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Parameters of protostrongylid infection of terrestrial snails on pastures used by chamois (Rupicapra rupicapra balcanica Bolkay, 1925) in the Western Rhodopes and Pirin Mountains

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Abstract. During the period 2018-2020, studies were conducted to determine the parameters of protostrongylid infection in intermediate hosts in pastures in the Western Rhodopes and Pirin Mountains. Of the eleven snail species identified, four were registered as intermediate hosts of protostrongylids. On the studied pastures – Mugla, Zabardo and Vihren SE, protostrongylid larvae were detected in *Xerolenta obvia* at the first and second sites and in *Xerolenta macedonica*, *Cattania polinscii* and *Cattania haberhaueri* at the third site. The differences in the periods of maximum risk of infection observed in the present study and the data of other authors are due to the high altitudes at which the studied pastures are located. The high values of certain infection parameters observed in summer are likely due to the significantly higher amount and frequency of precipitation in the mountainous areas, combined with favorable temperatures, which in a large part of the period are in the optimal range.

Key words: Protostrongylids, terrestrial snails, infection, chamois.

Introduction

The development of scientifically sound methods for the control of parasitic diseases requires systematic and detailed investigation, particularly in the context of current global environmental changes. In wild ungulates and in particular in wild goats, there are no great opportunities for chemoprophylaxis of parasitosis, which puts on the agenda the issue of monitoring and, where possible, controlling mountain pastures with regard to the most important helminthiasis. The importance of this issue is also emphasized by the frequent use of the same pastures by wild and domestic ruminants.

Parasitism with protostrongylids is often associated with the development of concomitant infections with bacterial, viral or fungal pathogens (Cabaret, 1984a).

Protostrongylids have a complex life cycle, which involves terrestrial gastropods as intermediate hosts. Two larval stages develop in the intermediate host, after which they become capable of invading the definitive host (Anderson, 2000; Boev, 1975; Schultz & Gvozdev, 1972). In Bulgaria, studies on the intermediate hosts of protostrongylids have been conducted mainly in domestic sheep and goats (Georgiev, 2020; Samnaliev, 1966, 1968; Trifonov, 1972; Zurliiski, 1994). The susceptibility of some snails to protostrongylid invasion was studied by Panayotova-Pencheva (2006a). According to earlier studies outside our country (Cabaret, 1984a,b, 1988; Cabaret et al., 1983, 1985;

Lahmar et al., 1990; Trushin, 1973) and in Bulgaria (Georgiev, 2020), the invasiveness of pastures and the periods with the greatest risk for infection of definitive hosts can be determined by analysing infection parameters in intermediate hosts.

The aim of the present study is to characterize the circulation of protostrongylids in chamois on pastures in the Western Rhodopes and Pirin Mts. and its dependence on environmental parameters, the species composition of intermediate hosts and their population characteristics.

Materials and methods

Field survey

As a result of the field research, two pastures were identified, designated as "Mugla" (N41° 37.843′ E24° 29.801′) and "Vihren SE" (N41° 45.709′ E23° 24.258′). Monthly quantitative collections were carried out on them to characterize the fauna of land snails and determine the population density and dynamics of protostrongylids on chamois in 2019 and 2020. The pastures were identified based on the constant presence of chamois on them; the presence of land snails and established protostrongylid larvae in the intermediate hosts. At a later stage, due to the establishment of a significantly increased density of the chamois population in the area, the pasture around the village of Zabardo (N41° 47.661' E24° 36.236') was also included.

Each pasture was visited monthly for 2 to 4 consecutive days. In the field, six transects were surveyed, which include the different altitudes and pasture characteristics. Each transect covered a strip with a length of 40 m and a width of 2 m. The aim was for each route to pass through different plant formations and associations, which are representative of the characteristic malacofauna of the pasture. The information from each transect was recorded on a GPS device in an appropriate coordinate system. At the same time, a field form was filled out.

All species of snails are collected along the routes, with samples not exceeding 100 individuals. The snails collected from one strip represent one primary sample and are placed in a separate plastic container with ventilation holes.

Laboratory analyses

In laboratory conditions, snails from each primary sample are separated by species and size groups with a step of 2 mm. Snails of each size group were dissected individually under laboratory conditions by carefully crushing the shell between two Petri dishes. Before dissection, the snails are submerged in water in glass flasks. In the case of large species, the leg is dissected and placed between two compression slides (for trichinoscopy). In the case of small species, the entire body is compressed. Specimens were examined under a microscope at 100× magnification.

Statistical analysis

The quantitative indicators of invasion used in this study correspond to the definitions adopted by Bush et al. (1997).

The sample variance was calculated using the formula:

$$s^2 = \frac{\sum(x - \bar{x})^2}{n - 1}$$

The population variance is calculated using the formula:

$$\sigma^2 = \frac{\sum(x - \bar{x})^2}{n}$$

The index of dispersion (ID) is defined as the ratio of the dispersion to the mean (Krebs, 1989).

Statistical data were processed with IPM SPSS Statistics version 26.

Results

Eleven species of terrestrial snails were found on the studied pastures: *Cattania haberhaueri, Xerolenta macedonica, Macedonica marthae, Cattania polinskii, Helix pomatia, Zebrina detrita, Chondrus zebra tantalus, Xerolenta obvia, Cattania rumelica, Caucasotachea vindobonensis* and *Euomphalia strigella.*

On the studied pastures - Mugla, Zabardo and Vihren SE, invasion with protostrongylid larvae was established as follows: in *X. obvia* for the first and second and in *X. macedonica, C. polinscii* and *C. haberhaueri* for the third of them.

The most widespread gastropod species, common to the three pastures, is *Z. detrita*. No protostrongylid infestation was recorded in it.

The second most widespread species, *X. obvia*, is the main host for pastures in the Western Rhodopes. *X. macedonica* is the third most widespread species. It has been identified as the main host of protostrongylid larvae in chamois for Pirin (Table 1). With the present study *C. polinskii* and *C. haberhaueri* are reported for the first time as intermediate hosts of protostrongylids.

Table 1. Invasion parameters by intermediate host species.

Species	N	N_{inf}	P %	I	Iav	A
X. obvia	548	151	27.55	1-19	2.55	0.70
X. macedonica	145	75	51.72	1-15	2.55	1.32
W. polinskii	42	7	16.67	2-3	1.86	0.31
C. haberhaueri	3	1	33.33	1	4.00	1.33

Parameters of protostrongylid infection in *X. obvia*, Mugla pasture: The pasture is located at 1550 m above sea level. Four species of snails have been identified on it, infection with protostrongylid larvae was detected only in *X. obvia* (Table 2). The seasonal density of snails is 0.26 individuals/m^2 in spring, increases to 0.39 individuals/m^2 in summer and decreases to 0.27 individuals/m^2 in autumn. The density of protostrongylids, expressed as the number of invading larvae per unit area, in spring is only 0.09 L_3/m^2, increases to 0.36 L_3/m^2 in summer and decreases to 0.25 L_3/m^2 in autumn.

The dispersion (s^2) of the intermediate hosts in the spring is 1550.33 and the dispersion index (ID) is 37.20 for the sample. Referred to the general population, the dispersion (σ^2) has a value of 1033.55, while ID is 24.80. In the summer, respectively, s^2 =557 and ID = 15.47 for the sample. For the population: σ^2= 461.60 and ID = 12.82. The dispersion of the sample in the autumn season is 670.25 with a dispersion index of 20.46. In the population, the dispersion is 502.69. The dispersion index is 15.35. These values correspond to aggregated distribution in the population of the intermediate hosts.

Invasion parameters of *X. obvia*, Zabardo pasture: This pasture is located in the Kamaka area, near the village of Zabardo at an altitude of 1400 m. The only species registered with invasion is *X. obvia*. Table 3 presents the available data on the invasion parameters of this pasture by month.

The dispersion has a value of 0.071 with a dispersion index of 0.95 in the summer, and in the autumn s^2 is 7.11, and ID = 7.48. Referred to the population, these values are respectively for summer and autumn: σ^2 = 0.069, ID = 0.92 and σ^2 = 7, ID = 7.36. The population density of the intermediate hosts in summer is 0.08 individuals/m^2, and in autumn 0.13 individuals/m^2. The density of nematode larvae is respectively for the two seasons: 0.06 L_3/m^2 and 0.13 L_3/m^2.

Parameters of the invasion of *Xerolenta macedonica*, Vihren SE pasture: Six snail species were recorded on this pasture during the study period, three of which were infected with protostrongylids. The most abundant was *X. macedonica*, which is also the species with the greatest importance for the transmission of protostrongylids. The invasion parameters are presented in Table 4.

The population density of the intermediate host is 0.05 individuals per m^2 in spring, reaches 0.2 individuals per m^2 in summer, and drops to 0.08 individuals per m^2 in autumn. The sample variance is 98 in spring, and the dispersion index is 8.17, 323.67 and 13.21 in summer, respectively, and in autumn the values are 41.33 and 3.35. The density of L_3 is 0.07 larvae per m^2 in the spring season, 0.24 larvae per m^2 in summer and 0.08 larvae per m^2 in autumn.

Table 2. Data on the invasion parameters of *Xerolenta obvia*, Mugla pasture.

Month	N	N_{inf}	P %	I	Iav	A
April	22	3	13.63	1-3	2.33	0.32
May	103	14	13.59	1-3	2.42	0.33
June	20	3	15.00	2-12	2.67	0.40
July	72	36	50.00	1-10	2.78	1.38
August	95	35	36.84	1-5	1.86	0.68
September	93	31	33.33	3-19	3.77	1.26
October	38	4	10.53	1	0.75	0.08

Table 3. Data on the invasion parameters of *X. obvia*, Zabardo pasture.

Month	N	N_{inf}	P %	I	Iav	A
April	-	-	-	-	-	-
May	-	-	-	-	-	-
June	4	0	-	-	-	-
July	13	2	15.38	1	1	0.15
August	23	1	4.35	1	1	0.04
September	23	4	17.39	1-3	1.75	0.30
October	40	13	32.5	1-14	4.08	1.33

Table 4. Data on the parameters of the invasion of *X. macedonica*, Vihren SE pasture.

Month	N	N_{inf}	P %	I	Iav	A
May	5	4	80.00	4-8	4.50	3.60
June	19	6	31.58	5-6	2.50	0.79
July	48	21	43.75	1-6	2.43	1.06
August	50	28	56.00	4-15	2.61	1.46
September	20	8	40.00	1-8	2.50	1.00
October	17	8	47.05	3-5	2.25	1.06

The dispersion of the invasive protostrongylid larvae has the following values for the sample: in spring 4.85, ID = 3.51, in summer 4.04, ID = 3.45 and in autumn 3.28, ID= 1.11. Referred to the population, s^2 and ID have the following values, respectively: spring, 4.65 and 3.37; summer, 4.00 and 3.41; autumn, 3.19 and 2.87. These parameters correspond to an aggregated distribution in the populations of intermediate hosts and parasites during the three seasons.

The age structure of the populations was described by the characteristic of their size structure. The established size groups were divided into seven size classes according to the shell diameter: class 1 = 4-6 mm, class 2 = 6-8 mm, class 3 = 8-10 mm, class 4 = 10-12 mm, class 5 = 12-14 mm, class 6 = 14-16 mm and class 7 = 16-18 mm. Different size classes during different seasons have different significance for the spread of the invasion (Table 5).

Spearman correlation analysis showed that there are strong positive and statistically significant relationships between all studied indicators ($p < 0.001$). The strongest relationship was found between the mean abundance and the variance relative to the mean ($\rho = 0.980$), which suggests that with higher abundance of the parasite, higher relative variability is observed.

The relationship between occurrence and mean abundance is also significant ($\rho = 0.939$), which indicates that the more common parasite is usually more numerous.

The Spearman correlation analysis performed showed that there is a strong and statistically significant positive relationship between prevalence (P%) and average abundance ($\rho = 0.886$, $p = 0.003$). This suggests that as the number of infected individuals increases, the quantitative accumulation (abundance) of the parasite also increases (Fig. 1). Spearman's correlation analysis between different measures of variation showed that all indicators are extremely strongly positively correlated with each other, with some reaching perfect correlation ($\rho = 1.000$, $p < 0.001$). This indicates that the variance, the dispersion index and their corresponding variants for the general population describe the same variational characteristics.

The conducted studies revealed the presence of clearly expressed seasonal dynamics of the population density of both *X. obvia* and *X. macedonica* and of the protostrongylids on the studied pastures (Fig. 2).

Table 5. Prevalence of nematode larvae by size classes during the three seasons in the pastures near Mugla, Vihren SE and Zabardo.

Size class		**Season**		
No	Size (mm)	Spring	Summer	Autumn
		Mugla *X. obvia*		
1	4-6	-	-	-
2	6-8	25	-	-
3	8-10	0.11	4.34	-
4	10-12	10.53	-	-
5	12-14	2.42	15.15	7.88
6	14-16	1.49	30.6	11.28
7	16-18	-	18.18	21.21
		Vihren SE *X.macedonica*		
1	4-6	-	-	-
2	6-8	36.36	9.1	-
3	8-10	-	34.21	6.58
4	10-12	3.85	34.62	13.46
5	12-14	20	26.67	6.67
6	14-16	25	-	75
7	16-18	-	-	-
		Zabardo *X. obvia*		
1	4-6	-	-	-
2	6-8	-	-	-
3	8-10	-	-	-
4	10-12	-	10	30
5	12-14	-	2.38	35.7
6	14-16	-	-	2.38
7	16-18	-	33.33	-

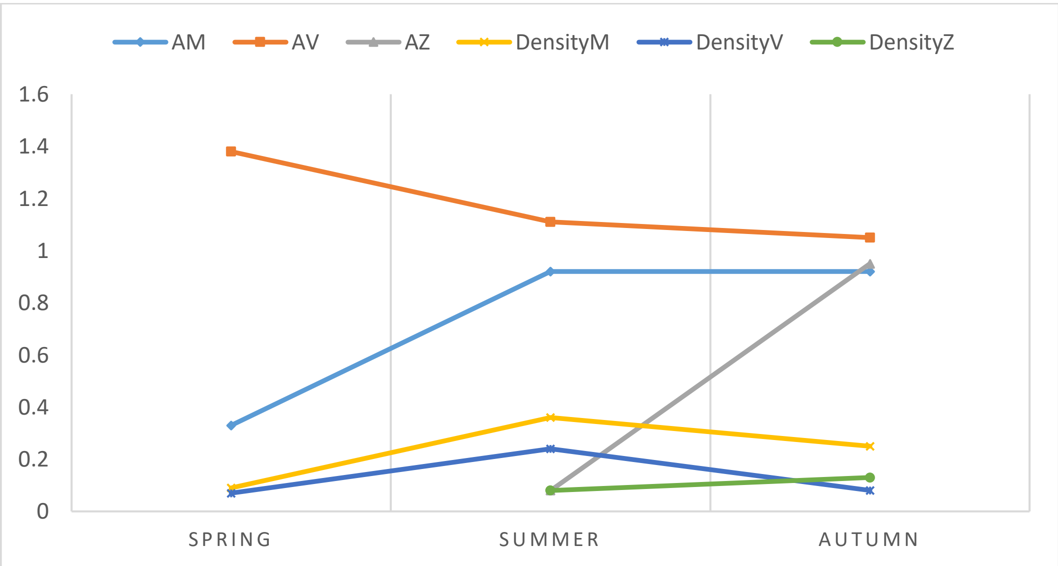

Fig. 1. Correlations between density and abundance of the three studied pastures (A- abundance, M - Mugla, V - Vihren SE, Z - Zabardo).

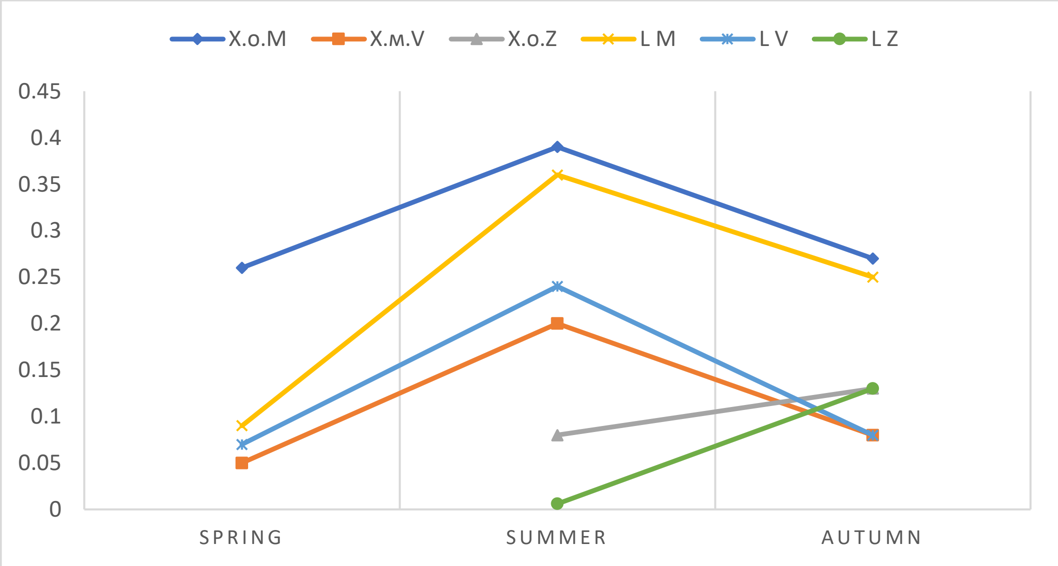

Fig. 2. Seasonal dynamics of population density of *Xerolenta* spp. and L_3 protostrongylids (X.o. - *Xerolenta obvia*, X.m. - *X. macedonica*, L - L_3, M - Mugla, V - Vihren SE, Z - Zabardo).

Discussion

The density of protostrongylids per unit area is an important indicator for assessing the invasiveness of pastures. Its maxima are noted as periods of maximum risk for infection of definitive hosts (Cabaret, 1983, 1984b, 1988; Lahmar et al., 1990). In this study, the density of protostrongylids, like the density of intermediate hosts, shows a pronounced seasonal maximum, which for the Mugla and Vihren SE pastures is in summer, and for the Zabardo pasture - in autumn. It should be noted that despite the seasonal summer maximum, the highest density of the pasture in Mugla was recorded in September - 0.25 L_3/m^2. This discrepancy is due to the negligibly low density in October - 0.006 L_3/m^2.

Of the regions in which studies have been conducted to determine the periods of maximum risk for infection of domestic sheep and goats, no picture close to the one we observed was found. On the pastures in the Stara Zagora villages of Oryahovitsa and Rakitnitsa, Georgiev (2020) observed two clearly expressed maxima. The first maximum covers the months of May and June, and the second is in September; the two maxima have comparable values. The trend is similar on the pasture near Rakitnitsa, where, however, the maximum in early summer is significantly higher than that in September. Corresponding observations have been made on pastures in Touraine, France (Cabaret, 1983), where two clearly expressed maxima of the density of protostrongylids per unit area are also observed, but they are shifted by about one month from those in Stara Zagora.

It can be assumed that the reason for the differences is the high altitude of the three studied pastures, two of them, Mugla and Zabardo, fall into the Rila-Rhodope, and Vihren SE in the High-mountainous Alpine-tundra Pirin biogeographical region (Asenov, 2006). These areas are characterized by prolonged periods of low temperatures and long-lasting snow cover, which determines a shorter period of activity of the intermediate hosts and limits the periods of maximum risk of infection between summer and autumn.

The observed maxima in the density of protostrongylid larvae in the present study differ significantly from those found in North African countries. In a pasture in Northern Tunisia, Lahmar et al. (1990) found a period of maximum risk of infection with protostrongylids in the winter months (December–February), and the density vlues were lowest in the summer months (June–September); the observed dynamics showed a correlation with the average monthly rainfall and the number of rainy days in the respective month. In a pasture in Morocco, the maxima in the density of invasive protostrongylid larvae were in April and July–September; according to the cited study, the density dynamics were determined mainly by the monthly rainfall (Cabaret, 1988).

In the present study, the observed maxima of nematode larval density did not coincide with the maximum monthly precipitation values all three pastures have a peak in precipitation in May and June. The discrepancies between the peaks of the two indicators for the Mugla and Vihren SE pastures are about a month, and for the Zabardo pasture - about three months and differ from the data presented by Georgiev (2020) for differences of about two months between the highest values of precipitation and the density of nematode larvae and contradict the coincidences of the risk periods for infection of the final hosts with the maximum precipitation documented by Cabaret (1988) and Lahmar et al. (1990), which gives grounds to support the claim that this coincidence cannot be absolute and used directly to predict the invasiveness of pastures (Georgiev, 2020).

Georgiev (2020) proposes the following hypothesis: During the months of April and May, when monthly rainfall is at its maximum, a period of increased activity of *X. obvia* occurs. This period coincides with the mass penetration of the snails by invasive first-stage larvae, which, as a free-living stage, are highly dependent on humidity (Cabaret, 1984b; Egorov, 1960; Rose, 1957; Trushin, 1973b). Development from L_1 to L_3 in *H. obvia* under favorable conditions lasts for 14-17 days for *M. capillaris*, 51-80 days for *N. linearis*, 30-40 days for *C. ocreatus* and 35-45 days for *Protostrongylus rufescens* (Urban, 1980). These periods determine the maximum density of third-stage larvae (Georgiev, 2020). The data from the present study correspond to this hypothesis for the Mugla pasture, with maxima in density occurring between July and September (Table 2). On Vihren SE, a very high average abundance was recorded in May - 3.6 specimens/snail, which dropped sharply in the following month to 0.79 and significantly increased its values in July - 1.06 specimens/snail and August - 1.46 specimens/snail. Despite the decline in September and October, the average abundance values remained relatively high, 1 and 1.06 specimens/snail, respectively (Table 4). The high abundance of L_3 in May is probably due to the fact that the collected specimens of *X. macedonica* were infected in the autumn of the previous year, and the development of the larvae was completed after the activation of the snails the following year, the high values in summer are due to the significantly higher amount and frequency of precipitation in the mountainous areas, combined with favorable temperatures, which for a large part of the period are in the optimal range, between 10 and 20°C (Damyanov & Likharev, 1975), which suggests shorter periods of aestivation. These periods, according to Damyanov & Likharev (1975), can last for several hours during the hot part of the day. On the pasture in Zabardo, the shift is greater and the highest abundance is recorded in October - 1.33 specimens/snail. In September it is 0.3 specimens/snail, and in the other months for which we have data, they have negligible low values (Table 3).

When comparing the maximum densities per unit area of different pastures for which this parameter was studied, it was found that the data obtained in the course of this study differ significantly from those of other authors. On a pasture in Morocco, the maximum monthly average value of the density of protostrongylids was 2.26 larvae/m^2 (Cabaret, 1988), on a pasture in France (Touraine) – 11 larvae/m^2 (Cabaret, 1983a), and on a pasture in Tunisia – over 100 larvae/m^2 (Lahmar et al., 1990). In all three cases, the authors consider the registered density levels to be risky in terms of infection of the final hosts. The maximum values of 49 larvae/m^2 at Oryahovitsa and 19 larvae/m^2 at Rakitnitsa recorded by Georgiev (2020) are lower than the recorded risk density levels in Tunisia, but significantly higher than those in Morocco and France. The results established in the present study show significantly lower densities in the studied populations. The maximum values recorded are in summer for Mugla and Vihren and in autumn for Zabardo, respectively for each of the three pastures: 0.36 larvae/m^2, 0.2 larvae/m^2 and 0.13 larvae/m^2. These density values are significantly lower than all those cited, but are probably capable of maintaining an infectious cycle of protostrongylids in chamois. Such a hypothesis is indirectly supported by previous studies in the Western Rhodopes that found protostrongylid invasion in autopsy lungs and examination of fecal samples from chamois (Panayova-Pencheva, 2006b; Panayotova-Pencheva, 2008; Panayotova-Pencheva et al, 2004; Panayotova-Pencheva & Mutafova, 2005).

According to Cabaret (1988), the invasiveness of a pasture is by definition related to the populartion density of the snails. This statement is also supported by the present studies, which show a high degree of correlation of the density of protostrongylids with the population density of the host (Fig. 2).

A number of studies in the past have used parasitological parameters for epizootological characterization of pastures with respect to protostrongylids: prevalence, intensity of invasion and (or) average abundance (Egorov, 1960; Golubev, 1963; Samnaliev, 1966, 1968; Trushin, 1973b; Žďárská, 1960; Zurliiski, 1994, etc.).

The comparison of the dynamics of the average abundance with that of the density of larvae per unit area, revealed in this study, shows a certain discrepancy in the Mugla pasture. A similar discrepancy in terms of the maximum values was also found by Georgiev et al. (2003). According to the authors, this fact determines the average abundance as an auxiliary criterion in identifying the risk periods for infection of the final hosts, which, however, cannot replace the assessment of the density of nematode larvae. The situation is different with regard to the occurrence indicator, whose seasonal maxima coincide in time with the maxima of the density.

Eight species of terrestrial snails have been reported as intermediate hosts of protostrongylids in previous studies (Georgiev et al., 2003; Georgiev & Georgiev, 2002; Samnaliev, 1968; Zurliyski, 1994). In two of these species, *Z. detrita* and *C. vindobonensis*, no protostrongylid invasion was found in the present study, and *X. obvia* emerged as one of the main intermediate hosts. The maximum sizes of *X. obvia* recorded in this study correspond to those established by Damyanov & Likharev, (1975) and Welter-Schultes (2012), but those of *X. macedonica* exceed them and reach over 14 mm. *X. macedonica* was found at an altitude of 2400 m, significantly higher than the 2000 m indicated by Damyanov & Likharev (1975) and by Welter-Schultes (2012) - 2200m.

Conclusions

The studied pastures have a poor species composition of intermediate hosts. A small number of mainly xerophilous species can be considered a typical element of this type of community. The mesophilic species, some of which are attached to forest and shrub communities, probably fall by chance into the ecotone zones of the pastures. The small number with which they were found in the collections made confirms their accidental nature.

The main intermediate host of the nematodes for the studied pastures are species of the genus *Xerolenta*, for the Western Rhodopes this is *X. obvia*, and for Pirin - *X. macedonica*.

The differences in the periods of maximum risk of infection observed in the present study and the data of other authors are due to the high altitudes at which the pastures we studied are located.

The high abundance of L_3 in May in Pirin is due to the fact that the collected specimens of *X. macedonica* were infected in the autumn of the previous year, and the development of the larvae was completed after the activation of the snails the following year, the high values in summer are due to the significantly higher amount and frequency of precipitation in the mountainous areas, combined with favorable temperatures, which in a large part of the period are in the optimal range.

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