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RESEARCH ARTICLE

Endoparasites of the European ground squirrel (Spermophilus citellus) (Rodentia: Sciuridae) in central Macedonia, Greece

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The European ground squirrel (Spermophilus citellus) is a small rodent categorized as vulnerable (IUCN). To investigate the parasitic fauna of this species in Greece, faecal samples from 125 animals belonging to six different populations were examined by standard parasitological methods. Parasites were found in 118 of the animals (94.4%). Oocysts of the coccidia Eimeria callospermophili were found in 92 animals (73.6%), Eimeria citelli in 76 (60.8%), Eimeria cynomysis in 41 (32.8%), Eimeria spp. (17.6%), Cryptosporidium spp. in 29 (23.2%), cysts of Entamoeba spp. in 32 animals (25.6%) and eggs of the trematode Brachylaima spp. in seven animals (5.6%). This is the first report of Entamoeba spp., Cryptosporidium spp. and Brachylaima spp. in S. citellus. The possible impact of these findings on the health status of S. citellus and the possible significance to domestic animals or public health is discussed.

Keywords: Spermophilus citellus; parasites; protozoa; trematoda; Greece

Introduction

The European ground squirrel or European souslik (Spermophilus citellus) is a small mammal belonging to the order Rodentia and the family Sciuridae. It is a diurnal animal, living in colonies of individual burrows, usually in short grass open habitats (Kryštufek and Vohralik 2005). The European ground squirrel is endemic to central and southeastern Europe (Figure 1) (Kryštufek 1999; Kryštufek and Vohralik 2005; Ozkurt et al. 2005), reaching its southernmost range in northern Greece, and has been categorized as vulnerable according to the International Union for the Conservation of Nature (Coroiu et al. 2008).

Although the ecology, morphology and physiology of the European ground squirrel have been adequately studied (e.g. Fragedakis-Tsolis 1977; Kryštufek 1993, 1995; Huber et al. 1999; Millesi et al. 1999; Hut et al. 2002; Katona et al. 2002; Ozkurt et al. 2002; Hoffmann et al. 2003; Everts et al. 2004; Vaczi et al. 2006; Youlatos et al. 2007), investigations concerning the parasites of this species are scant (Stefanov et al. 2001; Golemansky and Koshev 2007, 2009). However, the role of parasitism in wildlife could be of great importance, especially in peripheral populations that are seriously

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declining. This is the case for the species in northern Greece, where populations are isolated by intense urbanization and habitat degradation, allowing little or no contact, between previously adjacent units. Such units are subject to inbreeding and vulnerable to decline and extinction. Therefore, their welfare and health are of utmost importance for their protection and conservation.

In this context, the aim of the present study was to investigate the endoparasitic fauna of different populations of *S. citellus*, for the first time in Greece, to enrich our knowledge of the species’ health status and of potential threats to its conservation, as well as to consider any potential consequences of its parasites for domestic animals or public health.

**Material and methods**

**Study sites and animals**

The current study was confined to central Macedonia, Greece, where most of the populations of the species currently occur. For technical reasons, we initially located colonies of *S. citellus* in areas within the prefecture of Thessaloniki. The colonies
were identified by the presence of characteristic burrow entrances with signs of activity around them (freshly excavated soil, food remains and droppings). In this way, we identified and sampled six different populations from an equal number of areas from central Macedonia (Figure 2). These populations were selected mainly because the distance between them, as well as natural (e.g. deep rivers, forested areas) and/or anthropogenic (e.g. highways, frequently used roads, extended urban areas) barriers assured their differentiation. The number of sampled burrows per population varied between 5 and 37 (Table 1).

**Faecal samples**

Faecal samples were collected from the entrances of burrows. All the faecal pellets of a single entrance were considered an individual sample and were collected in a latex glove. As each burrow is used exclusively by one animal, each sample was considered to correspond to a single individual. For every burrow sampled, the exact location was recorded, using a portable GPS (Garmin etrex, Garmin Inc., Olathe, KS, USA).
Table 1. *Spermophilus citellus* sampling sites, number of burrows examined from each site and parasites found in each site.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>No. of burrows sampled</th>
<th>Parasites</th>
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<tbody>
<tr>
<td>1. University Agriculture Farm</td>
<td>36</td>
<td><em>Eimeria callospermophili</em> E. citelli E. cynomysis Cryptosporidium spp.</td>
</tr>
<tr>
<td>2. C Army Veterinary Hospital</td>
<td>33</td>
<td><em>E. callospermophili</em> E. citelli E. cynomysis Cryptosporidium spp.</td>
</tr>
<tr>
<td>6. Aggelochori salt pits</td>
<td>5</td>
<td><em>E. callospermophili</em> Entamoeba spp.</td>
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**Faecal examination**

All faecal samples were examined using standard parasitological methods, i.e. zinc sulphate flotation, Telemann sedimentation and Ziehl–Neelsen-stained smears as described before, with minor modifications (Henriksen and Pohlenz 1981; MAFF 1986; Thienpont et al. 1986). Initially the faecal material of each burrow was well mixed and homogenized. For the zinc sulphate flotation method, approximately 1 g of the homogenized faecal material was diluted with tap water and passed through a sieve (No. 150) in a centrifuge tube. The tube was centrifuged at 1500 rpm for 3 min, then the supernatant fluid was discharged down to approximately 1 cm above the sediment and zinc sulphate (ZnSO$_4$ · 7H$_2$O) solution 33.2% (weight/volume) was added to the sediment. After thorough dilution of the sediment, zinc sulphate solution was
added to just over the top of the tube and a cover slip was placed on the top of the tube. After centrifugation at 900 rpm for 1 min, the cover slip was carefully removed, placed on a microscope slide and examined under the optical microscope at 100× and 400× magnification. For the Teleman sedimentation method, approximately 1 g of the homogenized faecal material was diluted in HCl 16%, passed through a sieve (No. 150) in a centrifuge tube, 5 ml ether was added and the content of the tube was homogenized by vigorous shaking. After centrifugation at 1500 rpm for 3 min all the phases of the centrifuged material but the sediment were discharged. Drops of the sediment were examined under the optical microscope at 100× and 400× magnification. For the Ziehl–Neelsen-stained smears, faecal smears were prepared on glass slides, fixed by passing over the flame of a Bunsen burner and covered with carbol-fuchsin solution that was kept warm for 5 min. The smears were then decolourized with alcohol–acid mixture, rinsed with tap water and finally covered for 1–2 min with Malachite green. After a last rinse with tap water, the smears were allowed to dry and then examined under the microscope with oil lens (1000×) for the detection of Cryptosporidium spp. oocysts.

The identification of the parasites found was based on the morphology of their reproductive elements in the faeces (Levine and Ivens 1990; Duszynski et al. 2001; Haralabidis and Diakou 2001). The identification was feasible to species level for some parasites and to genus level for others. Identification to species level for all parasites would require either adult parasites (after necropsy) or molecular techniques, both being beyond the scope of the present study.

Sporulation of coccidian oocysts
The faecal samples that contained unsporulated coccidian oocysts were allowed to sporulate to fully develop their morphological characteristics, necessary for identification of the species. For that purpose, the faecal material was left in a solution of 2.5% (weight/volume) aqueous potassium dichromate (K2Cr2O7) at room temperature and checked every day for the completion of the sporulation (Boch and Supperer 1992).

Results
Parasites were found in 118 of the 125 sampled animals (94.4%). Coccidia of the genus Eimeria were detected in 116 of the animals (92.8%). More precisely, Eimeria callospermophili was found in 92 animals (73.6%), Eimeria citelli in 76 animals (60.8%), Eimeria cynomysis in 41 animals (32.8%) and two unidentified species of the genus Eimeria in 22 animals (17.6%). In addition, two more protozoan genera were found: cysts of Entamoeba spp. in 32 animals (25.6%) and oocysts of Cryptosporidium spp. in 29 animals (23.2%). Finally, eggs of the trematode Brachylaima spp. were found in seven animals (5.6%).

All the sampled colonies were infected with parasites. Mixed infections with more than one parasite species were common with various combinations of parasites. The most prevalent was the E. callospermophili and E. citelli co-infection, found in 34 animals (27.2%). The greatest variety of parasites, i.e. E. callospermophili, E. citelli, E. cynomysis, Cryptosporidium spp., Entamoeba spp. and Brachylaima spp., was found in two animals (1.6%). The parasites found in the different sampling areas
are presented in Table 1. In 20% of the *Eimeria*-positive samples, the oocysts were in abundance in the optic field (> 20 oocysts/o.f. in ×100 magnification).

**Discussion**

The range of the European ground squirrel is divided by the Carpathian Mountains into two main basins: the Pannonian and the Balkan. The northwestern area of its distribution (Pannonian basin) covers parts of the Czech Republic, Austria, Slovakia, Hungary, northern Serbia, Montenegro and western Romania, while the southeastern area of its range (Balkan basin) extends into parts of southern Serbia, Former Yugoslav Republic of Macedonia, northern Greece, Bulgaria, southern Romania, Turkish Thrace, Moldova and Ukraine (Panteleyev 1998; Kryštufek 1999).

Despite its relatively large range in central, eastern and southern Europe, population numbers appear to have rapidly declined by > 30% in the last 10 years, with regionally extinct populations in Croatia and Germany and successful reintroductions in Poland (Coroiu et al. 2008). However, Matějů et al. (2010) showed that most reintroduction projects in central Europe had little success. Given the current population status of the species across Europe and its range, Greek populations could be especially important, as they are found at the periphery of the southern border of the range of the species, and there is evidence of population decline in this area too (Youlatos 2009). Therefore, the health of these peripheral populations is fundamental for their survival, as well as for future action plans on protection and conservation of the species across Europe. In this context, parasitism is directly related to the health of established populations and therefore, identifying and evaluating the parasitic load is important in understanding the health robustness of a given population.

There are three main points when evaluating parasitism in wild animals: (1) the effect and importance of the parasites on the animal itself, (2) the importance of these parasites to domestic animals and, (3) the possible impact on public health (Choquette 1956). In free-living animals, parasites are almost always present but apparently in most cases are doing little damage. However, under certain circumstances the host–parasite relationship can be disturbed and then a clinical condition develops. Such circumstances may be malnutrition, extreme weather conditions and co-existence of other pathogens. In cases of vulnerable or threatened animal populations, the role of parasites becomes even more significant.

In the present study, the most prevalent parasites of *S. citellus* were the coccidian protozoa of the genus *Eimeria* (92.8%). The high percentage found in Greece is in accordance with the results of previous similar studies. In the Czech Republic and Slovakia coccidian oocyst were found in all (100%) of the examined European ground squirrels (Kvičerová 2008). In Bulgaria, *Eimeria* infection was found in 88.05% of *S. citellus*, with the most prevalent species being *E. citelli* (92.7%) and *E. callospermophili* (66.6%) (Golemansky and Koshev 2009).

Levine and Ivens (1990) described 19 species of *Eimeria* in *Spermophilus* spp. Of these species, *E. callospermophili* seems to be particularly common for the genus. Apart from *S. citellus*, this parasite has been reported in *Spermophilus xanthophrymnus* in Turkey (Ciçek et al. 2010), in *Spermophilus richardsonii*, *Spermophilus townsendii*, *Spermophilus lateralis* and *Spermophilus elegans* in North America (Stanton et al. 1992; Seville and Stanton 1993; Wilber et al. 1994) and in *Spermophilus parryii* in Alaska and Siberia (Seville et al. 2005).
The identification and taxonomy of *Eimeria* spp. has traditionally been based on the morphology of the sporulated oocyst and the host where the examined species is found (Levine and Ivens 1990). In the present study, apart from the three identified *Eimeria* species, two more types of oocysts with different morphologies were detected in 22 animals. Unfortunately, the number of morphological characteristics of *Eimeria* spp. is limited whereas the number of species is large (Motriuk-Smith et al. 2011). Development and application of molecular methods for the precise identification of these parasites are planned and would solve this problem.

*Eimeria* is a protozoan parasite with a direct life cycle that invades the epithelial cells of the small and large intestine where it reproduces. There are no data in the literature about the pathogenicity of *Eimeria* in European ground squirrels. Generally, coccidiosis in sciurids seems to cause few or no clinical signs (Joseph 1975; Cohn et al. 1986; Sainsbury and Gurnell 1995). However, in red squirrels (*Sciurus vulgaris*) signs such as loss of general condition, loss of appetite, diarrhoea and even a fatal outcome have been reported (Pellerdy 1954, 1974).

The immune response of *Spermophilus* spp. to *Eimeria* infection has not been clarified. There is evidence that immunity depends on the species of both the parasite and the host. Moreover, the fact that juveniles are infected more than adults due to age-developed immunity has not been proven (Wilber et al. 1994). However, Golemansky and Koshev (2009) found heavier infection with *E. callospermophilii* and *E. cynomysis* in subadult and juvenile *S. citellus* and Seville and Stanton (1993) found greater variety of *Eimeria* species in juvenile *S. richardsonii* than in adults. Taking into consideration, on the one hand, the high prevalence and heavy infection with *Eimeria* spp. found in the present study, and on the other hand, the potential pathogenicity of the parasite and the evidence of declining populations of *S. citellus* in Greece, it can be suggested that this infection could represent a threat for ground squirrels and a risk for the stability of the populations in the study area. More investigations towards this end are currently underway, as this may affect populations of the species that inhabit generally favourable habitats in central Greece.

*Cryptosporidium* is another genus of the subclass Coccidia. It has a direct life cycle and the host becomes infected by ingestion or inhalation of the oocysts found in food, water and the environment in general. It is a parasite of the epithelial cells of the gastrointestinal tract (less commonly of the respiratory tract) and is an important cause of gastrointestinal illness for humans and animals (Dubey et al. 1990). Without having strict host specificity, *Cryptosporidium* species are recognized to differ principally in their host range but some genotypes are considered zoonotic (Hunter and Thompson 2005).

In the present study, *Cryptosporidium* spp. was found in 23.2% of the examined animals. To the authors’ best knowledge, this is the first report of this parasite in *S. citellus*. However, 35.5% of the *S. suslicus* examined by direct immunofluorescence assay in Poland were shedding *Cryptosporidium* oocysts (Kloch and Bajer 2012). Moreover, *Cryptosporidium parvum* has been found in 16% of the *Spermophilus beecheyi* examined in California (USA), where two different genotypes were circulating within a single host population (Atwill et al. 2001). In a more recent survey, the parasite was found in two more *Spermophilus* species (*Spermophilus beldingi* and *Spermophilus lateralis*) in California and the phylogenetic analyses of the isolates revealed that *Spermophilus* squirrels shed novel *Cryptosporidium* species (Pereira et al. 2010). As cross-species transmission of *Cryptosporidium* spp. is a fact (Monis and Thompson 2003), molecular
characterization of isolates of this parasite would be useful in the investigation of the possible transmission of the *S. citellus* genotypes to other animal species or humans. *Entamoeba* is also a protozoan parasite with a direct life cycle. It parasitizes the intestines of animals and humans and includes non-pathogenic and pathogenic species. The reports of *Entamoeba* infection in sciurids are scant. The species reported to infect *Spermophilus* are *Entamoeba muris* and *Entamoeba citelli*. The cystic form of *Entamoeba citelli* (Becker 1926) is about 15 μm in diameter with eight nuclei and a thick cystic wall. In the present study, the *Entamoeba* sp. cysts, found in morphologically good condition in very dry faeces, had all the characteristics described for *Entamoeba citelli*. This parasite has also been reported in *S. tridecemlineatus*, *S. townsendii*, *S. beldingi* and *S. lateralis* (Becker 1926; Davis 1969; Rickard 1987), and this is the first report for *S. citellus*. The significance of this parasite to the health status of the sciurids is not known, as there are no relative published data.

*Brachylaima* is a trematode parasite of the intestines of mammals and birds. It has an indirect life cycle with land snails and slugs as first and second intermediate hosts (Yamaguti 1958; Butcher 2003). This parasitic trematode has been previously reported from two species of the family Sciuridae, *Atlantoxerus getulus* (López-Darias et al. 2008) and *Sciurus carolinensis* (Kennedy 1988). To our knowledge, this report is a new host record for *Brachylaima* spp. This trematode infection is related to the fact that *S. citellus* often feeds on land snails (Coroiu et al. 2008), which act as an intermediate host. This parasite was found only in Axios – eastern coast (site No. 3; Figure 2), where seven of the 19 examined animals (36.8%) excreted eggs of this parasite in their faeces. Although the investigation of the epizootiology of the parasite is beyond the aim of the present study, the fact that this infection was limited to only one population is interesting. This isolated occurrence could be due to incidental infection of the particular population, failure of infection transmission because of short dispersal movements, and/or predation of higher numbers of snails acting as intermediate hosts (Helicidae) compared with the rest of the sampling sites. Since the species *B. criibi* has been incidentally identified in humans, in Australia (Butcher and Grove 2001), its presence in the site requires further investigations related to human health.

The small number of helminth parasites found in *S. citellus* is consistent with the general findings in sciurids, which indicate that this family is parasitized at a lower level than predicted (Morand and Poulin 1998). Moreover, the relatively limited variation of the parasitic fauna found in the present study could be attributed to the fact that our study was restricted to non-invasive faecal examination. Eventually, necropsies would reveal more parasitic species, but euthanasia is not an option, because of the vulnerable status of *S. citellus*. Reports of helminth parasites in *S. citellus* are scant. Stefanov et al. (2001) reported four helminth species, the cestodes *Hymenolepis magaloon* and *Ctenotaenia marmotae* and the nematodes *Streptopharus kutasii* and *Trichostrongylus colubriformis*, from Bulgaria.

Despite the asocial behaviour of *S. citellus*, transmission of parasites is facilitated between individuals due to the density of the colonies, which was relatively high in all the sampled sites. Furthermore, the resistance of the reproductive elements of the parasites to the environmental conditions is also a factor that contributes to the infection of the animals. In Greece, *S. citellus* inhabits low vegetation, relatively dry habitats that are exposed to intense heat and solar radiation, especially during summer months. Under such conditions, particularly resistant helminth eggs and protozoan cysts and
oocysts are vital for their survival. This may explain the very good condition of the parasitic elements found in the present study, despite the dry faeces often examined.

This study provides the first report on the parasitic fauna for S. citellus in Greece. The high prevalence and in some cases heavy infection of the animals with Eimeria should be further investigated to evaluate the significance of the specific parasite to the population health status and conservation. Close monitoring of the animals and regular parasitological examinations would provide additional information towards this end. The species of Eimeria and most probably Entamoeba reported in this study are exclusive parasites of sciurids. In this way, no expected risk for domestic animals and public health exists. On the other hand, Cryptosporidium spp. and Brachylaima spp. may possibly affect other animals and humans. In every case, genotyping of the parasites found in S. citellus would provide data for more accurate identification and would facilitate the investigation of any probable genetic relation to species derived from other wild or domestic animals and humans. Furthermore, similar investigations on hosts that share the same habitats with S. citellus would be necessary for any consideration of actions plans for the conservation of S. citellus in Greece and across Europe.

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