Low occurrence and limited zoonotic potential of *Cryptosporidium* spp., *Giardia duodenalis*, and *Balantioides coli* infections in free-ranging and farmed wild ungulates in Spain

Running Head: Zoonotic protist parasites in Spanish wild ungulates

Alejandro Dashti[1,†], Pamela C. Köster[1,†], Begoña Bailo[1], Ana Sánchez de las Matas[1], Miguel Ángel Habela[2], Antonio Rivero-Juarez[3,4], Joaquín Vicente[5], Emmanuel Serrano[6,‡], Maria C. Arnal[7], Daniel Fernández de Luco[7], Patrocinio Morrondo[8], José A. Armenteros[9], Ana Balseiro[10,11], Guillermo A. Cardona[12], Carlos Martínez-Carrasco[13], José Antonio Ortiz[14], Antonio José Carpio[15,16], Rafael Calero-Bernal[17], David González-Barrio[1*], David Carmena[1,4*]

[†]These first authors contributed equally to this article.

[‡]On behalf on the WE&H group. Members of the group are listed at the end of this work.

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

[2]Department of Animal Health, Veterinary Sciences Faculty, Extremadura University, Caceres, Spain

[3]Infectious Diseases Unit, Maimonides Institute for Biomedical Research (IMIBIC), University Hospital Reina Sofía, University of Córdoba, Córdoba, Spain

[4]Center for Biomedical Research Network in Infectious Diseases (CIBERINFEC), Health Institute Carlos III, Madrid, Spain

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

[6]Wildlife Ecology & Health Group (WE&H), Wildlife Environmental Pathology Service (SEFaS), Department of Animal Medicine and Surgery, Autonomous University of Barcelona, Bellaterra, Spain

[7]Department of Animal Pathology, Veterinary Faculty, University of Zaragoza, Zaragoza, Spain

[8]INVESAGA Group, Department of Animal Pathology, Faculty of Veterinary, University of Santiago de Compostela, Lugo, Spain

[9]Council of Development, Territory Planning and the Environment of the Principado de Asturias, Oviedo, Spain

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

[11]Animal Health Department, Mountain Livestock Institute (CSIC-University of León), León, Spain

[12]Livestock Laboratory, Regional Government of Álava, Vitoria-Gasteiz, Spain

[13]Animal Health Department, University of Murcia, Regional Campus of International Excellence "Campus Mare Nostrum", Espinardo, Murcia, Spain

[14]Medianilla S.L., Department of Veterinary and Research, Benalup-Casas Viejas, Spain

[15]Institute for Research on Hunting Resources, IREC (UCLM-CSIC-JCCM), Ciudad Real, Spain

[16]Department of Zoology, University of Cordoba, Campus de Rabanales, Cordoba, Spain

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

Correspondence

David González-Barrio, Parasitology Reference and Research Laboratory, National Centre for Microbiology, Ctra. Majadahonda-Pozuelo Km 2, 28220 Majadahonda, Madrid, Spain.
Email: dgonzalezbarrio@gmail.com
David Carmena, Parasitology Reference and Research Laboratory, National Centre for Microbiology, Ctra. Majadahonda-Pozuelo Km 2, 28220 Majadahonda, Madrid, Spain.
Email: dacarmena@isciii.es

SUMMARY

Little information is currently available on the occurrence and molecular diversity of the enteric protozoan parasites *Cryptosporidium* spp., *Giardia duodenalis*, and *Balantioides coli* in wild ungulates and the role of these host species as potential sources of environmental contamination and human infections. The presence of these three pathogens was investigated in eight wild ungulate species present in Spain (genera *Ammotragus*, *Capra*, *Capreolus*, *Cervus*, *Dama*, *Ovis*, *Rupicapra*, and *Sus*) by molecular methods. Faecal samples were retrospectively collected from free-ranging (n = 1,058) and farmed (n = 324) wild ungulates from the five Spanish bioregions. Overall infection rates were 3.0% (42/1,382; 95% CI: 2.1–3.9%) for *Cryptosporidium* spp., 5.4% (74/1,382; 95% CI: 4.2–6.5%) for *G. duodenalis*, and 0.7% (9/1,382; 95% CI: 0.3–1.2%) for *B. coli*. *Cryptosporidium* spp. was detected in roe deer (7.5%), wild boar (7.0%) and red deer (1.5%), and *G. duodenalis* in southern chamois (12.9%), mouflon (10.0%), Iberian wild goat (9.0%), roe deer (7.5%), wild boar (5.6%), fallow deer (5.2%) and red deer (3.8%). *Balantioides coli* was only detected in wild boar (2.5%, 9/359). Sequence analyses revealed the presence of six distinct *Cryptosporidium* species: C. *ryanae* in red deer, Roe deer, and wild boar; *C. parvum* in red deer and wild boar; *C. ubiquitum* in roe deer; *C.*

scrofarum in wild boar; *C. canis* in roe deer; and *C. suis* in red deer. Zoonotic assemblages A and B were detected in wild boar and red deer, respectively. Ungulate-adapted assemblage E was identified in mouflon, red deer, and southern chamois. Attempts to genotype samples positive for *B. coli* failed. Sporadic infections by canine- or swine-adapted species may be indicative of potential cross-species transmission, although spurious infections cannot be ruled out. Molecular evidence gathered is consistent with parasite mild infections and limited environmental contamination with (oo)cysts. Wild ungulate species would not play a significant role as source of human infections by these pathogens. Wild ruminants are not susceptible hosts for *B. coli*.

KEYWORDS

Enteric protozoan parasites, wildlife, disease transmission, occurrence, molecular diversity, genotyping, zoonoses

1. INTRODUCTION

Ungulates are suitable hosts for numerous zoonotic pathogens representing a public health concern (Palmer et al., 2017; Trimmel & Walzer, 2020). In Europe, wild ungulate species have increased their densities and expanded their habitat ranges during the last few decades due to land-use changes (Carpio et al., 2021). Human-driven overabundance of wild ungulate populations might increase the interaction with other animals and the risk of zoonotic transmission. Among parasitic agents, the enteric protozoa *Cryptosporidium* spp. and *Giardia duodenalis*, and, to a lesser extent, the ciliate *Balantioides coli* can infect a wide variety of ungulate species (Feng & Xiao, 2011; Ponce-Gordo & García-Rodriguez 2021; Santín 2020; Zahedi et al., 2015). Indeed, diarrhoea caused by both *Cryptosporidium* spp. and *G. duodenalis* infections has been

99 associated with significant economic losses due to growth retardation and mortality in
100 infected livestock (Hatam-Nahavandi et al., 2019; Santín, 2020). *Balantioides coli* is the
101 only ciliate known to infect humans. In addition, this parasite is commonly found in
102 domestic and wild swine populations, suggesting that these animals are the main reservoir
103 of this pathogen (Ponce-Gordo & García-Rodriguez, 2021). All three parasite species are
104 faecal-orally transmitted through cysts (*G. duodenalis* and *B. coli*) or oocysts
105 (*Cryptosporidium* spp.) either by direct contact with faeces of infected humans or animals
106 or indirectly by ingestion of contaminated water or foodstuffs.

107 Currently, at least 46 *Cryptosporidium* species are considered taxonomically valid
108 (Ježková et al., 2021; Ryan et al., 2021; Zahedi et al., 2021), of which ten
109 species/genotypes have been reported in cervids: *C. parvum*, *C. bovis*, *C. ryanae*, *C.*
110 *ubiquitum*, *C.* deer genotype, *C. muris*, *C. andersoni*, *C. parvum* genotype II, *C.* cervine
111 genotype, and *C. suis*-like genotype (Hatam-Nahavandi et al., 2019). On the other hand,
112 only *C. parvum*, *C. suis* and *C. scrofarum* have been reported in wild boar (*Sus scrofa*) to
113 date (García-Presedo et al., 2013b; Ryan et al., 2021).

114 *Giardia duodenalis* is one of the most common enteric parasites that infect
115 humans as well as domestic and wild mammals (Feng & Xiao, 2011). This pathogen is
116 considered as a species complex that comprises eight (A to H) genetic variants or
117 assemblages (Cai et al., 2021). Assemblages A and B are able to infect a wide range of
118 hosts including humans, livestock, companion animals, and wildlife and are considered
119 zoonotic. Assemblages C and D are mainly identified in canids, E in domestic and wild
120 hoofed animals, F in felids, G in rodents, and H in marine mammals (Cai et al., 2021).
121 Besides assemblage E, assemblages A and to a lesser extent B and C have been reported
122 in ungulate species (Cai et al., 2021).

123 Comparatively, the molecular diversity of *B. coli* is more limited than that
124 observed in the *Cryptosporidium* and *Giardia* genera. To date, three (A to C) genotypes
125 have been reported. Genotypes A and B are found in various host species, whereas
126 genotype C seems to be restricted to non-human primates (Ponce-Gordo et al., 2011).
127 However, little is known on the role of non-human primates or non-swine animals as
128 suitable hosts for *B. coli*.

129 In Spain, data on the occurrence and molecular diversity of *G. duodenalis*,
130 *Cryptosporidium* spp., and *B. coli* in ungulate species is limited. Most studies have
131 reported the presence of these parasites in farmed ungulate species, whereas few studies
132 have been carried out in wild animal populations (Table S1). *Cryptosporidium* infections
133 have been identified in wild ungulates of the genera *Capreolus*, *Cervus*, and *Sus* with
134 prevalence rates ranging from 4–17%. *Cryptosporidium bovis* and *C. ryanae* have been
135 detected in roe deer (*Capreolus capreolus*), and *C. parvum*, *C suis*, and *C. scrofarum* in
136 wild boar (Table S1). *Giardia duodenalis* has been reported in members of the genera
137 *Capreolus*, *Cervus*, and *Sus* at prevalence rates of 1–23%. Only assemblage A has been
138 documented in roe deer (García-Presedo et al., 2013a). A single study reported the
139 presence of *B. coli* in wild boar in Córdoba (Rivero-Juarez et al., 2020). Given the scarcity
140 of molecular epidemiological data on these enteric parasites in ungulate species in Spain,
141 the purpose of the current study was to investigate the occurrence, genetic diversity, and
142 zoonotic potential of *Cryptosporidium* spp., *G. duodenalis*, and *B. coli* in free-ranging
143 and farmed wild ungulates at the national scale.
144

145 ## 2. MATERIALS AND METHODS

146 ### 2.1. Ethical statement

147 Sampled animals were legally hunted under Spanish (RD 8/2003, RD 138/2020) and EU
148 (RD 53/2013) legislation. All the hunters had hunting licenses. Professional personnel
149 collected the faecal samples from hunter-harvested wild ruminants and boar during the
150 regular hunting seasons.
151
152 **2.2. Study area and sampling strategy**
153 Between 1999 and 2021, a retrospective nationwide survey was performed. Faecal
154 samples from the eight wild ungulate species present in Spain: Barbary sheep
155 (*Ammotragus lervia*), Iberian wild goat (*Capra pyrenaica*), roe deer, red deer (*Cervus*
156 *elaphus*), fallow deer (*Dama dama*), mouflon (*Ovis aries musimon*), southern chamois
157 (*Rupicapra pyrenaica*), and wild boar, were collected throughout the five bioregions
158 (BRs, see below) of mainland Spain (Table 1).
159 Based on landscape structure, major ecosystems, game management practices,
160 and socio-political aspects, the Spanish Wildlife Disease Surveillance Scheme splits
161 mainland Spain into five different BRs sharing similar epidemiological features
162 (PNVSFS, 2020). BR1 comprises the Northern areas of temperate Atlantic climate with
163 almost no game management; meanwhile, the remaining BRs present a Mediterranean
164 climate with an increasing drought gradient from BR2 to BR4. In the Mediterranean BRs,
165 game management is not the norm except for BR3 and the Southwest of BR5, where the
166 highly productive savannah-like or oak forest landscapes are frequently profited for large
167 game production. Mountain habitats are more dominant in BRs 1, 2, and 5, while cereal
168 plains are predominant in BR4. This zoning has been previously exploited to facilitate
169 disease surveillance efforts in wild ungulates in Spain (García-Bocanegra et al., 2016;
170 González-Barrio et al., 2015; Jiménez-Ruiz et al., 2021; Lorca-Oró et al., 2014; Muñoz
171 et al., 2010). From each sampling site, that is, hunting estates or game reserves (n = 63,

172 Table S2) selected by simple random sampling throughout the study area, the animals
173 (15–20 whenever possible) were also randomly sampled. Figure 1 shows the map of the
174 Iberian Peninsula showing the sampling areas and the geographical distribution of
175 protozoan DNA detected according to the bioregion of origin in all free-ranging and
176 farmed wild ungulate species (Panel A), in free-ranging and farmed wild cervid (fallow
177 deer, red deer, and roe deer) species only (Panel B), in free-ranging wild bovid (Barbary
178 sheep, Iberian wild goat, mouflon, and suthern chamois) species only (Panel C), and in
179 wild boar only (Panel D).

180 Faecal samples were collected directly from the rectum of each animal during field
181 necropsies after hunting using disposable gloves and placed in individual sterile tubes
182 with records of the date, location, and host species. Collected samples were transported
183 in cooled boxes to each participating institution responsible for the sampling and stored
184 at –20°C. Aliquots of these faecal samples were shipped to the Spanish National Centre
185 for Microbiology, Majadahonda (Spain) for subsequent molecular analyses.

186 For comparative purposes, aliquots of faecal samples from a farmed wild red deer
187 population located in southern Spain (Figure 1, Panel B) were obtained from a previous
188 work (González-Barrio et al., 2017). These red deer were semi-extensively bred in a
189 forest-shrub prairie habitat divided into different plots by high-wire fencing. The animals
190 were kept in separate batches according to their sex and productive status. Batches of 60–
191 80 reproductive females were kept within large-fenced areas (6–8 ha), whereas males
192 were kept in separate enclosures. The animals were identified with individual ear tags.
193 Faecal material was collected directly from the rectum using sterile disposable latex
194 gloves during routine health veterinary inspections.

195

196 **2.3. DNA extraction and purification**

Genomic DNA was isolated from about 200 mg of each faecal specimen of free-ranging or farmed wild ungulate by using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except that samples mixed with InhibitEX buffer were incubated for 10 min at 95°C. Extracted and purified DNA samples were eluted in 200 μl of PCR-grade water and kept at 4°C until further molecular analysis.

Molecular detection and characterisation of *Cryptosporidium* spp., *Giardia duodenalis,* and *Balantioides coli*

Detailed information on the PCR cycling conditions and oligonucleotides used for the molecular identification and/or characterisation of the protozoan parasites investigated in the present study is presented in Tables S3 and S4, respectively.

The presence of *Cryptosporidium* spp. was assessed using a nested PCR protocol to amplify a 587-bp fragment of the small subunit of the rRNA (*ssu* rRNA) gene of the parasite (Tiangtip and Jongwutives, 2002). Specific subtyping tools targeting the partial 60-kDa glycoprotein gene (*gp60*) were used in those samples that tested positive for *Cryptosporidium* by *ssu*-PCR including *C. canis* (Jiang et al., 2021), *C. parvum* (Feltus et al., 2008), *C. ryanae* (Yang et al, 2020), and *C. ubiquitum* (Li et al., 2014) to ascertain intra-species genetic diversity.

The presence of *G. duodenalis* was investigated using a real-time PCR (qPCR) method targeting a 62-bp region of the *ssu* rRNA gene of the parasite as initial screening methods (Verweij et al., 2003). For assessing the molecular diversity of *G. duodenalis* at the assemblage level, a nested PCR was used to amplify a 300-bp fragment of the *ssu* rRNA gene in those samples that yielded cycle threshold (C_T) values <35 in qPCR (Appelbee et al., 2003). For assessing the molecular diversity of the parasite at the sub-assemblage level we adopted a sequence-based multilocus genotyping (MLST) scheme

222 targeting the genes encoding for the glutamate dehydrogenase (*gdh*), β-giardin (*bg*), and
223 triose phosphate isomerase (*tpi*) proteins of the parasite. Only samples that yielded qPCR
224 C_T values <32 were assessed under the MLST scheme. A semi-nested PCR was used to
225 amplify a 432-bp fragment of the *gdh* gene (Read et al., 2004), and nested PCRs were
226 used to amplify 511 and 530 bp fragments of the *bg* and *tpi* genes, respectively (Lalle et
227 al., 2005 Sulaiman et al., 2003).

228 *Balantioides coli* detection was attempted by a direct PCR assay to amplify the
229 complete ITS1–5.8s-rRNA–ITS2 region and the last 117 bp (3′ end) of the *ssu*-rRNA
230 sequence of this ciliate using (Ponce-Gordo et al., 2011).

231 All the direct, semi-nested and nested PCR protocols described above were
232 conducted on a 2720 Thermal Cycler (Applied Biosystems). Reaction mixes always
233 included 2.5 U of MyTAQ™ DNA polymerase (Bioline GmbH, Luckenwalde, Germany)
234 and 5–10 μl 5× MyTAQ™ Reaction Buffer containing 5 mM deoxynucleotide
235 triphosphates and 15 mM $MgCl_2$. Negative and positive controls were included in every
236 PCR run. PCR amplicons were visualized on 2% D5 agarose gels (Conda, Madrid, Spain)
237 stained with Pronasafe (Conda nucleic acid staining solutions. A 100 bp DNA ladder
238 (Boehringer Mannheim GmbH, Mannheim, Germany) was used for the sizing of the
239 obtained amplicons.

240

241 **2.4. Sequence analysis**

242 All amplicons of the expected size were directly sequenced in both directions with the
243 corresponding internal primer pair (see Table S4) in 10 μl reactions using Big Dye™
244 chemistries and an ABI 3730xl sequencer analyser (Applied Biosystems, Foster City,
245 CA). Raw sequences were examined with Chromas Lite version 2.1 software
246 (http://chromaslite.software.informer.com/2.1) to generate consensus sequences. These

sequences were compared with reference sequences deposited at the National Center for Biotechnology Information (NCBI) using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences generated in the present study were deposited in the GenBank public repository database under accession numbers OP164760 (*C. canis*), OP164761–OP164762 (*C. parvum*), OP164763–OP164767 (*C. ryanae*), OP164768–OP164773 (*C. scrofarum*), OP164774 (*C. suis*), OP164775 (*C. ubiquitum*), and OP888101-OP888103 (*G. duodenalis*).

2.5. Statistical analysis

The pathogens community was analysed with a Permutational Multivariate Analysis of Variance (PERMANOVA). Two fixed factors were entered into the analysis: bioregion (with five levels) and species (with eight levels). Type III Sum of Squares was used since it is appropriate in the case of an unbalanced design. All the tests were performed with 999 permutations to increase the power and precision of analysis (Anderson et al., 2008) of residuals under a reduced model (Anderson and Braak, 2003). The differences in community structure among the bioregion and species were investigated using a posteriori pair-wise test with 999 permutations. The advantage of the permutation approach is that the resulting test is "distribution-free" and not constrained by many of the typical assumptions of parametric statistics (Walters and Coen, 2006). The relative contribution of each pathogen species to determine differences in the composition of the communities between bioregion and species were evaluated using a similarity percentage analysis (SIMPER). In this study, SIMPER was employed to identify those pathogen species that were responsible for more than 90% of dissimilarity among bioregion and species. All the analyses were performed using PRIMER v6 software (Clarke and Gorley, 2006), including the PERMANOVA+ add-on package (Anderson et al., 2008).

272

273 ## 3. RESULTS

274 ### 3.1. Occurrence of enteric protozoa

275 The full dataset of this study showing sampling, diagnostic, and molecular data can be
276 found in Table S5. A total of 1,382 samples from free-ranging ungulates (76.6%,
277 1058/1,382) and farmed wild red deer (23.4%, 324/1,382) were included in the survey.
278 Overall, *G. duodenalis* was the most prevalent enteric parasite found in the wild and
279 farmed ungulate species (5.4%, 74/1,382; 95% CI: 4.2–6.5%), followed by
280 *Cryptosporidium* spp. (3.0%, 42/1,382; 95% CI: 2.1–3.9%), and *B. coli* (0.7%, 9/1,382;
281 95% CI: 0.3–1.2%).

282 *Cryptosporidium* spp. was only detected in roe deer (7.5%, 7/93), wild boar (7.0%,
283 25/359) and red deer (1.5%, 10/653). This protozoan was detected in BR3 (7.5%, 25/335),
284 BR1 (6.8%, 7/103), BR2 (4.3%, 7/164) and BR5 (0.4%, 3/748) (Table 1, Figure 1). Unlike
285 the Barbary sheep (n = 20), *G. duodenalis* was detected in seven of the studied host
286 species including southern chamois (12.9%, 8/62), mouflon (10.0%, 1/10), Iberian wild
287 goat (9.0%, 8/89), roe deer (7.5%, 7/93), wild boar (5.6%, 20/359), fallow deer (5.2%,
288 5/96) and red deer (3.8%, 25/653) (Figure 1, Table 1). The infection rate of *G. duodenalis*
289 detected in farmed wild red deer (5.2%, 17/324) was higher than in free-ranging red deer
290 (2.4%, 8/329). According to the spatial distribution in the bioregion of origin, *G.*
291 *duodenalis* infections were more frequent in BR1 (12.6%, 13/103) than in BR4 (12.5%,
292 4/32), BR5 (4.9%, 37/748), BR2 (4.3%, 7/164) and BR3 (3.9%, 13/335). The ciliate *B.*
293 *coli* was only detected in wild boar (2.5%, 9/359) from the BR5 (1.2%, 9/748) (Figure 1,
294 Table 1).

295

296 ### 3.2. Molecular diversity

297 Six *Cryptosporidium* species were identified in the ungulate population investigated: *C.*
298 *scrofarum* (52.4%, 22/42), *C. ryanae* (31.0%. 13/42), *C. parvum* (7.1%, 3/42), *C. canis*
299 (2.4%, 1/42), *C. suis* (2.4%, 1/42), and *C. ubiquitum* (2.4%, 1/42) (Table 2).
300 *Cryptosporidium scrofarum* was identified exclusively in wild boar, whereas *C. ryanae*
301 was found infecting red deer (*n* = 7), roe deer (*n* = 5), and wild boar (*n* = 1).
302 *Cryptosporidium parvum* was detected in red deer (*n* = 2) and wild boar (*n* = 1), *C.*
303 *ubiquitum* and canine-adapted *C. canis* in roe deer (one each), and swine-adapted *C. suis*
304 in red deer. An additional wild boar sample (very likely belonging to *C. scrofarum*) could
305 not be assigned to a given *Cryptosporidium* species due to insufficient sequence quality.
306 *Cryptosporidium scrofarum*, *C. ryanae,* and *C. parvum* had a wide geographical
307 distribution, being identified in two or more bioregions. *C. suis* was only detected in BR3,
308 *C. canis* in BR2, and *C. ubiquitum* in BR1 (Table S5).

309 None of the *Cryptosporidium*-positive samples could be genotyped at the *gp60*
310 gene. Remarkably, the *ssu*-PCR used for initial *Cryptosporidium* detection yielded many
311 unspecific amplification reactions (*n* = 113). Sanger sequencing analysis of these products
312 revealed the presence of bacterial (Aeromonas), fungal (genera *Alternaria*, *Bipolaris*,
313 *Cercophora*, *Fusarium*, *Gnomoniopsis*, *Preussia*, *Sirococcus*, *Sordaria*, *Thelebolus*),
314 plant (genera *Fagus*, *Pelargonium*), algae (genus *Desmodesmus*) or other protist (genera
315 *Adelina*, *Colpodella*, *Cyclotella*, *Platyophrides*, *Stylonychia*, *Theileria*) organisms (Table
316 S5).

317 *Giardia duodenalis*-positive samples from free-ranging and farmed wild ungulate
318 species (*n* = 74) generated cycle threshold (C_T) values ranging from 20.9 to 39.5 (median:
319 33.7; SD: 3.6). To maximize resources and time, only samples with C_T values ≤35 (*n* =
320 35) were attempted to be genotyped at the *ssu* rRNA locus for assemblage determination.
321 Sequence analyses revealed the presence of zoonotic assemblages A (*n* =1) and B (*n* = 1)

and ungulate-adapted assemblage E (n = 4) (Table 2). Additionally, samples with C_T values ≤32 (n = 21) were assessed at the *gdh*, *bg*, and *tpi* loci for sub-assemblage determination, but none of them could be successfully amplified at these makers.

Balantioides coli was unmistakably identified in eight wild boar samples, but sequence data of insufficient quality precluded the possibility of determining the genotype of this parasite species. Four additional amplicons (also from swine origin) yielded faint bands on gel electrophoresis that could not be confirmed by Sanger sequencing. These four samples were conservatively regarded as *B. coli*-negative.

Pathogen community The PERMANOVA analysis revealed significant differences in the composition of pathogen species across bioregion (P = 0.001) and host species (P = 0.002). Pair-wise test results revealed significant differences in pathogen compositions according to bioregions (between BR2 *vs*. BR1, BR2 *vs*. BR5, and BR3 *vs*. BR5) and host species (between *C. elaphus vs*. *S. scrofa* and *C. elaphus vs*. *R. pyrenaica* (Table 3).

The SIMPER analysis showed considerable average dissimilarity between bioregions: BR2 *vs*. BR1 (96.8%), BR2 *vs*. BR5 (98.4%), and BR3 *vs*. BR5 (98.7%). The species with the highest dissimilarity contribution between bioregions was *G. duodenalis* (Table 4). The SIMPER analysis also showed considerable average dissimilarity between species: *C. elaphus vs*. *Sus scrofa* (98.5%) and *C. elaphus vs*. *R. pirenaica* (97.2%). The species with the highest dissimilarity contribution values between host species also was *G. duodenalis*.

4. DISCUSSION

This study represents the largest attempt to assess the occurrence, molecular diversity, and zoonotic potential of *Cryptosporidium* spp., *G. duodenalis*, and *B. coli* in wild hoofed

347 animals conducted in Spain to date. A molecular-based study specifically devoted to the
348 Microsporidia *Enterocytozoon bieneusi* was previously published in this very same
349 population (Dashti et al., 2022). Our study had several strengths, including large sample
350 size, representativeness of all eight wild ungulate species present in the country, national
351 coverage, and molecular-based diagnosis and genotyping approaches. The survey is also
352 timely because information on the wild ungulate contribution to environmental
353 *Cryptosporidium* oocysts and *Giardia* cysts is scarce (Hatam-Nahavandi et al., 2019).
354 Regarding *B. coli*, little is known about the role of non-swine species as potential suitable
355 hosts for this ciliate parasite (Ponce-Gordo & García-Rodríguez, 2021).

356 Our data revealed an overall *Cryptosporidium* infection rate of 3%, peaking at 7–
357 8% in roe deer and wild boar, respectively. These figures agree with that (8%, 484/9,480)
358 estimated in a recent systematic review and meta-analysis of *Cryptosporidium* prevalence
359 in deer worldwide (Lv et al., 2021). In Spain, most of the epidemiological studies on
360 protozoan infections in domestic and wild ruminants have been conducted in Galicia
361 (north-western areas of the country) (see Table S1). In this region *Cryptosporidium*
362 infections have been reported in 7% of red deer, in 1–4% of roe deer, and in 7–17% of
363 wild boar (Castro-Hermida et al., 2011a,b; García-Presedo et al., 2013a,b). A prevalence
364 rate of 6% has also been documented in wild boar in the south of Spain (Rivero-Juarez et
365 al., 2020). At the European scenario, *Cryptosporidium* infections have been identified in
366 nine different species of wild ungulate species. These include 2% of Alpine chamois
367 (*Rupicapra rupicapra*) in Italy (Trogu et al., 2021), 6% of fallow deer in the UK (Sturdee
368 et al., 1999), 3% of moose (*Alces alces*) in Norway (Hamnes et al., 2006), 2% of mouflons
369 (*Ovis musimon*) in the Czech Republic (Kotkova et al., 2016), 0.3–80% of red deer in the
370 Czech Republic (Kotkova et al., 2016), Ireland (Skerrett & Holland, 2001), Italy (Trogu
371 et al., 2021), Poland (Paziewska et al., 2007), Norway (Hamnes et al., 2006), and UK

(Wells et al., 2015), 10% of Reeve´s muntjac (*Muntiacus reevesi*) in the UK (Sturdee et al., 1999), 3–33% of roe deer in Italy (Trogu et al., 2021), Poland (Paziewska et al., 2007), and Norway (Hamnes et al., 2006), 12% of white-tailed deer (*Odocoileus virginianus*) in the Czech Republic (Kotkova et al., 2016), and 13–17% of wild boar in central Europe (Němejc et al., 2012, 2013).

Our sequence analyses confirmed the occurrence of six distinct *Cryptosporidium* species circulating within the surveyed wild ungulate population. Of them, *C. ryanae* showed the widest host range, being detected in red deer, roe deer, and wild boar. *Cryptosporidium parvum* was identified in red deer and wild boar, but less frequently than *C. ryanae*. *Cryptosporidium ubiquitum* was observed only in one roe deer sample, whereas *C. scrofarum* (formerly known as pig genotype II) was exclusively detected in wild boar. The findings of *C. canis* in one roe deer and *C. suis* in one red deer are interesting. These *Cryptosporidium* species are adapted to infect canids and swine, respectively, suggesting that their presence in wild cervids could be the result of overlapping sylvatic and/or domestic transmission cycles of the parasite in habitats where different host species live sympatrically. Whether these findings correspond to true or spurious (mechanical carriage) infections remain to be elucidated.

In the only previous Spanish study reporting molecular data on wild cervids, *C. ryanae* and *C. bovis* were described infecting roe deer in Galicia (García-Presedo et al., 2013a). At the European level, *C. ubiquitum* is also the predominant *Cryptosporidium* species circulating in wild ruminants, being identified in red deer in the Czech Republic (Kotkova et al., 2016), roe deer in Italy and the UK (Trogu et al., 2021), and Alpine chamois in Italy (Trogu et al., 2021). *Cryptosporidium* deer genotype has also been reported in white-tailed deer in the Czech Republic (Kotkova et al., 2016) and in red deer and roe deer in the UK (Robinson et al., 2011; Wells et al., 2015), whereas sporadic cases

of infections by murine-adapted *C. muris* have been identified in mouflon, red deer and white-tailed deer (one each) in the Czech Republic (Kotkova et al., 2016). Neither *C.* deer genotype and *C. muris* nor *C. bovis* were identified in the Spanish population surveyed in the present study. Remarkably, *C. parvum* is a common finding in wild cervids in the UK (Wells et al., 2015), likely as a result of spill over events from infected livestock. Regarding wild boar, both *C. scrofarum* and *C. suis* have been documented at similar proportions in Central European countries including Austria, Czech Republic, Poland, and Slovakia (Němejc et al., 2012, 2013). This is not the case in Spain, where *C. scrofarum* is far more prevalent than *C. suis* in this host species (Rivero-Juarez et al., 2020; present study).

In our study *G. duodenalis* was the most prevalent protozoan parasite found (5%), with peaks of 10–13% in mouflons and southern chamois. In Spain, this parasite has been previously identified at infection rates of 8% in red deer, 5–9% in roe deer, and 1–4% in wild boar in Galicia (Castro-Hermida et al., 2011a,b; García-Presedo et al., 2013a). A higher infection rate of 23% was documented in wild boar in the southern province of Córdoba (Rivero-Juarez et al., 2020). In other European countries, *G. duodenalis* has been described in six genera of wild ungulates including *Alces*, *Capreolus*, *Cervus*, *Dama*, *Rangifer*, and *Sus*. Prevalences of 1–2% and 4–24% have been obtained in red deer and roe deer, respectively, in Croatia (Beck et al., 2011), Norway (Hamnes et al., 2006), and Poland (Majewska et al., 2012; Paziewska et al., 2007). *Giardia* infections have also been documented at rates of 12% in fallow deer in Italy (Lalle et al., 2007), of 5–12% in moose and reindeer (*Rangifer tarandus*) in Norway (Hamnes et al., 2006; Robertson et al., 2007), and in 2–4% in wild boar in Croatia and Switzerland (Beck et al., 2011; Spieler & Schnyder, 2021).

421 *Ssu* rRNA sequence analyses revealed the presence of zoonotic assemblages A
422 and B in single samples from wild boar and red deer, whereas ungulate-adapted
423 assemblage E was identified in mouflon, red deer, and southern chamois. We failed to
424 amplify any of these isolates at the *gdh*, *bg* or *tpi* loci, so information at the sub-
425 assemblage level is still lacking. Similar negative sub-genotyping results have been
426 obtained in previous studies by our research group when investigating wild boar (Rivero-
427 Juarez et al., 2020). Information on the molecular diversity of *G. duodenalis* in wild
428 ungulate European populations is also limited. Assemblage A seems to be the most
429 prevalent genetic variant of the parasite, being detected in red deer, roe deer, and wild
430 boar in Croatia (Beck et al., 2011), in fallow deer in Italy (Lalle et al., 2007), in moose
431 and reindeer in Norway (Idland et al., 2021; Robertson et al., 2007), and in roe deer in
432 Spain (García-Presedo et al., 2013a). Interestingly, canine-adapted *G. duodenalis*
433 assemblages C/D and murine-adapted *G. muris* have been sporadically detected in
434 Croatian roe deer and wild boar (Beck et al., 2011), indicative of cross-species
435 transmission or overlapping of transmission cycles of these pathogens. As in the case of
436 *C. canis* and *C. suis*, at present it is unclear whether these findings correspond to true or
437 spurious infections.

438 Swine-adapted *B. coli* was identified at low frequency (<3%) in wild boar only.
439 This finding suggests that wild ruminants are not suitable hosts for this ciliate parasite,
440 which in Europe seems naturally restricted to pigs and, to a lesser extent, wild boar. A *B.*
441 *coli* infection rate of 2% has been previously reported in wild boar in Switzerland (Spieler
442 & Schnyder, 2021).

443 Our study provided also interesting data when protozoan infections were analysed
444 according to host species and bioregion of origin. Because higher interaction rates
445 (facilitating pathogen transmission) are more likely to occur among animals living in

446 restricted areas than among wandering animals, one would expect that wild farmed red
447 deer would bear higher protozoan infection rates than their free-ranging wild
448 counterparts. Although this was the case for *G. duodenalis* infections (2.4% *vs*. 5.2%),
449 exactly the opposite trend was observed for *Cryptosporidium* infections (2.7% *vs*. 0.3%).
450 Interestingly, free-living wild red deer were infected by a higher diversity of
451 *Cryptosporidium* species (*C. ryanae*, *C. parvum*, *C. suis*) than farmed wild red deer (in
452 which only *C. parvum* was found), suggesting that the former were exposed to a wider
453 range of infective sources. Regarding bioregion of origin, wild boar living in BR5 were
454 less likely to be infected by *Cryptosporidium* spp., but not by *G. duodenalis*. A potential
455 explanation for this finding is that BR5 comprises all the Spanish Mediterranean littoral,
456 highly developed and urbanized and, at least in theory, less suitable for sustaining large
457 populations of wild animals. Remarkably, all the *B. coli*-positive samples were collected
458 in the Doñana National Park, a natural reserve extending over three provinces (Huelva,
459 Cádiz, and Seville) in southern Spain, BR5. At present we do not have a clear explanation
460 for the apparent geographical segregation observed for *B. coli* in wild boar.

461 This study had four potential limitations that may have biased the results obtained.
462 First, its retroactive nature implied that some of the faecal samples analysed were stored
463 at –20°C for up to 12 years prior DNA extraction and molecular testing. Long-term
464 storage may have altered the quantity/quality of parasite DNA, compromising the
465 performance of the PCRs used. Second, sample size for some wild ungulate species (e.g.,
466 mouflon and Barbary sheep) may be underrepresented. Of note, the natural populations
467 of these ruminants in Spain are relatively low in numbers (mouflon: near 15,000
468 individuals; Barbary sheep: near 1,300 individuals) making difficult obtaining
469 representative sample numbers. Third, the genetic markers used in our genotyping PCR
470 protocols had intrinsic limited sensitivities. In practical terms this means that samples

with little amount of parasite DNA would be amplified by the detection PCRs (targeting highly sensitive, multi-copy genes such as *ssu* rRNA) but not by the genotyping PCRs (targeting low sensitive, single-copy genes such as *gdh*, *bg*, and *tpi* in *Giardia* or *gp60* in *Cryptosporidium*). Lack or insufficient genotyping data make difficult the assessment of zoonotic potential and the public health significance of the results obtained. Finally, direct comparison of results among bioregions should be interpreted with caution because of differences not only in environmental and epidemiological factors, but also in methodological approaches including sampling periods and strategies and diagnostic methods.

5. CONCLUSIONS

This is the largest molecular epidemiological study investigating the presence and genetic diversity of *Cryptosporidium* spp., *G. duodenalis*, and *B. coli* in wild ungulate species conducted in Spain to date. Overall infection rates were relatively low (≤5%) and, in the case of *Cryptosporidium* and *Balantiodes* infections, mostly caused by ungulate-adapted species/genotypes. Sporadic infections by canine- or swine-adapted species may be indicative of potential cross-species transmission, although spurious infections cannot be ruled out. Taken together, these data would indicate that wild ungulate species pose a limited role as source of human infections by *Cryptosporidium* spp., *G. duodenalis*, or *B. coli*. Wild ruminants are not suitable hosts for *B. coli*, whose main ungulate host species is the wild boar.

ACKNOWLEDGEMENTS

This study was funded by Health Institute Carlos III (ISCIII), Spanish Ministry of Economy and Competitiveness, under project PI19CIII/00029. Sample collection in

Catalonia was supported by the Council of Barcelona through contracts 13/051, 15/0174, 16/0243 and 16/0243-00-PR/01, from the Spanish Ministry of Economy and Competitiveness under research grants CGL2012- 40043-C02-01, CGL2012-40043-C02-02, and from the Spanish Ministry of Science and Innovation under research grant CGL2016-80543-P PID2020-115046GB-I00. Sampling in the Basque Country was conducted by members of the Association for the Defence of the Game Natural Heritage of the Basque Country (ARTIO). We thank the Dirección General del Medio Natural y Planificación Rural del Principado de Asturias (Oviedo, Spain). Antonio Rivero-Juárez is the recipient of a Miguel Servet Research Contract by the Ministerio de Ciencia, Promoción y Universidades of Spain (CP18/00111). David González-Barrio is the recipient of a Sara Borrell Research Contract funded by the Spanish Ministry of Science, Innovation and Universities (CD19CIII/00011). AJC is supported by 'Juan de la Cierva' contract (IJC2020-042629-I) funded by MCIN/AEI/ 10.13039/501100011033 and by the European Union Next Generation EU/PRTR. Alejandro Dashti is the recipient of a PFIS contract (FI20CIII/00002) funded by the Spanish Ministry of Science and Innovation and Universities. The funders did not play any role in the design, conclusions, or interpretation of the study.

WILDLIFE ECOLOGY & HEALTH GROUP (WE&H)

The following investigators (in alphabetic order) participated in the Wildlife Ecology & Health Group (WE&H): Carles Conejero, Carlos González-Crespo, Cristina Garrido, Diana Gassó, Emmanuel Serrano, Gregorio Mentaberre, Helena Martínez-Torres, Irene Torres-Blas, Josep Estruch, Josep Pastor, Jorge Ramón López-Olvera, María Escobar-González, Marta Valldeperes, Montse Mesalles, Raquel Álvarez, Rafaela Cuenca, Roser Velarde, Santiago Lavín.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available within the main body of the manuscript.

AUTHORS CONTRIBUTIONS

MAH, ARJ, JV, MCA, DFL, PM, JAA, AB, GAC, CMC, JAO, RCB, DGB and ES (on behalf of the WE&H group) collected the samples. AD, PCK, BB and ASM carried out the laboratory experiments. DC and DGB designed and supervised the experiments. AD, DGB and DC writing—original draft preparation. ARJ, AB, RCB, ES, DGB and DC writing—review and editing. The final version was read and approved by all authors.

ORCID

Alejandro Dashti https://orcid.org/0000-0001-8707-5731

Pamela C. Köster https://orcid.org/0000-0001-5963-8824

Begoña Bailo https://orcid.org/0000-0002-9453-9431

Ana Sánchez de las Matas https://orcid.org/0000-0003-2606-6342

Miguel Ángel Habela https://orcid.org/0000-0002-0019-5798

Antonio Rivero-Juarez https://orcid.org/0000-0002-5813-6889

Joaquín Vicente https://orcid.org/0000-0001-8416-3672

Emmanuel Serrano https://orcid.org/0000-0002-9799-9804

Maria C. Arnal https://orcid.org/0000-0002-6770-3400

Daniel Fernández de Luco https://orcid.org/0000-0003-3289-4267

Patrocinio Morrondo https://orcid.org/0000-0001-7171-7162

José A. Armenteros https://orcid.org/0000-0001-8776-1299

Ana Balseiro https://orcid.org/0000-0002-5121-7264

Guillermo A. Cardona https://orcid.org/0000-0002-4156-4174

Carlos Martínez-Carrasco https://orcid.org/0000-0002-8742-0109

Antonio José Carpio https://orcid.org/0000-0002-6989-970X

Rafael Calero-Bernal https://orcid.org/0000-0003-2323-0135

David González-Barrio https://orcid.org/0000-0001-5083-8854

David Carmena https://orcid.org/0000-0002-4015-8553

REFERENCES

Anderson, M., & Braak, C. T. (2003). Permutation tests for multi-factorial analysis of variance. *Journal of Statistical Computation and Simulation*, *73*, 85–113. https://doi.org/10.1080/00949650215733

Anderson, M. J., Gorley, R. N., & Clarke, K. R. (2008). PERMANOVA+ for PRIMER: Guide to software and statistical methods. PRIMER-E Ltd., Plymouth.

Appelbee, A. J., Frederick, L. M., Heitman, T. L., & Olson, M.E. (2003). Prevalence and genotyping of *Giardia duodenalis* from beef calves in Alberta, Canada. *Veterinary Parasitology*, *112*, 289–294. https://doi.org/10.1016/s0304-4017(02)00422-3

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

570 Cai, W., Ryan, U., Xiao, L., & Feng, Y. (2021). Zoonotic giardiasis: an update.
571 *Parasitology Research*, *120*, 4199–4218. https://doi.org/10.1007/s00436-021-07325-
572 2
573 Clarke, K. R., & Gorley, R. N. (2006). PRIMER v6: Use manual/Tutorial. PRIMER-E
574 Ltd., Plymouth.
575 Carpio, A. J., Apollonio, M., & Acevedo, P. (2021). Wild ungulate overabundance in
576 Europe: contexts, causes, monitoring and management recommendations. *Mammal*
577 *Review*, *51*, 95–108. https://doi.org/10.1111/mam.12221
578 Castro-Hermida, J. A., García-Presedo, I., Almeida, A., González-Warleta, M., Correia
579 Da Costa, J. M., & Mezo, M. (2011a). *Cryptosporidium* spp. and *Giardia duodenalis*
580 in two areas of Galicia (NW Spain). *Science of the Total Environment*, *409*, 2451–
581 2459. https://doi.org/10.1016/j.scitotenv.2011.03.010
582 Castro-Hermida, J. A., García-Presedo, I., González-Warleta, M., & Mezo, M. (2011b).
583 Prevalence of *Cryptosporidium* and *Giardia* in roe deer (*Capreolus capreolus*) and
584 wild boar (*Sus scrofa*) in Galicia (NW, Spain). *Veterinary Parasitology*, *179*, 216–219.
585 https://doi.org/10.1016/j.vetpar.2011.02.023
586 Dashti, A., Santín, M., Köster, P. C., Bailo, B., Ortega, S., Imaña, E., Habela, M. A.,
587 Rivero-Juarez, A., Vicente, J., WE&H group, Arnal, M. C., Fernández de Luco, D.,
588 Morrondo, P., Armenteros, J. A., Balseiro, A., Cardona, G. A., Martínez-Carrasco, C.,
589 Ortiz, J. A., Calero-Bernal, R., Carmena, D., & González-Barrio, D. (2022). Zoonotic
590 *Enterocytozoon bieneusi* genotypes in free-ranging and farmed wild ungulates in
591 Spain. *Medical Mycology*, in press.
592 Efstratiou, A., Ongerth, J. E., & Karanis, P. (2017). Waterborne transmission of protozoan
593 parasites: Review of worldwide outbreaks - An update 2011-2016. *Water Research*,
594 *114*, 14–22. https://doi.org/10.1016/j.watres.2017.01.036

595 Feltus, D. C., Giddings, C. W., Schneck, B. L., Monson, T., Warshauer, D., & McEvoy,
596 J. M. (2006). Evidence supporting zoonotic transmission of *Cryptosporidium* spp. in
597 Wisconsin. *Journal of Clinical Microbiology*, *44*, 4303–4308.
598 https://doi.org/10.1128/JCM.01067-06
599 Feng, Y., & Xiao L. (2011). Zoonotic potential and molecular epidemiology of *Giardia*
600 species and giardiasis. *Clinical Microbiology Reviews*, *24*, 110–140.
601 https://doi.org/10.1128/CMR.00033-10
602 García-Bocanegra, I., Paniagua, J., Gutiérrez-Guzmán, A. V., Lecollinet, S., Boadella,
603 M., Arenas-Montes, A., Cano-Terriza, D., Lowenski, S., Gortázar, C., & Höfle, U.
604 (2016). Spatio-temporal trends and risk factors affecting West Nile virus and related
605 flavivirus exposure in Spanish wild ruminants. *BMC Veterinary Research*, *12*, 249.
606 https://doi.org/10.1186/s12917-016-0876-4
607 García-Presedo, I., Pedraza-Díaz, S., González-Warleta, M., Mezo, M., Gómez-Bautista,
608 M., Ortega-Mora, L. M., & Castro-Hermida, J. A. (2013a). The first report of
609 *Cryptosporidium bovis*, *C. ryanae* and *Giardia duodenalis* sub-assemblage A-II in roe
610 deer (*Capreolus capreolus*) in Spain. *Veterinary Parasitology*, *197*, 658–664.
611 https://doi.org/10.1016/j.vetpar.2013.07.002
612 García-Presedo, I., Pedraza-Díaz, S., González-Warleta, M., Mezo, M., Gómez-Bautista,
613 M., Ortega-Mora, L. M., & Castro-Hermida, J. A. (2013b). Presence of
614 *Cryptosporidium scrofarum*, *C. suis* and *C. parvum* subtypes IIaA16G2R1 and
615 IIaA13G1R1 in Eurasian wild boars (*Sus scrofa*). *Veterinary Parasitology*, *196*, 497–
616 502. https://doi.org/10.1016/j.vetpar.2013.04.017
617 González-Barrio, D., Almería, S., Caro, M. R., Salinas, J., Ortiz, J. A., Gortázar, C., &
618 Ruiz-Fons, F. (2015). *Coxiella burnetii* shedding by farmed red deer (*Cervus elaphus*).

619 *Transboundary and Emerging Diseases*, *62*, 572–574.
620 https://doi.org/10.1111/tbed.12179
621 González-Barrio, D., Ortiz, J. A., & Ruiz-Fons, F. (2017). Estimating the efficacy of a
622 commercial phase I inactivated vaccine in decreasing the prevalence of *Coxiella*
623 *burnetii* infection and shedding in red deer (*Cervus elaphus*). *Frontiers in Veterinary*
624 *Science*, *4*, 208. https://doi.org/10.3389/fvets.2017.00208
625 Hamnes, I. S., Gjerde, B., Robertson, L., Vikøren, T., & Handeland, K. (2006).
626 Prevalence of *Cryptosporidium* and *Giardia* in free-ranging wild cervids in Norway.
627 *Veterinary Parasitology*, *141*, 30–41. https://doi.org/10.1016/j.vetpar.2006.05.004
628 Hatam-Nahavandi, K., Ahmadpour, E., Carmena, D., Spotin, A., Bangoura, B., & Xiao,
629 L. (2019). *Cryptosporidium* infections in terrestrial ungulates with focus on livestock:
630 a systematic review and meta-analysis. *Parasites & Vectors*, *12*, 453.
631 https://doi.org/10.1186/s13071-019-3704-4
632 Hopkins, R. M., Meloni, B. P., Growth, D. M., Wetherall, J. D., Reynoldson, J.A.,
633 Thompson, & R. C. (1997). Ribosomal RNA sequencing reveals differences between
634 the genotypes of *Giardia* isolates recovered from humans and dogs living in the same
635 locality. *Journal of Parasitology*, *83*, 44–51.
636 Idland, L., Juul, A. M., Solevåg, E. K., Tysnes, K. R., Robertson, L. J., & Utaaker, K. S.
637 (2021). Occurrence of faecal endoparasites in reindeer (*Rangifer tarandus*) in two
638 grazing areas in northern Norway. *Acta Veterinaria Scandinavica*, *63*, 13.
639 https://doi.org/10.1186/s13028-021-00578-y
640 Ježková, J., Limpouchová, Z., Prediger, J., Holubová, N., Sak, B., Konečný, R.,
641 Květoňová, D., Hlásková, L., Rost, M., McEvoy, J., Rajský, D., Feng, Y., & Kváč, M.
642 (2021). *Cryptosporidium myocastoris* n. sp. (Apicomplexa: Cryptosporidiidae), the

643 species adapted to the Nutria (*Myocastor coypus*). *Microorganisms*, *9*, 813.
644 https://doi.org/10.3390/microorganisms9040813
645 Jiang, W., Roelling, D. M., Guo, Y., Li, N., Feng, Y., & Xiao, L. (2021). Development
646 of a subtyping tool for zoonotic pathogen *Cryptosporidium canis*. *Journal of Clinical*
647 *Microbiology*, *59*, e02474-20. https://doi.org/10.1128/JCM.02474-20
648 Jiménez-Ruiz, S., Vicente, J., García-Bocanegra, I., Cabezón, Ó., Arnal, M. C., Balseiro,
649 A., Ruiz-Fons, F., Gómez-Guillamón, F., Lázaro, S., Escribano, F., Acevedo, P.,
650 Domínguez, L., Gortázar, C., Fernández de Luco, D., & Risalde, M. A. (2021).
651 Distribution of Pestivirus exposure in wild ruminants in Spain. *Transboundary and*
652 *Emerging Diseases*, *68*, 1577–1585. https://doi.org/10.1111/tbed.13827
653 Kotkova, M., Nemejc, K., Sak, B., Hanzal, V., Kvetonova, D., Hlaskova, L., Condlova,
654 S., McEvoy, J., & Kvac, M. (2016). *Cryptosporidium ubiquitum*, *C. muris* and
655 *Cryptosporidium* deer genotype in wild cervids and caprines in the Czech Republic.
656 *Folia Parasitologica (Praha)*, *63*, 2016.003. https://doi.org/10.14411/fp.2016.003
657 Lalle, M., Frangipane di Regalbono, A., Poppi, L., Nobili, G., Tonanzi, D., Pozio, E., &
658 Cacciò, S. M. (2007). A novel *Giardia duodenalis* assemblage A subtype in fallow
659 deer. *Journal of Parasitology*, *93*, 426–428. https://doi.org/10.1645/GE-983R.1
660 https://doi.org/10.1016/j.micpath.2021.105009
661 Lalle, M., Jimenez-Cardosa, E., Cacciò, S. M., & Pozio, E. (2005). Genotyping of *Giardia*
662 *duodenalis* from humans and dogs from Mexico using a beta-giardin nested
663 polymerase chain reaction assay. *Journal of Parasitology*, *91*, 203–205.
664 https://doi.org/10.1645/GE-293R
665 Li, N., Xiao, L., Alderisio, K., Elwin, K., Cebelinski, E., Chalmers, R., Santin, M., Fayer,
666 R., Kvac, M., Ryan, U., Sak, B., Stanko, M., Guo, Y., Wang, L., Zhang, L., Cai, J.,
667 Roellig, D., & Feng, Y. (2014). Subtyping *Cryptosporidium ubiquitum*, a zoonotic

668 pathogen emerging in humans. *Emerging Infectious Diseases*, *20*, 217–224.
669 https://doi.org/10.3201/eid2002.121797
670 Lorca-Oró, C., López-Olvera, J. R., Ruiz-Fons, F., Acevedo, P., García-Bocanegra, I.,
671 Oleaga, Á., Gortázar, C., & Pujols, J. (2014). Long-term dynamics of bluetongue virus
672 in wild ruminants: relationship with outbreaks in livestock in Spain, 2006-2011. *PLoS*
673 *One*, *9*, e100027. https://doi.org/10.1371/journal.pone.0100027
674 Lv, X. Q., Qin, S. Y., Lyu, C., Leng, X., Zhang, J. F., & Gong, Q. L. (2021). A systematic
675 review and meta-analysis of *Cryptosporidium* prevalence in deer worldwide.
676 *Microbial Pathogenesis*, *157*, 105009. https://doi.org/10.1016/j.micpath.2021.105009
677 Majewska, A. C., Solarczyk, P., Moskwa, B., Cabaj, W., Jankowska, W., & Nowosad, P.
678 (2012). *Giardia* prevalence in wild cervids in Poland. *Annals of Parasitology*, *58*, 207–
679 209.
680 Muñoz, P. M., Boadella, M., Arnal, M., de Miguel, M. J., Revilla, M., Martínez, D.,
681 Vicente, J., Acevedo, P., Oleaga, A., Ruiz-Fons, F., Marín, C. M., Prieto, J. M., de la
682 Fuente, J., Barral, M., Barberán, M., de Luco, D. F., Blasco, J. M., & Gortázar, C.
683 (2010). Spatial distribution and risk factors of Brucellosis in Iberian wild ungulates.
684 *BMC Infectious Diseases*, *10*, 46. https://doi.org/10.1186/1471-2334-10-46
685 Němejc, K., Sak, B., Květoňová, D., Hanzal, V., Janiszewski, P., Forejtek, P., Rajský, D.,
686 Ravaszová, P., McEvoy, J., & Kváč, M. (2013). *Cryptosporidium suis* and
687 *Cryptosporidium scrofarum* in Eurasian wild boars (*Sus scrofa*) in Central Europe.
688 *Veteterinary Parasitology*, *197*, 504–508.
689 https://doi.org/10.1016/j.vetpar.2013.07.003
690 Němejc, K., Sak, B., Květoňová, D., Hanzal, V., Jeníková, M., & Kváč, M. (2012). The
691 first report on *Cryptosporidium suis* and *Cryptosporidium* pig genotype II in Eurasian

692 wild boars (*Sus scrofa*) (Czech Republic). *Veterinary Parasitology*, *184*, 122–125.
693 https://doi.org/10.1016/j.vetpar.2011.08.029
694 Palmer, M. V., Cox, R. J., Waters, W. R., Thacker, T. C., & Whipple, D. L. (2017). Using
695 white-tailed Deer (*Odocoileus virginianus*) in infectious disease research. *Journal of*
696 *the American Association for Laboratory Animal Science: JAALAS*, *56*, 350–360.
697 Paziewska, A., Bednarska, M., Niewegłowski, H., Karbowiak, G., & Bajer, A. (2007).
698 Distribution of *Cryptosporidium* and *Giardia* spp. in selected species of protected and
699 game mammals from North-Eastern Poland. *Annals of Agricultural and*
700 *Environmental Medicine*, *14*, 265–270.
701 PNVSFS, Plan Nacional de Vigilancia Sanitaria en Fauna Silvestre, MAPA, Ministerio
702 de Agricultura, Pesca y Alimentación. 2020. Retrieved from
703 https://www.mapa.gob.es/es/ganaderia/temas/sanidad-animal-higiene-
704 ganadera/pvfs2020_tcm30-437517.pdf. Accessed on July 10 2022.
705 Ponce-Gordo, F., Fonseca-Salamanca, F., & Martínez-Díaz, R. A. (2011). Genetic
706 heterogeneity in internal transcribed spacer genes of *Balantidium coli* (Litostomatea,
707 Ciliophora). *Protist*, *162*, 774–794. https://doi.org/10.1016/j.protis.2011.06.008
708 Ponce-Gordo, F., & García-Rodríguez, J. J. (2021). *Balantioides coli*. *Research in*
709 *Veterinary Science*, *135*, 424–431. https://doi.org/10.1016/j.rvsc.2020.10.028
710 R Core Team. (2012). R: A language and environment for statistical computing. R
711 Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0.
712 Retrieved from http://www.R-project.org. Accessed on July 10 2022.
713 Read, C. M., Monis, P. T., & Thompson, R. C. (2004). Discrimination of all genotypes
714 of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP.
715 *Infection, Genetics and Evolution*, *4*, 125–130.
716 https://doi.org/10.1016/j.meegid.2004.02.001

717 Rivero-Juarez, A., Dashti, A., López-López, P., Muadica, A. S., Risalde, M., Köster, P.
718 C., Machuca, I., Bailo, B., de Mingo, M. H., Dacal, E., García-Bocanegra, I., Saugar,
719 J. M., Calero-Bernal, R., González-Barrio, D., Rivero, A., Briz, V., & Carmena, D.
720 (2020). Protist enteroparasites in wild boar (*Sus scrofa ferus*) and black Iberian pig
721 (*Sus scrofa domesticus*) in southern Spain: a protective effect on hepatitis E
722 acquisition? *Parasites & Vectors*, *13*, 281. https://doi.org/10.1186/s13071-020-04152-
723 9
724 Robertson, L. J., Forberg, T., Hermansen, L., Hamnes, I. S., & Gjerde, B. (2007). *Giardia*
725 *duodenalis* cysts isolated from wild moose and reindeer in Norway: genetic
726 characterization by PCR-rflp and sequence analysis at two genes. *Journal of Wildlife*
727 *Diseases*, *43*, 576–585. https://doi.org/10.7589/0090-3558-43.4.576
728 Robinson, G., Chalmers, R. M., Stapleton, C., Palmer, S. R., Watkins, J., Francis, C., &
729 Kay, D. (2011). A whole water catchment approach to investigating the origin and
730 distribution of *Cryptosporidium* species. *Journal of Applied Microbiology*, *111*, 717–
731 730. https://doi.org/10.1111/j.1365-2672.2011.05068.x
732 Ryan, U., Zahedi, A., Feng, Y., & Xiao, L. (2021). An update on zoonotic
733 *Cryptosporidium* species and genotypes in humans. *Animals*, *11*, 3307.
734 https://doi.org/10.3390/ani11113307
735 Santin, M. (2020). *Cryptosporidium* and *Giardia* in ruminants. *The Veterinary Clinics of*
736 *North America. Food Animal Practice*, *36*, 223–238.
737 https://doi.org/10.1016/j.cvfa.2019.11.005
738 Skerrett, H. E., & Holland, C. V. (2001). Asymptomatic shedding of *Cryptosporidium*
739 oocysts by red deer hinds and calves. *Veterinary Parasitology*, *94*, 239–246.
740 https://doi.org/10.1016/s0304-4017(00)00405-2

741 Solarczyk, P., Majewska, A. C., Moskwa, B., Cabaj, W., Dabert, M., & Nowosad, P.
742 (2012). Multilocus genotyping of *Giardia duodenalis* isolates from red deer (*Cervus*
743 *elaphus*) and roe deer (*Capreolus capreolus*) from Poland. *Folia Parasitologica*
744 *(Praha)*, *59*, 237–240. https://doi.org/10.14411/fp.2012.032
745 Spieler, N., & Schnyder, M. (2021). Lungworms (*Metastrongylus* spp.) and intestinal
746 parasitic stages of two separated Swiss wild boar populations north and south of the
747 Alps: Similar parasite spectrum with regional idiosyncrasies. *International Journal for*
748 *Parasitology. Parasites and Wildlife*, *14*, 202–210.
749 https://doi.org/10.1016/j.ijppaw.2021.03.005
750 Sturdee, A. P., Chalmers, R. M., & Bull, S. A. (1999). Detection of *Cryptosporidium*
751 oocysts in wild mammals of mainland Britain. *Veterinary Parasitology*, *80*, 273–280.
752 https://doi.org/10.1016/s0304-4017(98)00226-x
753 Sulaiman, I. M., Fayer, R., Bern, C., Gilman, R. H., Trout, J. M., Schantz, P. M., Das, P.,
754 Lal, A. A., & Xiao, L. (2003). Triosephosphate isomerase gene characterization and
755 potential zoonotic transmission of *Giardia duodenalis*. *Emerging Infectious Diseases*,
756 *9*, 1444–1452. https://doi.org/10.3201/eid0911.030084
757 Tiangtip, R., & Jongwutiwes, S. (2002). Molecular analysis of *Cryptosporidium* species
758 isolated from HIV-infected patients in Thailand. *Tropical Medicine and International*
759 *Health*, *7*, 357–364. https://doi.org/10.1046/j.1365-3156.2002.00855.x
760 Trimmel, N. E., & Walzer, C. (2020). Infectious wildlife diseases in Austria-A literature
761 review from 1980 until 2017. *Frontiers in Veterinary Science*, *7*, 3.
762 https://doi.org/10.3389/fvets.2020.00003
763 Trogu, T., Formenti, N., Marangi, M., Viganò, R., Bionda, R., Giangaspero, A.,
764 Lanfranchi, P., & Ferrari, N. (2021). Detection of zoonotic *Cryptosporidium*

ubiquitum in alpine wild ruminants. *Pathogens*, *10*, 655. https://doi.org/10.3390/pathogens10060655

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

Walters, K., & Coen, L. D. (2006). A comparison of statistical approaches to analyzing community convergence between natural and constructed oyster reefs. *Journal of Experimental Marine Biology and Ecology*, *330*, 81–95. https://doi.org/10.1016/j.jembe.2005.12.018

Wells, B., Shaw, H., Hotchkiss, E., Gilray, J., Ayton, R., Green, J., Katzer, F., Wells, A., & Innes, E. (2015). Prevalence, species identification and genotyping *Cryptosporidium* from livestock and deer in a catchment in the Cairngorms with a history of a contaminated public water supply. *Parasites & Vectors*, *8*, 66. https://doi.org/10.1186/s13071-015-0684-x

Zahedi, A., Bolland, S. J., Oskam, C. L., & Ryan, U. (2021). *Cryptosporidium abrahamseni* n. sp. (Apicomplexa: Cryptosporidiiae) from red-eye tetra (*Moenkhausia sanctaefilomenae*). *Experimental Parasitology*, *223*, 108089. https://doi.org/10.1016/j.exppara.2021.108089

Zahedi, A., Paparini, A., Jian, F., Robertson, I., & Ryan, U. (2015). 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

Yang, X., Huang, N., Jiang, W., Wang, X., Li, N., Guo, Y., Kváč, M., Feng, Y., & Xiao, L. (2020). Subtyping *Cryptosporidium ryanae*: A common pathogen in bovine animals. *Microorganisms*, *8*, 1107. https://doi.org/10.3390/microorganisms8081107

FIGURE LEGENDS

FIGURE 1 Map of the Iberian Peninsula showing the sampling areas and the geographical distribution of protozoan DNA detected according to established bioregions (BR1-5) in Spain (Muñoz et al., 2010). Coloured circles indicate the presence of the protozoan species (red: *Cryptosporidium* spp.; green: *G. duodenalis*; yellow: *Balantioides coli*) investigated in each sampling area. Black filled circles indicate a negative PCR result. The area of the represented circles is proportional to the sample size (see figure legend). Panel A: all free-ranging and farmed wild ungulate species. Panel B: Free-ranging and farmed wild cervid species only. Panel C: Free-ranging wild bovid species only. Panel D: Free-ranging wild boar only.

TABLE LEGENDS

TABLE 1. Infection rates of the protozoan intestinal parasites investigated in the present survey according to host species

TABLE 2. Frequency and molecular diversity of *Cryptosporidium* spp. and *G. duodenalis* identified at the ssu rRNA gene in the free-ranging and farmed wild ungulate species investigated in the present study.

812 **TABLE 3**. Results of multivariate PERMANOVA (A) main test and pairwise tests
813 assessing for the presence/absence of pathogens for each pair of bioregions (B) and host
814 species (C). *P*-values in bold indicate statistical significance.
815
816 **TABLE 4** SIMPER results of pathogen species that contribute at dissimilarity between
817 bioregions and host species
818
819
820 **SUPPLEMENTARY MATERIAL**
821 **TABLE S1** Infection rates and molecular diversity of *Cryptosporidium* spp., *Giardia*
822 *duodenalis*, and *Balantioides coli* reported in wild and farmed ungulate (ruminant and
823 wild boar) species in Spain.
824 TABLE S2 Number and relative frequencies of faecal samples from free-ranging and
825 farmed wild ungulates (n = 1,382) analysed in the present survey according to the
826 bioregion of origin.
827 TABLE S3 PCR cycling conditions used for the molecular identification and/or
828 characterization of the microeukaryotic parasites investigated in the present study.
829
830 **TABLE S4** Oligonucleotides used for the molecular identification and/or
831 characterization of *Cryptosporidium* spp., *Giardia duodenalis*, and *Balantioides coli* in
832 the present study.
833
834 **TABLE S5** Full dataset generated in the present study showing epidemiological,
835 diagnostic, and genotyping results.
836