



INFECTIONS WITH *DICROCOELIUM DENDRITICUM*,
CYSTICERCUS PISIFORMIS, *MICIPSELLA NUMIDICA* AND
SEROPREVALENCE OF *TOXOPLASMA GONDII* IN EUROPEAN
BROWN HARES (*LEPUS EUROPEUS* L.) FROM BULGARIA

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Summary

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During the period 2009–2016, brown hares (*Lepus europeus* L.) from different regions of Bulgaria were investigated for infections with liver parasites as well as for seroprevalence of protozoan *Toxoplasma gondii*. Three helminth species were found in the liver: one trematode – *Dicrocoelium dendriticum*, one cestode – larval form of *Taenia pisiformis* – *Cysticercus pisiformis* and one nematode – *Micipsella numidica*. Dicrocoeliasis was found in all parts of the country with infected hares originating mainly from the western regions. The total prevalence of infection with *D. dendriticum* was 10.59%. The intensity of infection ranged from 1 to 184, 26.67 on the average. The total prevalence of infection with *C. pisiformis* was 16.8%. The prevalence of infection was highest among hares from the southern parts of the country, and no cysticercosis was registered in the western parts. The extent of infestation with cysticerci was different – from single cysticerci on the omentum or serosa of some organs in the abdominal cavity to innumerable ones that affected also the organs in the thoracic cavity. However, cases in which the animals were slightly affected, predominated. The total prevalence of infection with *M. numidica* was 5.31%, and the intensity of infection varied between 1 and 6 parasites in one animal. The nematodiasis was established mainly in hares from southern Bulgaria. The total seroprevalence of *T. gondii* was 6.15% and the protozoan-positive animals originated from three districts: Stara Zagora, Plovdiv, and Burgas. The current studies also showed that the prevalence of *D. dendriticum* infection among animals increased with age. No correlation was observed between prevalence of infection with *C. pisiformis* and *M. numidica* and host age. No correlation was found between the prevalence of the helminth infections and sex of the animals.

Key words: Bulgaria, *Cysticercus pisiformis*, *Dicrocoelium dendriticum*, European brown hare, *Micipsella numidica*, *Toxoplasma gondii* seropositivity

INTRODUCTION

The European brown hare (*Lepus europaeus* L.) is listed as species of Least Concern by the International Union for Conservation of Nature and Natural Resources. However, its population in mainland Europe is declining. In Bulgaria, the number of hares has decreased permanently in recent years. In 2012, the number of brown hares in the country was about 34% of this in 1974 and the use of hares as hunting objects was reduced from 5.9% in 2004 to 3.7% in 2011 (Trifonova & Mirchev, 2013). There are different reasons for reduced populations of the European brown hare. Many researchers believe that this is due to changes in farmland management practices, resulting in the loss of crop/landscape diversity affecting nutrition (Edwards *et al.*, 2000).

Various diseases, including parasitoses, also have a negative effect in this regard. The spreading of parasitic diseases in the animals reveals a number of negative effects. On one hand massive infections with certain dangerous species can cause death to the affected animals and on the other the parasites could appear as a pre-disposing factor for the development of secondary deficiency and infectious diseases.

The present study is a part of extensive research on the problems and prospects for sustainable development of European brown hare, in which hares from all over the country were studied (Zhelev, 2015).

Our aim was to determine the infection with liver helminths and seroprevalence of the protozoan parasite *Toxoplasma gondii* in this game species, as well as to study some epidemiological aspects of the respective parasitoses.

MATERIALS AND METHODS

A total of 1,161 brown hares were admitted to the National Research Station of Game Management, Biology and Pathology for various studies in the period 2009–2016. Of these, 868 were investigated for *Dicrocoelium dendriticum*, 1,024 for *Cysticercus pisiformis*, 866 for *Micipsella numidica*, and 65 for presence of antibodies to *Toxoplasma gondii*.

The animals were shot on the territory of various hunting enterprises from 25 districts of the country: Blagoevgrad, Burgas, Varna, Veliko Tarnovo, Vidin, Vratsa, Dobrich, Kardzhali, Lovech, Montana, Pazardzhik, Plevan, Plovdiv, Razgrad, Ruse, Silistra, Smolyan, Sofia, Sofia region, Stara Zagora, Targovishte, Haskovo, Shumen and Yambol. Hares from twelve districts (Blagoevgrad, Burgas, Vidin, Vratsa, Lovech, Pazardzhik, Plevan, Plovdiv, Silistra, Sliven, Stara Zagora and Yambol) were studied for seroprevalence of *T. gondii*.

The age of the animals was determined as described by Zhelev (2015) and according to this they were divided into young – up to 1 year, middle-aged – from 1 to 3 years and adult – over 3 years.

Nematode infections were detected by conventional methods for complete or partial helminthological necropsy (Koinarski *et al.*, 2009). Helminths found were collected and cleaned in saline solution and after that stored in 70% ethanol. They were studied after clearing in lactophenol. The identification of the helminths was carried out on the basis of their localisation and morphological characteristics.

Extent of infection with *C. pisiformis* was subjectively classified according to the dissemination and number of cysticerci as followed: weak (single cysticerci

on the omentum or serosa of some of the organs in the abdominal cavity, up to 10), mild (more cysticerci on the omentum or serosa of some of the organs in the abdominal cavity, which can be counted, up to 100), strong (a lot of cysticerci in the abdominal cavity, cannot be counted), massive (countless cysticerci that affect both the organs in the abdomen and chest cavity).

Blood samples from 65 hares were obtained *post mortem* and sent to the laboratory for serological testing for *T. gondii* antibodies. Blood sera were separated following centrifugation immediately after their receipt in the laboratory and stored at -20°C until their study. Serum samples were tested for the presence of specific *T. gondii* IgG antibodies in the passive haemagglutination test (PHAT) with toxoplasmic erythrocyte diagnosticum (commercial kit BUL BIO – Ltd. Bulgaria), according to the manufacturer's instructions. Samples with an agglutination from 4+ to 2+ with a dilution of the sera over 1:10 were considered positive.

The data obtained from the studies were processed with MS Excel software.

RESULTS

During the necropsies, three types of helminths were found in the liver of hares: one trematode – *D. dendriticum*, one cestode – the larval form of *Taenia pisiformis* – *C. pisiformis* and one nematode – the filaria *M. numidica*.

Dicrocoelium dendriticum

The lancet liver fluke was found in the small bile ducts of the liver. The total prevalence of infection with it was 10.59%. The prevalence of the infection in the various administrative districts of

the country is shown on Fig. 1. The highest prevalence (over 20%) was found in the districts of Lovech, Sofia region, Smolyan, Vidin and Burgas. Between 10% and 20% was the prevalence of this parasitic infection in brown hares from the districts of Vratsa, Razgrad, Dobrich, Varna, Yambol and Pleven. The prevalence of dicrocoeliasis was low (less than 10%) among hares from the districts of Blagoevgrad, Stara Zagora, Pazardzhik, Sliven, Plovdiv, Silistra and Haskovo.

The prevalence of dicrocoeliosis in the different regions of the country was as followed: 6.23% for southcentral Bulgaria, 9.85% for northeastern, 11.29% for northcentral, 13.5% for southeastern. The highest prevalence of infection was found in southwestern (17.24%) and northwestern (20%) Bulgaria.

The intensity of *D. dendriticum* infection ranged from 1 to 184 trematodes in one animal, with a mean intensity of 26.67. In 39.6% of hares the intensity of infection varied from 1 to 10 specimens in animal, in 20.9% – from 10 to 20 specimens, in 18.7% – from 20 to 30 specimens, in 4.4% – from 40 to 50 specimens, in 3.3% – from 50 to 60 specimens. Intensity of infection with 30–40, 60–70, 70–80, 100–110, 180–190 specimens was found in 2.2% of the hares. In 1.1% of the hares, 110–120 and 160–170 parasites per animal were found.

Cysticercus pisiformis

The total prevalence of *C. pisiformis* infection was 16.8%. Cysticercosis of different prevalence was found in 10 districts: Burgas, Veliko Tarnovo, Dobrich, Pazardzhik, Plovdiv, Silshstra, Sliven, Stara Zagora, Haskovo and Yambol (Fig. 2). The prevalence of infection was highest among brown hares in the southern parts of the country – 26.5% in

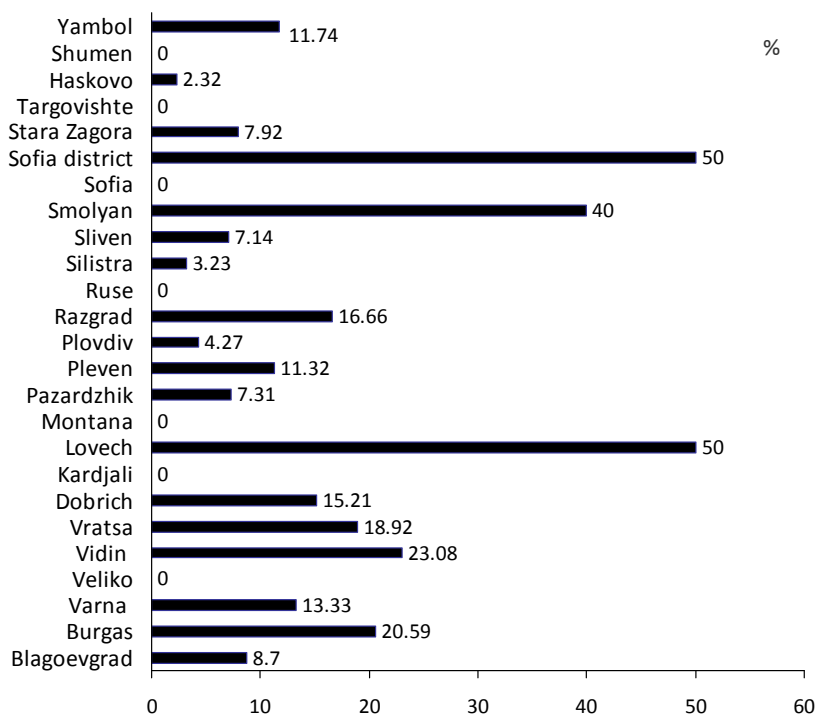


Fig. 1. Prevalence of *Dicrocoelium dendriticum* infection in hares from different districts of Bulgaria.

southeastern, and 21.45% in southcentral regions. It was 5.65% in northeastern and 1.28% in northcentral Bulgaria. In the western parts this parasitosis was not recorded.

In most cases (47.09%) only single cysticerci were observed on the omentum or serosa of some of the organs in the abdominal cavity, thus according to accepted subjective criteria for assessing the extent of infection, it was defined as weak. In 25% of the hares, the infestation was in mild extent. Relatively close was the number of animals with strong (14.53%) and massive (13.37%) infection, in which the cysticerci were in such quantities that their number could not be determined (Fig. 3) and affected even organs in the thoracic cavity (Fig. 4).

Micipsella numidica

Infection with *M. numidica* was found in brown hares from 7 districts of the country: Blagoevgrad, Dobrich, Plovdiv, Sliven, Stara Zagora, Haskovo, and Yambol. The total prevalence of infection with this filarial nematode was 5.31%, and the intensity of infection varied between 1 and 6 parasites in one animal. The parasitosis was found mainly in the hares from southern regions with prevalence 3.31% for southeastern, 9.03% for southcentral, and 24.14% for southwestern region. In northern Bulgaria, *M. numidica* was found only in the northeastern region with a prevalence of 0.76%.

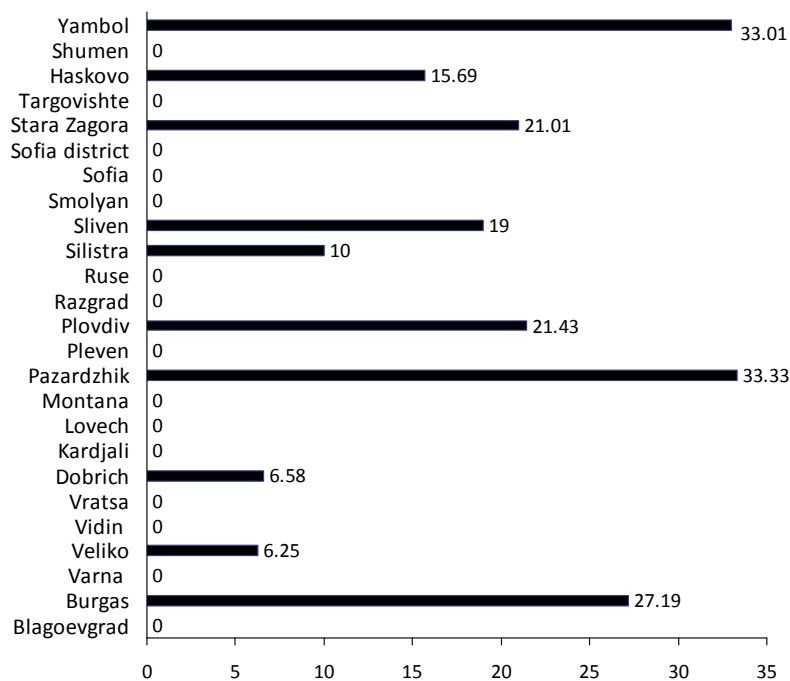


Fig. 2. Prevalence of *Cysticercus pisiformis* infection in hares from different districts of Bulgaria.



Fig. 3. Necropsy of a brown hare with strong *C. pisiformis* infection.

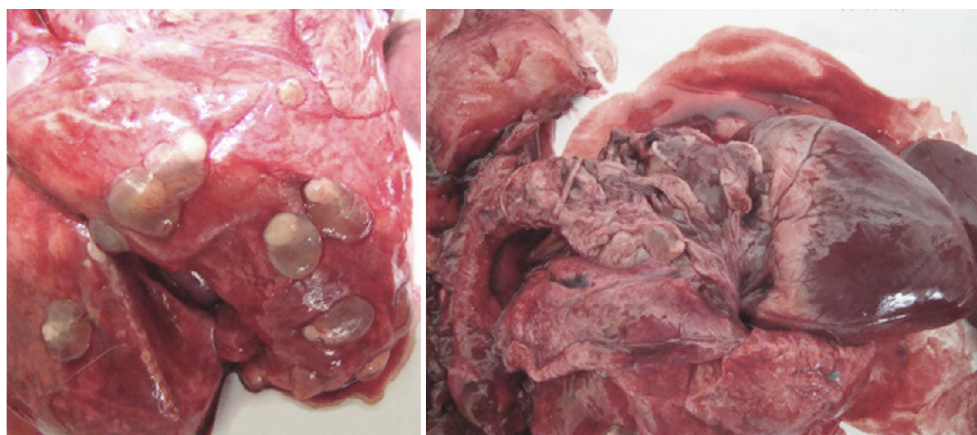


Fig. 4. Thoracic organs of a brown hare affected by massive *C. pisiformis* infection.

Table 1. Results of serological examination of serum samples from brown hares obtained from different regions of Bulgaria

Regions	Number of tested samples	Positive samples	Titre			Seropositivity (%)
			1:20	1:40	1:80	
Blagoevgrad	1	0	0	0	0	0
Burgas	8	1	1	0	0	12.5
Vidin	1	0	0	0	0	0
Vratsa	4	0	0	0	0	0
Lovech	2	0	0	0	0	0
Pazardzik	1	0	0	0	0	0
Pleven	1	0	0	0	0	0
Plovdiv	12	1	0	0	1	8.33
Silistra	3	0	0	0	0	0
Sliven	11	0	0	0	0	0
Stara Zagora	18	2	2	0	0	11.11
Yambol	3	0	0	0	0	0
Total	65	4	3	0	1	6.15

Epidemiological data

Infection with *D. dendriticum* was not found in the juvenile hares. In the middle-age hares, its prevalence was 5.4% and in the adults – 15.85%. Prevalence of infection with *C. pisiformis* was 15.38%, 14.78% and 19.37% in juvenile, middle-age and adult hares, and this with *M.*

numidica was 2.7%, 5.68% and 5.62% respectively.

Prevalence of infection with *C. pisiformis* in male and female brown hares was 17.52% and 17.72%, with *D. dendriticum*: 10.82% and 9.39%, and with *M. numidica*: 5.57% and 5.88% respectively.

Toxoplasma gondii

Examination of 65 serum samples from brown hares showed that four of them had specific IgG antibodies to *T. gondii* with a titre over 1:10. The obtained rate of seropositivity in the group was 6.15%. Hares originating from four hunting regions from three districts of Bulgaria were serologically positive (Table 1).

Agglutination at a test serum titre of 1:20 was obtained in 3 hares (two of them from hunting areas in Stara Zagora, one of them from Burgas). Positive reactions with a serological titre of 1:20 were 4.62% of the total studied. The positive result with the highest serological titre (1:80) was found in a hare from the surroundings of the village Belozem, Plovdiv district. Of the four animals that responded positively to the test, 3 were female and one male.

DISCUSSION

Cases of dicrocoeliasis in European brown hares have been reported in different parts of Europe – England (Bailenger *et al.*, 1965), Lithuania (Kazlauskas & Arnastauskene, 1969), Germany (Nickel & Gottwald 1979), Finland (Soveri & Valtonen 1983), Greece (Diakou *et al.*, 2014). *Dicrocoelium dendriticum* is among the common representatives of the helminthic fauna of the European brown hare in Bulgaria too. This is evidenced by the findings of a number of scientists (Yanchev, 1963a,b; 1970; 1973; Genov, 1973; Tsolov & Gechev, 1990; Todev *et al.*, 2003). According to the more recent data (Todev *et al.*, 2003), this parasitosis was found in the region of Pernik in more than half of the studied brown hares with a mean intensity of infection of 19.66. These results are close to those estab-

lished by us in Sofia region, which confirms the widespread prevalence of dicrocoeliasis among brown hares in western Bulgaria.

Pisiform cysticercosis is also a common helminthosis among the brown hare in Europe. It has been registered in Germany (Nickel & Gottwald, 1979), Belarus – 22.5% prevalence of infection (Dubina & Subbotin, 2000), Austria (Chroust *et al.*, 2012), Romania – 9.52% prevalence of infection (Hora *et al.*, 2015), Italy (Stancampiano *et al.*, 2019). In our country the parasitosis has been established in different parts of the country (Yanchev, 1963a, b; 1970; 1973; Trifonov & Meshkov, 1964; Genov, 1973). The parameters of infection found among hares in the Burgas district by Trifonov & Meshkov (1964): prevalence of infection from 6.5% to 18.3%, and infection intensity - from 7 to 261 cysticerci are lower than those established by us for the same region: prevalence of infection 27.19%, and so large number of cysticerci in some animals that it could not be counted. Given these facts, attention should be paid to the human factor, such as the responsibility of people for the appropriate disposal of carcasses with established cysticercosis. In this connection, Soveri & Valtonen (1983) explained the absence of cysticerci of *T. pisiformis* in their survey with the impact of improved hygiene practices instituted by hunters.

In Europe, *M. numidica* has been recorded in the European brown hare from the former Czechoslovakia (Erhardova, 1956) and Italy (Gabrielli *et al.* 2015), in the Iberian hare (*Lepus granatensis*) from Spain (Segovia *et al.*, 2014) and in rabbits (*Oryctolagus cuniculus*) from Italy (Cancrini *et al.*, 1988). It has been also found in hares of Central and Eastern European origin, imported to repopulate an area in

the Lyon region of France (Graber & Le-brette, 1971). Until now, in Bulgaria the parasite has been also registered mainly in southern regions (Yanchev, 1963a,b; 1970; 1973). This geographical distribution is most likely due to the more suitable climatic conditions for the development and spread of arthropods – vectors of the parasite in the southern parts of the country.

Our results showed that the prevalence of lancet liver fluke infection increases with the age, which is probably a result of an accumulation of infection with time. However, this tendency was not observed with the other two parasitoses. Keith *et al.* (1986) have also found no relationship between the host age and *C. pisiformis* prevalence. These results are in support of the conclusion of Beck *et al.* (2014) about the complexity and context-dependent nature of epidemiological processes in multi-host systems.

A tendency for similar prevalence rates in both sexes in all three parasitoses was observed. Our results are in agreement with those of Keith *et al.* (1986), affirming that the extensity of *T. pisiformis* infection was neither associated with hosts' sex. Sex of the host had no important role on the prevalence of some parasites, including *M. numidica* in Iberian hares (Segovia *et al.*, 2014). According to Kornaś *et al.* (2014) the frequency of hares infected by nematodes vs. non-infected ones was also similar in both sexes.

The established *T. gondii* seroprevalence among hares in present study (6.15%) was close to rates found in our southern neighbour Greece – 5.7%, Slovakia – 6% and some dry regions in Spain – 6.1% (Almeria *et al.*, 2004; Bártova *et al.*, 2010; Tsokana *et al.*, 2019). It was significantly lower from those reported from Germany – 46%, Czech Republic – 21%, Austria – 13% and forest areas in

Spain – 14.2% (Frölich, 2003; Bártova *et al.*, 2010; Almeria *et al.*, 2004). Our data show lower prevalence of *T. gondii* among brown hares in our country in comparison with the levels established by Nankov (1968): 15.7% and Nankov (1971): 14.8%. The difference is probably due to the long period of time between the two surveys, methods of testing, changes in the game management practice, and a serious reduction of the number of this game species in Bulgaria. It should be remembered that hares infected with *T. gondii* can be a potential source of toxoplasmosis for other animals, especially carnivores but also for humans (Tsokana *et al.*, 2019). Hunters should be aware of *T. gondii* infection and advised to take precautions when skinning hares and cook or freeze meat before consumption.

CONCLUSIONS

The infestation of the European brown hare with *D. dendriticum* is widespread in Bulgaria. The trematode is found in most of the studied regions. It can be assumed that the European brown hare is one of the factors for maintaining the infection of lancet liver fluke in nature and its spreading in our country.

Pisiform cysticercosis is a common helminthosis among brown hares in Bulgaria. In most cases, the animals were slightly affected – single cysticerci on the omentum or serosa of organs in the abdominal cavity were observed.

Infection with *M. numidica* was registered only in 7 of the studied 25 districts of the country. Affected animals were mainly from the southern regions, probably due to the geographical and climatic factors in these areas that favoured the development of insects – vectors of this filarial parasite.

The seroprevalence of *T. gondii* among brown hares in the country was low. However, it should be borne in mind that this game species may be a potential source of toxoplasmosis for other animals and humans.

The prevalence of *D. dendriticum* infection increased with age of the hosts. No correlation was observed between prevalence of infection with *C. pisiformis* and *M. numidica* and the host age. There was no sex-related correlation between the prevalence of infections with *D. dendriticum*, *C. pisiformis* and *M. numidica* among brown hares.

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