Helminth infections in faecal samples of Apennine wolf (*Canis lupus italicus*) and Marsican brown bear (*Ursus arctos marsicanus*) in two protected national parks of central Italy

Barbara Paoletti¹, Raffaella Iorio¹, Donato Traversa¹, Cristina E. Di Francesco¹, Leonardo Gentile², Simone Angelucci³, Cristina Amicucci¹, Roberto Bartolini¹, Marianna Marangi⁴, Angela Di Cesare¹

¹Faculty of Veterinary Medicine, University of Teramo, Piano D’accio, 64100-Teramo, Italy
²Abruzzo Lazio and Molise National Park, Viale Santa Lucia, 67032 Pescasseroli, Italy
³Veterinary Office, Majella National Park, Sulmona, Italy
⁴Department of Production and Innovation in Mediterranean Agriculture and Food Systems, University of Foggia, Via A. Gramsci, 72122-Foggia, Italy

Corresponding Author: Barbara Paoletti; e-mail: bpaoletti@unite.it

ABSTRACT. This article reports the results of a copromicroscopic and molecular investigation carried out on faecal samples of wolves (n=37) and brown bears (n=80) collected in two protected national parks of central Italy (Abruzzo Region). Twenty-three (62.2%) samples from wolves were positive for parasite eggs. Eight (34.78%) samples scored positive for single infections, i.e. *E. aerophilus* (21.74%), *Ancylostoma/Uncinaria* (4.34%), *Trichuris vulpis* (4.34%), *T. canis* (4.34%). Polyspecific infections were found in 15 samples (65.21%), these being the most frequent association: *E. aerophilus* and *Ancylostoma/Uncinaria*. Thirty-seven (46.25%) out of the 80 faecal samples from bears were positive for parasite eggs. Fourteen (37.83%) samples were positive for *B. transfuga*, and six (16.21%) of them also contained *Ancylostoma/Uncinaria*, one (2.7%) *E. aerophilus* and one (2.7%) both *E. aerophilus* and *Ancylostoma/Uncinaria*. Of the other samples, 19 (51.35%) were positive for *Ancylostoma/Uncinaria*, two (5.4%) for *E. aerophilus* and two (5.4%) for both. Molecular analysis found the roundworm and capillariid eggs found in wolves and bear samples to be *Toxocara canis*, *Baylisascaris transfuga* and *Eucoleus aerophilus* (syn. *Capillaria aerophila*). Considering the high prevalence of zoonotic intestinal helminths detected in this study, it is important to improve the knowledge and awareness of the general public and park operators regarding the potential health risk associated with infections in wildlife.

Key words: intestinal helminths, wolves, bears, faeces, zoonosis

Introduction

Wild animals play an important role in the epidemiology of several diseases of veterinary and zoonotic concern, as they may act as reservoirs and spreaders of pathogens capable of infecting animals and human beings. Various diseases are increasingly associated with human-modified ecological transition zones [1], and urbanization is an ongoing global phenomenon that has a significant impact on ecosystems and host-parasite interactions [2]. Additionally, several parasites pose a risk for the health and welfare of wildlife, as disease epidemics can severely reduce and isolate animal populations within protected areas [3] where pathogen transmission from domestic species is believed to have affected wild species [4]. In fact, the threat of disease transmission from domestic animals to wildlife has become recognized as an increasing concern within the conservation community in recent years [5].

In Europe, wolves are hosts and frequent spreaders of various helminths of zoonotic concern and/or veterinary importance, e.g. taeniid
tapeworms [6–8], roundworms [9], hookworms [10,11], whipworms [12], Trichinella spp. [13] and extraintestinal nematodes [14–18]. Roundworms and hookworms are the most frequent intestinal helminths affecting wolves. For instance, Toxocara canis has been found in wolves from Poland, Hungary, Latvia, Spain and Italy with prevalence rates up to ~20% [10,19–22]. Similarly, ancylostomatid hookworms, e.g. Ancylostoma caninum and Uncinaria stenocephala, have been recorded in wolves in eastern Europe, the Iberian peninsula and Italy, with a prevalence of up to ~30% [13,19,23].

These nematodes are able to cause subclinical diseases in wolves but some of them, e.g. T. canis, can also infect humans and domestic animals [23]. For instance, when humans accidentally ingest larvated eggs from the environment, migrating Toxocara larvae can cause severe damage (i.e. larva migrans syndromes) [24]. Also, zoonotic hookworms may infect humans and cause skin, enteric and pulmonary diseases, such as cutaneous lesions and eosinophilic enteritis [25]. While the role of A. caninum and U. stenocephala as a cause of cutaneous larva migrans is still unclear [26], in humans this parasite has been associated with folliculitis, ephemeral and papular/pustular eruptions [27] and also with the penetration of muscle fibres and lung infiltrates [28]. As wolves mark their territories by scats and urine [29] and sporadically prey on livestock [30], the possibility of parasite transmission between wolves and humans or domestic animals may become a risk factor for public health due to increased contact with human settings.

Wild bears may harbour different zoonotic helminths e.g. tapeworms, flukes, roundworms and hookworms [31]. Nonetheless, the most important endoparasite affecting all species of bears worldwide is the roundworm Baylisascaris transfuga [32], which has been widely described in North America [33,34] and in some areas of Europe [35–37]. Roundworms of the Baylisascaris genus have been implicated in clinical and subclinical diseases in a range of natural hosts including bears; importantly, they are also known to play a role in life-threatening larva migrans syndrome in a number of domestic animals and humans [38–41]. There is no unequivocal evidence of natural infections by B. transfuga in non-ursid animals or humans [42], although migrating larvae have been reported to experimentally invade the central nervous system causing visceral, neural, and ocular disease in some animals, including mice [40,43–45].

The mountainous Abruzzo Region of central Italy encompasses four protected regional or national parks, and numerous others natural reserves and protected areas. These territories host large wild endangered predators, such as the Apennine wolf (Canis lupus italicus) and the Marsican brown bear (Ursus arctos marsicanus) [46]. The wolf population inhabiting some parks of the Abruzzo Region is currently stable, comprising about seven reproductive packs with a total number of about 30 wolves in Abruzzo, Lazio and Molise Park [47] and 10 reproductive packs with a total amount of about 70-80 individuals in Majella Park [48]. Importantly, the Marsican bear population comprises an isolated population of a subspecies [49] of the European brown bear (Ursus arctos, Linnaeus, 1758). The population is small, with an estimated number of about 40 individuals, and threatened (95% CI: 37–52) [50].

At present, scattered information is available on parasites of wolves in Italy, although intestinal helminths have been reported in some surveys from limited areas [8,19,51,52]. For instance, studies carried out on illegally killed wolves in the Apennine mountainous chain have found them to harbour various parasites, the most frequent being T. canis, Trichuris vulpis and Echinococcus granulosus [8].

Little information is available in Italy on the occurrence of parasites in free-ranging bears living in natural reserves. One study conducted in the Abruzzo, Lazio and Molise National Park and from its adjacent buffer zone found that B. transfuga was identified in a relatively large number of faecal samples (14.8%) followed by Trichuris spp. (1.8%) and Strongyloides (1.4%) [53]. Elsewhere, Baylisascaris spp. and Trichinella spp. were recorded in captive bears in Italy [37,54].

Therefore, given the importance of parasitosis of veterinary and zoonotic concern for endangered wildlife of Italian national parks, the aim of the present study was to obtain novel information on the distribution of intestinal helminths in wolves and bears living in two national parks of the Abruzzo mountainous territory of the country.

Materials and Methods

From May 2013 to October 2014, faecal samples from wolves (n=37) were collected from different areas of two national protected parks of central
Italy: 11 from the “Abruzzo, Lazio and Molise Park” (41°48’N, 13°47’E), and 26 from the “Majella Park” (42°10’4”N, 14°1’48”E). Eighty samples were collected from brown bears in an area within the “Abruzzo, Lazio and Molise Park”.

The two study areas are located at similar latitudes, share a mountainous backcountry, this being a mosaic of forested and open habitats, and are exposed to similar seasonal climatic variations. Wolf and bear scats were discriminated from each other and from those of other animal species by size, shape, smell, composition and location. In particular, wolf adult droppings are 10–15 cm long and 3–5.5 cm thick, cylindrical, with sub-divisions, and are tapered at one of the extremities. The secretion produced by the anal gland, which is atrophied in most dog breeds, adheres to the faeces during defecation and gives it an acrid and characteristic smell. Moreover, wolves usually use faeces in territorial marking. In the wild, faeces are more common along trails and roads, particularly at junctions, and are placed on conspicuous objects. In addition, undigested prey remains (i.e. fur, snails and parts of bones), especially from large wild ungulates such as wild boar and deer, were taken into account [8]. Samples were stored at 5°C in labelled plastic bags and put in a cooler for transportation. Wolf samples were then frozen at –80°C for four days for safety precautions [55], and then placed at refrigeration temperature. All samples were also included in a monitoring program aimed at identifying the major enteropathogenic viruses in wildlife. Molecular screening against Canine parvovirus type 2 (CPV-2), Canine adenovirus type 1 and 2 (CAV-1 and CAV-2) and Canine distemper virus (CDV) was negative for all samples (unpublished data).

For parasitological exams, faecal samples were divided into aliquots for copromicroscopic and molecular analysis, respectively. Copromicroscopic examination was made with a classical flotation procedure using a saturated NaNO₃ solution (specific gravity 1.300) [56]. Parasite elements were identified according to their morphological features and micrometric measurements at 40× and 100× magnifications [38, 57].

The identity of roundworm eggs and capillariids found in bear and wolf faeces was genetically confirmed as follows. Genomic DNA was extracted from an aliquot of each positive sample using the commercial NORGEN Stool DNA Isolation Kit (Norgen Biotek Corp. Canada). Roundworm eggs from wolves were identified with primers specific for the ribosomal internal transcribed spacer (ITS-2) of Toxocara [58], while a primer set internal to the mitochondrial gene encoding for the cytochrome c

<table>
<thead>
<tr>
<th>Animal</th>
<th>Total positive samples</th>
<th>Monospecific infection</th>
<th>Two species</th>
<th>Three species</th>
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<tbody>
<tr>
<td>Wolves (N=37)</td>
<td>23 (62.2%)</td>
<td>8</td>
<td>12</td>
<td>3</td>
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<td>34.78% on positive</td>
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<td>E. aerophilus + E. aerophilus +</td>
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<td>T. vulpis + T. canis</td>
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<td>T. canis</td>
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<td>Bears (N=80)</td>
<td>37 (46.25%)</td>
<td>28</td>
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<td>21.61% on positive samples:</td>
<td>2.7% on positive samples:</td>
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<td>samples:</td>
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<td>– 2 (1.7%)</td>
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<td>B. transfuga + B. transfuga</td>
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<td>Ancylostoma/Uncinaria E. aerophilus +</td>
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<td>Ancylostoma/Uncinaria E. aerophilus</td>
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<td>Ancylostoma/Uncinaria</td>
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oxidase subunit I (cox1) of the Baylisascaris genus was chosen for the bear faeces [59]. Capillariid eggs were genetically identified with a semi-nested PCR amplifying diagnostic regions within the cox1 gene [60]. The amplicons obtained were purified and sequenced, and the sequences were aligned using Data Analysis in Molecular Biology and Evolution version 4.5.55 (DAMBE); the results were compared to sequences available in the GenBank™ database using the Nucleotide-Nucleotide “Basic Local Alignment Search Tool” (BLAST).

Results

Wolves

Twenty-three (62.2%) out of the 37 faecal samples from wolves were positive for parasite eggs (Table 1). Roundworms, capillariids, Ancylostoma/Uncinaria and Trichuris vulpis eggs were identified. Roundworm and capillariid eggs were molecularly confirmed to be T. canis and Eucœlus aerophilus (syn. Capillaria aerophila), i.e. 100% homology with sequence AB110034 and KC341988 deposited in the Genbank™ database, respectively. Monospecific infections were found in eight (34.78%) samples: E. aerophilus (21.74%) being the most frequent, followed by Ancylostoma/Uncinaria, T. vulpis and T. canis, all with a proportion of 4.34%. Polyspecific infections were found in 15 samples (65.21%), 12 of which scored positive for two parasites and three for three parasites at the same time. The most frequent association was represented by Ancylostoma/Uncinaria and E. aerophilus (39%) (Table 1).

Bears

Thirty-seven (46.25%) out of the 80 faecal samples were positive for parasite eggs (Table 1). Roundworm, capilliard and Ancylostoma/Uncinaria eggs were identified. Roundworm and capillariid eggs were molecularly confirmed to be Baylisascaris transfuga and E. aerophilus (syn. Capillaria aerophila), i.e. 100% homology with sequence AB125694 and 100% homology with KC341988 deposited in the Genbank™ Database respectively.

Of the 37 positive samples, 14 (37.8%) were positive for B. transfuga eggs; of these, six (16.21%) were positive also for Ancylostoma/Uncinaria, one for E. aerophilus and one (2.7%) for both. Of the other positive samples, nineteen (51.35%) were positive for Ancylostoma/Uncinaria, two (5.4%) for E. aerophilus, and two (5.4%) for both E. aerophilus and Ancylostoma/Uncinaria (Table 1).

Discussion

The present results show that wolves and bears living in the national parks of central Italy are infected with different nematodes, some of which have zoonotic potential. As parasites found in examined wolves and bears have a direct life cycle, the study of parasitic communities in wildlife can provide valuable information on the health status, dietary habits, and other ecological aspects of these animals, such as their home range and population dispersion: the high number of parasites being an indication of a large home range and wide dispersion in the habitat.

The nematodofauna recorded from the wolves in the Abruzzo Region is similar to that described in other countries from Eastern Europe, such as Latvia [13]; in addition, the rate of infection by the most frequent parasites, i.e. E. aerophilus and hookworms, are in accordance with another study performed in Poland [22]. This is the first report of E. aerophilus in wolves from Italy, this parasite being previously recorded in wolves from Poland [22] and Latvia [13]. Importantly, this nematode also infects the lungs of a range of domestic and wild animals and, sometimes, people [61]. In the present study, T. canis and T. vulpis eggs were found with a lower percentage in comparison with rates recorded for hookworms, as reported previously [19]. While the prevalence of T. canis agrees with data from other European countries, i.e. Greece [62], Belarus [63], Poland [21] and Spain [23], the rates recorded for T. vulpis are lower than those recorded in eastern countries, e.g. Poland (38.5%) [21].

Infection of wolves with the nematode species reported here could result from direct transmission from other wild or domestic canids (e.g. T. vulpis, E. aerophilus) [64] or, in the case of Toxocara, through predation of infected small animals acting as paratenic hosts [65]. No taeniid eggs were found in the wolf faeces; however, this cannot suggest a true negativity to these parasites. In fact, previous studies based on necroscopic or genetic examinations have shown that the Italian wolf may act as a definitive host of many taeniids, i.e. Taenia spp. and Echinococcus granulosus [8,19,66]. The
absence of tapeworm eggs in the samples can be attributed to the fact that cestode occurrence is often underestimates by conventional microscopy in comparison with other more sensitive methods. Therefore, infection by tapeworms in wolves from the studied areas should not be ruled out, especially considering that the examined geographic areas are endemic for *E. granulosus* [8], a tapeworm that can infect various canids, including wolves, and can cause potentially fatal infections in humans.

Few reports are available on the distribution of parasites of brown bears in wild territories of Europe. The present results confirm the frequent occurrence of *B. transfuga* in brown bears, as recorded in previous surveys in Italy [53] and Croatia [67]. While it is possible that samples from the same animal were examined, resulting in an overestimation of the infection rate, the values could also be underestimated due to possible false negative results at the copromicroscopy. The prevalence of ascarids based on faecal examinations is, in general, significantly lower than the real prevalence at necropsy [22]; in fact, this technique has some limitations, including variable sensitivity depending on parasite species [68], the inability to identify eggs morphologically beyond family or genus level [69], and is beset by potential temporal mismatches, i.e. the samples might be collected during the prepatent period [70] or outside egg excretion peaks, when the number of eggs excreted is markedly reduced [71].

Infected bears often eliminate a large number of eggs in the environment [44]; therefore, the high number of samples that scored positive for *B. transfuga* indicate that bears may highly contaminate the area where they live and may potentially represent a threat for human health. In fact, until the role of *B. transfuga* as a zoonotic agent is ultimately clarified, this parasite should be considered as a potential threat for people. The presence of *Ancylostoma/Uncinaria* and *E. aerophilus* is also not surprising as these parasites have already been found in species of bears in Europe and other parts of world [31,34,72].

In the present study, wolves and bears were positive to *T. canis, B. transfuga* and *E. aerophilus*, i.e. nematodes able to cause intestinal and respiratory diseases in animals and with zoonotic potential. Humans become infected by ingesting larvated ascarid eggs from the environment, the eggs hatch in the intestine and the migrating larvae cause a number of clinical syndromes, e.g. *ocular* and *visceral larva migrans*, especially in toddlers and children [73–75]. In addition, when swallowing infective eggs of *E. aerophilus*, humans may display an infection characterized by fever, bronchitis, cough, haemoptysis and dyspnoea, and which can mimic bronchial carcinoma [76].

The present survey provides an update on the occurrence of parasites in free-ranging wolves and bears living in two national parks in central Italy. The identification of parasites with a zoonotic potential indicates the risk of transmission, for tourists, especially children, and local workers in different National Parks. Educational programs for the prevention of zoonotic parasitoses should be implemented to protect visitors in the National Park and continuous monitoring of areas potentially contaminated with wolf and bear faeces should be initiated. Also, veterinary control programs should be implemented to reduce the parasitic burden on wildlife to achieve improvements in animal health and welfare.

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Helminth infections in faecal


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