi, G., Ortega-Mora, L. M., & Manfredi, M. T. (2017). Serological dynamics and risk factors of *Besnoitia besnoiti* infection in breeding bulls from an endemically infected purebred beef herd. *Parasitology Research*, *116*, 1383–1393. https://doi.org/10.1007/s00436-017-5418-x

González-Barrio, D., & Ruiz-Fons, F. (2019). *Coxiella burnetii* in wild mammals: A systematic review. 2019. *Transboundary and Emerging Diseases*, *66*, 662–671. https://doi.org/10.1111/tbed.13085

González-Barrio, D., Diezma-Díaz, C., Tabanera, E., Aguado-Criado, E., Pizarro, M., González-Huecas, M., Ferre, I., Jiménez-Meléndez, A., Criado, F., Gutiérrez-Expósito, D., Ortega-Mora, L. M., & Álvarez-García, G. (2020). Vascular wall injury and inflammation are key pathogenic mechanisms responsible for early testicular degeneration during acute besnoitiosis in bulls. *Parasites and Vectors*, *13*, 113. https://doi.org/10.1186/s13071-020-3959-9

Gutiérrez-Expósito, D., Ortega-Mora, L. M., Marco, I., Boadella, M., Gortázar, C., San Miguel-Ayanz, J. M., García-Lunar, P., Lavín, S., & Álvarez-García, G. (2013). First serosurvey of *Besnoitia* spp. infection in wild European ruminants in Spain. *Veterinary Parasitology*, *197*, 557–564. https://doi.org/10.1016/j.vetpar.2013.05.017

Gutiérrez-Expósito, D., Esteban-Gil, A., Ortega-Mora, L. M., García-Lunar, P., Castillo, J. A., Marcén, J. M., & Alvarez-García, G. (2014). Prevalence of *Besnoitia besnoiti* infection in beef cattle from the Spanish Pyrenees. *Veterinary Journal*, *200*, 468–470. https://doi.org/10.1016/j.tvjl.2014.02.008

Gutiérrez-Expósito, D., Arnal, M. C., Martínez-Durán, D., Regidor-Cerrillo, J., Revilla, M., Fernández de Luco, D., Jiménez-Meléndez, A., Calero-Bernal, R., Habela, M. A., García-Bocanegra, I., Arenas-Montes, A., Ortega-Mora, L. M., & Álvarez-García, G. (2016). The role of wild ruminants as reservoirs of *Besnoitia besnoiti* infection in cattle. *Veterinary Parasitology*, *223*, 7–13. https://doi.org/10.1016/j.vetpar.2016.04.005

Gutiérrez-Expósito, D., Ortega-Mora, L. M., Ara, V., Marco, I., Lavín, S., Carvajal-Valilla, J., Morale, A., & Álvarez-García, G. (2017). Absence of antibodies specific to *Besnoitia* spp. in European sheep and goats from areas in Spain where bovine besnoitiosis is endemic. *Parasitolgy Research*, *116*(1), 445-448. https://doi.org/10.1007/s00436-016-5311-z

Hatam-Nahavandi, K., Calero-Bernal, R., Rahimi, M. T., Pagheh, A. S., Zarean, M., Dezhkam, A., & Ahmadpour, E. (2021) *Toxoplasma gondii* infection in domestic and wild felids as public health concerns: A systematic review and meta-analysis. *Scientific Reports*, *11*, 9509. https://doi.org/10.1038/s41598-021-89031-8

Hornok, S., Estók, P., Kováts, D., Flaisz, B., Takács, N., Szoke, K., Krawczyk, A., Kontschán, J., Gyuranecz, M., Fedák, A., Farkas, R., Haarsma, A.-J., & Sprong, H. (2015a). Screening of bat faeces for arthropod-borne apicomplexan protozoa: *Babesia canis* and *Besnoitia besnoiti*-like sequences from Chiroptera. *Parasites and Vectors*, *8*, 441. https://doi.org/10.1186/s13071-015-1052-6

Hornok, S., Fedák, A., Baska, F., Basso, W., Dencső, L., Tóth, G., Szeredi, L., Abonyi, T., & Dénes, B. (2015b). Vector-borne transmission of *Besnoitia besnoiti* by blood-sucking and secretophagous flies: Epidemiological and clinicopathological implications. *Parasites & vectors*, *8*, 450. https://doi.org/10.1186/s13071-015-1058-0

Jacquiet, P., Liénard, E., & Franc, M. (2010). Bovine besnoitiosis: Epidemiological and clinical aspects. *Veterinary Parasitology*, *174*, 30–36. https://doi.org/10.1016/j.vetpar.2010.08.013

Macdonald, D. W., & Reynolds, J. C. (2004). Red fox *Vulpes vulpes* Linnaeus, 1758. In (C. Sillero-Zubiri, M. Hoffmann, & D. W. Macdonald Eds.), *Canids: Foxes, wolves, jackals and dogs. Status survey and conservation action plan* (pp. 129–136). Gland, Switzerland: IUCN/SSC Canid Specialist Group

Mazuz, M. L., Alvarez-García, G., King, R., Savisky, I., Shkap, V., Ortega-Mora, L. M., & Gutiérrez-Expósito, D. (2018). Exposure to *Neospora spp.* and *Besnoitia* spp. in wildlife from Israel. *International Journal for Parasitology: Parasites and Wildlife*, *7*, 317–321. https://doi.org/10.1016/j.ijppaw.2018.08.002

Millán, J., Sobrino, R., Rodríguez, A., Oleaga, T., Gortazar, C., & Schares, G. (2012). Large-scale serosurvey of *Besnoitia besnoiti* in free-living carnivores in Spain. *Veterinary Parasitology*, *190*, 241–245. https://doi.org/10.1016/j.vetpar.2012.06.014

Nieto-Rodríguez, J. M., Calero-Bernal, R., Álvarez-García, G. Gutiérrez-Expósito, D., Redondo-García, E., Fernández-García, J. L., & Habela Martínez-Estéllez, M. A. (2016). Characterization of an outbreak of emerging bovine besnoitiosis in southwestern Spain.

Parasitology Research, *115*, 2887–2892. https://doi.org/10.1007/s00436-016-5050-1

Olias, P., Schade, B., & Mehlhorn, H. (2011). Molecular pathology, taxonomy and epidemiology of *Besnoitia* species (Protozoa: Sarcocystidae). *Infection. Genetics and Evolution*, *11*, 1564–1576. https://doi.org/10.1016/j.meegid.2011.08.006

Peteshev, V. M., Galuzo, I. G., & Polomoshnov, A. P. (1974). Koshki – definitivnye khoziaeva besnoitii (*Besnoitia besnoiti*), [Cats – definitive hosts of Besnoitia (*Besnoitia besnoiti*)]. *Izvest. Akad. Nauk. Kazakh. Ser. Biol*, *1*, 33–38.

Rommel, M., (1975). Neue Erkenntnisse zur Biologie der Kokzidien, Toxoplasmen, Sarkosporidien und Besnoitien. *Berliner Und Munchener Tierarztliche Wochenschrift*, *88*, 112–117.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*, 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Sarmento, P., Cruz, J., Eira, C., & Fonseca, C. (2009). Evaluation of camera trapping for estimating red fox abundance. *Journal of Wildlife Management*, *13*, 1207–1212. https://doi.org/10.1371/journal.pone.0207545

Schares, G., Joeres, M., Rachel, F., Tuschy, M., Czirják, G. Á., Maksimov, P., Conraths, F. J., & Wachter, B. (2021). Molecular analysis suggests that Namibian cheetahs (*Acinonyx jubatus*) are definitive hosts of a so far undescribed *Besnoitia* species. *Parasites and Vectors*, *14*, 201. https://doi.org/10.1186/s13071-021-04697-3

Seltmann, A., Schares, G., Aschenborn, O. H. K., Heinrich, S. K., Thalwitzer, S., Wachter, B., & Czirják, G. A. (2020). Species-specific differences in *Toxoplasma gondii*, *Neospora caninum* and *Besnoitia besnoiti* seroprevalence in Namibian wildlife. *Parasites and Vectors*, *13*, 7. https://doi.org/10.1186/s13071-019-3871-3

Waap, H., Nunes, T., Cortes, H., Leitão, A., & Vaz, Y. (2014). Prevalence and geographic distribution of *Besnoitia besnoiti* infection in cattle herds in Portugal. *Parasitology Research*, *113*, 3703–3711. https://doi.org/10.1007/s00436-014-4035-1

Waap, H., Nunes, T., Vaz, Y., & Leitão, A. (2016). Serological survey of *Toxoplasma gondii* and *Besnoitia besnoiti* in a wildlife conservation area in southern Portugal. *Veterinary Parasitology: Regional Studies and Reports*, *3-4*, 7–12. https://doi.org/10.1016/j.vprsr.2016.05.003

How to cite this article: González-Barrio, D., Köster, P. C., Habela, M. A., Martín-Pérez, M., Fernández-García, J. L., Balseiro, A., Barral, M., Nájera, F., Figueiredo, A. M., Palacios, M. J., Mateo, M., Carmena, D., Álvarez-García, G., Calero-Bernal, R. (2021). Molecular survey of *Besnoitia* spp. (Apicomplexa) in faeces from European wild mesocarnivores in Spain. *Transboundary and Emerging Diseases*, 1–11. https://doi.org/10.1111/tbed.14206