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Morphometric studies of Protostrongylidae's third stage larvae (Nematoda: Protostrongylidae) found in *Helicella obvia* in the pastures of Central South Bulgaria

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Abstract

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The purpose of the current study was to investigate the morphometric characteristics of L_3 protostrongylids, found commonly in intermediate hosts in Central South Bulgaria and to reveal the most common intermediate host in the studied region. Three terrestrial snaisls species: *Helicella obvia, Zebrina detrita* and *Monacha cartusiana* were collected from pastures primarily used by sheep and goats in the region of Stara Zagora city during the period from March to June and from September to November in 2017. *Helicella obvia* was the most widely distributed in all studied pastures. The highest general parameters of invasion with protostrongylids were observed in *Helicella obvia*. A total of 150 larvae of protostrongylids were identified, belonging to the following species: *Mullerius capillaris*, *Neostrongulys linearis* and *Cystocaulus ocreatus.* The established morphometric parameters on L_3 of these species were as follows: *M. capillaris* - average body length 0.624 mm and average length of the tail (the distance between the anal orifice and the tail tip) - 0.038 mm; *N. linearis* - 0.577 mm and 0.030 mm, respectively; *C. ocreatus* were 0.676 mm and 0.032 mm, respectively. The seasonal dynamics of the protostrongylids showed that the biggest invasion observed in all investigated pastures was during autumn and spring. The abundance of the nematode`s populations through different sites was low. The most common protostrongylid species was *M. capillaris*.

Keywords: sheep and goats; snails; *M. capillaris; N. linearis; C. ocreatus*

Introduction

The nematodes from Family Protostrongylidae (Leiper, 1926) parasitize in the lungs of wild and domestic ruminants and rabbits (Boev, 1975; Vasilev et al., 1986; Kamburov et al., 1994; Anderson, 2000). Among the complex of parasitic worms on sheep and goats, protostrongylids occupy an important place (Zurliyski & Rusev, 1990; Halacheva et al., 2001). It is well known that multiple terrestrial and freshwater snails have been registered as intermediate hosts of those parasites.

Lungworms are widely distributed throughout the world but are particularly common in countries with temperate climates, and in the highlands of tropical and sub-tropical countries. Protostrongylidae were recorded in Mexico, Peru, Brasil, Morocco, Turkey, Iraq, Iran also in wild sheep Pakistan and India (Over et al., 1992), in North America (Kutz et al., 2012). Protostrongylus species have been reported also in Germany, Finland, Sweden, England, Austria, Italy, Iberian Peninsula, and Czech Republic (Lesage et al., 2014). However, that the geographical distribution of the Protostrongylidae is mainly determined by the distribution of the snail intermediate hosts.

The occurrence of protostrogylids in Bulgaria in livestock and in some wild animals has been well studied (Bratanov et al., 1987; Panayotova-Pencheva, 2006; 2014), but the studies on L_3 (third-stage larvae) morphology are scarce. Due to intensive livestock breeding in Bulgaria and animal transport morphology studies of third-stage larvae are required. This will improve the species differentiation. Unfortunately, the studies in this field have not been conducted in the last 50 years, including of some of the most widespread protostrongylids, such as *M. capillaris*, *N. linearis* and *C. ocreatus*. Thus the purpose of the current study was to investigate the morphometric characteristics of L_3 protostrongylids found commonly in intermediate hosts in South Bulgaria.

Material and Methods

Sample collection

The gastropods were collected from four pastures in South Bulgaria based on quality composition of the snails (Figure 1, Table 1) For many years the studied area is well known as a developed agricultural and animal husbandry region. Goats and sheep prevail among livestock and the problem with their health is of particular importance.

Determining of terrestrial snails was done by shell characteristics and by identification keys (Angelov et al., 1963; Welter-Schultes, 2012).

The sampling was carried out one day per month in two periods (March - June and September – November) in 2017. At least 100 specimens were collected at each sampling. The procedure was conducted in conditions of rain or after the rain, between 7.00 a.m and 9.00 a.m. Quantitative sampling has been performed by a method (Georgiev et al., 2003) which is a modification of the method described by Gilyarov (1987).

Parasite examination

The gastropods were drowned in an airtight container (300 ml) full of water. The snail's leg was removed and placed between two compression glasses (for trichinoscopy). The compressed tissue was examined under a microscope at 100 × magnification on a Leica DM 2500 microscope with a Leica DFC 295 camera. The larvae were then isolated mechanically or by grinding the feet in artificial gastric juice (Cabaret et al., 1980). Larvae samples were prepared on microscope glass slides and examined using the method of Seinhorst (1959), then they were identified using Cabaret’s key (Cabaret, 1981).

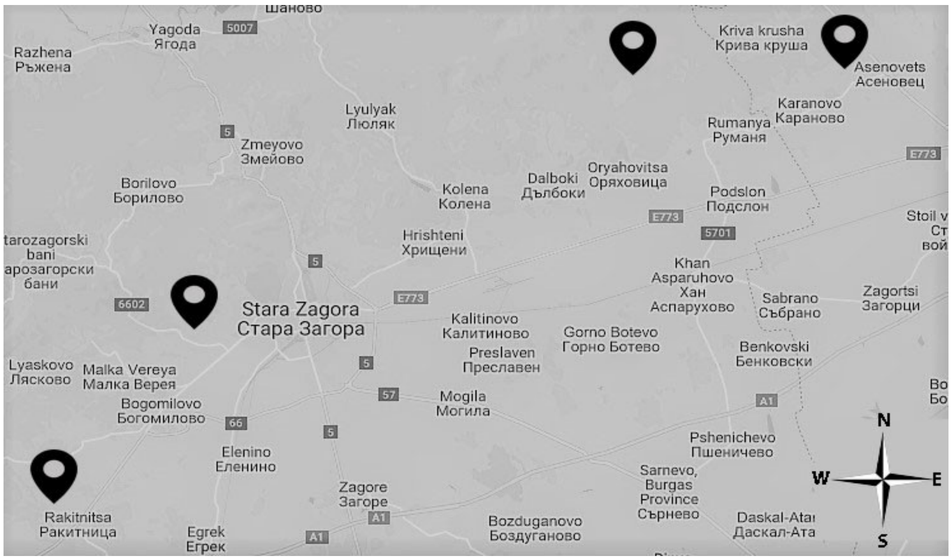

Fig. 1. Studied pastures in the Upper Thracian Plain and Sarnena Sredna Gora

Table 1. GPS coordinates of the sampling sites

Sample side	DD (decimal degrees)	
	Latitude	Longitude
Rakitnitsa	42.34991	25.50004
Zheleznik	42.4194	25.57607
Oryahovitsa	42.52959	25.81803
Karanovo	42.53312	25.93336

Quantitative analysis

The quantitative parameters of invasion used in this study were in line with the definitions perceived by Bush et al. (1997):

- Occurrence (P%) (prevalence, extent of invasion): the ratio of the number of individuals invaded with a parasitic species to the total number of host species examined, expressed as a percentage.
- Mean intensity: the ratio of the total number of certain parasite species found in the sample to the number of hosts infected with that parasite species.
- Mean abundance: the ratio of the total number of specimens of certain parasite species in the sampled host species to the total number of host species examined (infected + uninfected).

As a base for comparison of our results, the data reported by other authors on the morphology of L_3 protostrongylid larvae were used (Gerichter, 1951; Pohl, 1960; Zdarska, 1960; Samnaliev, 1968). According to these authors, the following parameters are considered to be essential for proper identification: total body length, length of the esophagus, length and characteristics of the tail and the location of the excretory pore. In the present study an additional parameter was included – body width.

Results

Morphometric characteristics

For the purpose of the present study, three species of terrestrial snails - *Helicella obvia, Zebrina detrita* and *Monacha cartusiana* were collected from pastures primarily used by sheep and goats in the region of Stara Zagora city. *Helicella obvia* was the most widely distributed species in all studied pastures. The individuals of this species showed the highest general parameters of invasion with protostrongylids.

A total of 150 larvae of protostrongylids were prepared on microscope glass slides. Three species were found in livestock in Stara Zagora region (*Mullerius capillaris, Neostrongulys linearis, Cystocaulus ocreatus.* Such results for the distribution of nematodes from the family Protostrongylidae were previously described by Georgiev & Georgiev (2002), Georgiev et al. (2003; 2005).

Morphology of L_3 Mullerius capillaris

The body of the isolated larvae of *Muellerius capillaris* is almost transparent with pale, yellow pigmentation. L3 of *M. capillaris* has a thick cuticle consisting of two layers – the external one (clearly striated) and the internal one (with smooth surface) (Figure 2). The structure of the tail and the shape of its end are of great importance for the identification of these species. The end of the tail has no appendages; it is gently undulated on the dorsal side and has a slightly sharpened tip. All of the observed characteristics match with those described by Boev (1975). The morphometric characteristics of the collected specimens are presented in Table 2.

Fig. 2. Microscope image of L_3 of *M. capillaris*

Table 2. Morphometric data of L_3 of *M. capillaris* (mm)

Indicator	n/ L_3	Mean	Min - Max	$S^2 \pm SD$
body length	28	0.624	0.465 - 0.875	0.015 ± 0.124
length of the esophagus	28	0.169	0.105 – 0.255	0.004 ± 0.065
length of the tail	28	0.038	0.025 – 0.050	0.000006 ± 0.002
body width	28	0.034	0.027 – 0.045	0.0002 ± 0.015

Morphology of L_3 Neostrongylus linearis

The larvae of *N. linearis* are whitey, almost transparent, covered with delicately striated cuticle which is smooth at the end of the head (Muller, 1934 citation by Boev, 1975). The excretory pore is located in the middle of the esophagus (Cabaret, 1981). The end of the tail is softly sharpened and has no appendages (Fig. 3.). Its dorsal side is slightly beveled. The morphometric characteristics of the collected specimens are presented in Table 3.

Fig. 3. Microscope images of L_3 of *N. linearis*

Table 3. Morphometric data of L_3 of *N. linearis* (mm)

Indicator		n/ L_3	Mean	Min - Max	$S^2 \pm SD$
body length		10	0.577	0.485 – 0.880	0.0002 ± 0.015
length of the esophagus		10	0.154	0.112 – 0.190	0.0008 ± 0.028
length of the tail		10	0.030	0.020 – 0.050	0.000003 ± 0.001
body width		10	0.030	0.025 – 0.037	0.00001 ± 0.003

Morphology of L_3 Cystocaulus ocreatus

The body of L_3 of *Cystocaulus ocreatus* is transparent, covered with dense and thick cuticle (Fig. 4.) which has two layers: external – striated and internal – soft and stuck to the body (Davtyan, 1934 cited by Boev, 1975). There is a cuticle edge laterally along the body. The excretory pore is located between the second and the third part of the esophagus (Cabaret, 1981).

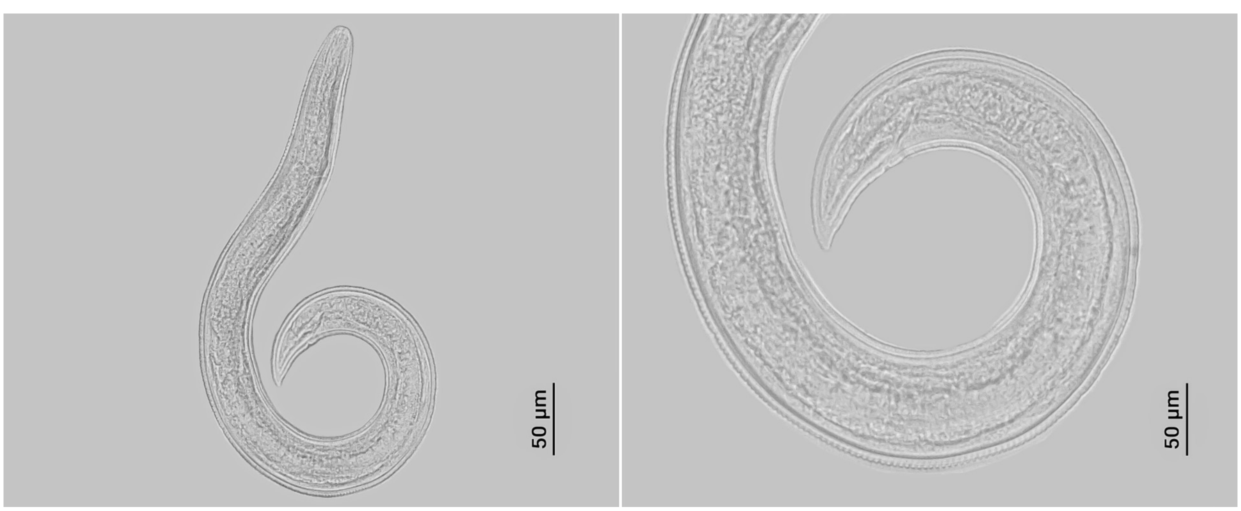

Fig. 4. Microscope images of L_3 of *C. ocreatus*

The larvae of *C. ocreatus* are easily distinguished from the other two species because of their bigger size and the fact that their cuticle is highly striated. The tail of this nematode ends with a blunt tip, slightly undulated dorsal side and highly undulated ventral side. The morphometric characteristics of the collected specimens are presented in Table 4.

Table 4. Morphometric data of L_3 of *C. ocreatus* (mm)

Indicator		n/ L_3	Mean	Min - Max	$S^2 \pm SD$
body length		6	0.676	0.555 – 0.822	0.004 ± 0.065
length of the esophagus		6	0.199	0.150 – 0.265	0.001 ± 0.040
length of the tail		6	0.032	0.022 – 0.047	0.0001 ± 0.010
body width		6	0.043	0.031 – 0.055	0.00001 ± 0.003

Quantitative indicators of invasion

Quantitative indicators of invasion were estimated for different pastures and seasons. The data collected in March and April 2017 represented spring results, these from June to September - summer results and these in October and November (2017) - autumn results.

The abundance of nematode`s populations in Oryahovitsa village pasture was low (Table 5). The most protostrongylids larvae were isolated in snails from Rakitnitsa village (Table 6), in autumn.

From all of the sampling sites Karanovo village (Table 7) was the one with the majority of infected snails, especially in spring. The highest level of invasion with protorstrongylids was found in all pastures during autumn and spring. Zheleznik sampling site (Table 8) was the one with the minimum infected snails. *Helicella obvia* showed the highest general parameters of invasion with protostrongylids.

Detailed information about the nematode`s and snail`s abundance and occurrence is displayed in the tables below.

Table 5. Invasion of land snails by protostrongylid larvae in pastures near Oryahovitsa village, 2017

Gastropod species	N	Nematode species	N_{inf}	P%	I	I_{av}±SD	A±SD
Spring							
Helicella obvia	88	Protostrongylidae	11	12.3	1-6	1.90±1.8	0.24±0.9
		M. capillaris	6	6.7	1-4	1.66±1.2	0.11±0.5
		N. linearis	2	2.2	1-2	1.50±0.7	0.03±0.2
		C. ocreatus	5	5.6	1-3	1.60±1.0	0.09±0.3
Zebrina detrita	93	Protostrongylidae	-	-	-	-	-
summer							
Helicella obvia	63	Protostrongylidae	9	14.3	1-6	2.66±1.6	0.38±1.1
		M. capillaris	7	11.1	1-3	2.00±0.8	0.22±0.7
		N. linearis	3	4.8	1-2	1.33±0.6	0.06±0.3
		C. ocreatus	1	1.6	2-2	2.00	0.03±0.3
Zebrina detrita	68	Protostrongylidae	-	-	-	-	-
Autumn							
Helicella obvia	100	Protostrongylidae	18	18.0	1-3	1.22±0.5	0.22±0.5
		M. capillaris	1	1.0	1-3	3.00	0.03±0.3
		N. linearis	1	1.0	1	1.00	0.01±0.1
		C. ocreatus	1	1.0	1	1.00	0.01±0.1

N - sample size; Ninf. - number of snails infected; P% - occurrence; I - intensity of invasion;

Iav - average intensity; A - average abundance

Table 6. Invasion of land snails by protostrongylid larvae in pastures near Rakitnitsa village, 2017

Gastropod species	N	Nematode species	N_{inf}	P%	I	I_{av}±SD	A±SD
Spring							
Helicella obvia	26	Protostrongylidae	10	39.5	1-26	6.50±8.0	2.50±5.8
		M. capillaris	10	38.5	1-19	4.80±6.0	1.85± 4.3
		N. linearis	4	15.4	1-4	2.00±1.0	0.37±0.9
		C. ocreatus	3	11.5	1-6	3.00±2.7	0.35±1.2
Zebrina detrita	45	Protostrongylidae	-	-	-	-	-
Monacha cartusiana	1	Protostrongylidae	-	-	-	-	-
summer							
Helicella obvia	69	Protostrongylidae	9	13.0	1-3	1.89±0.6	0.25±0.6
		M. capillaris	4	5.8	1-2	1.25±0.5	0.07±0.3
		N. linearis	1	1.4	2	2.00	0.03±0.2
		C. ocreatus	3	4.3	1-3	2.00±1.0	0.09±0.5
Zebrina detrita	3	Protostrongylidae	1	1/2	2	2.00	1.30
		C. ocreatus	1	1/2	2	2.00	1.30
autumn							
Helicella obvia	100	Protostrongylidae	30	19.5	1-43	3.13±7.6	0.61±3.5
		M. capillaris	19	12.3	1-43	3.52±9.6	0.44±3.5
		N. linearis	10	6.5	1-2	1.50±0.5	0.09±0.4
		C. ocreatus	6	3.9	1-4	2.00±1.3	0.11±0.6

N - sample size; Ninf. - number of snails infected; P% - occurrence; I - intensity of invasion; Iav - average intensity;
A - average abundance

Table 7. Invasion of land snails by protostrongylid larvae in pastures near Karanovo village, 2017

Gastropod species	N	Nematode species	N_{inf}	P%	I	I_{av}±SD	A±SD
Spring							
Helicella obvia	71	Protostrongylidae	48	67.6	1-24	5.20±6.1	3.54±5.6
		M. capillaris	5	7	1-8	4.80±2.8	0.37±1.4
		N. linearis	1	1.4	1	1.00	0.01±0.1
		C. ocreatus	5	7	1-4	2.40±1.5	0.20±0.7
Zebrina detrita	60	Protostrongylidae	4	6.7	1-3	1.50±1.0	0.10±0.4
		M. capillaris	3	5.0	1-3	1.70±1.2	0.08±0.4
		C. ocreatus	1	1.7	1	1.00	0.02±0.1
Summer							
Helicella obvia	91	*Protostrongylidae*	11	19.1	1-4	2.00±1.0	0.24±0.7
		M. capillaris	6	6.6	1-4	2.00±1.1	0.13±0.6
		N. linearis	1	1.1	3	3.00	0.03±0.3
		C. ocreatus.	5	5.5	1-2	1.40±0.5	0.07±0.4
Autumn							
Helicella obvia	100	*Protostrongylidae*	18	18.0	1-3	1.22±0.5	0.22±0.5
		M. capillaris	7	7.0	1-2	1.28±0.5	0.09±0.4
		C. ocreatus	4	4	1	1.00	0.04±0.2
		Protostrongylus sp.	7	7.0	1-3	1.28±0.8	0.09±0.4

N - sample size; Ninf. - number of snails infected; P% - occurrence; I - intensity of invasion; Iav - average intensity; A - average abundance

Table 8. Invasion of land snails by protostrongylid larvae in pastures near Zheleznik district, 2017

Gastropod species	N	*Nematode species*	N_{inf}	P%	I	I_{av}±SD	A±SD
spring							
Helicella obvia	41	Protostrongylidae	8	19.5	1-4	2.00±1.1	0.40±0.9
		M. capillaris	6	14.6	1-4	1.83±1.2	0.27±0.8
		N. linearis	1	2.4	1-1	1.00	0.02±0.2
		C. ocreatus	3	7.3	1-2	1.33±0.6	0.10±0.4
Monacha cartusiana	13	Protostrongylidae	-	-	-	-	-
summer							
Helicella obvia	83	Protostrongylidae	9	10.7	1-4	1.33±1.0	0.14±0.5
		M. capillaris	5	14.6	1-3	1.40±0.9	0.09±0.4
		N. linearis	1	1.2	1	1.00	0.02±0.2
		C. ocreatus	5	6.0	1	1.0	0.06±0.2
autumn							
Helicella obvia	92	Protostrongylidae	12	13.0	1-5	1.50±1.2	0.19±0.7
		M. capillaris	8	8.7	1-2	1.12±0.4	0.09±0.3
		N. linearis	7	7.7	1.12	2.71±4.1	0.2±1.3
		C. ocreatus	6	6.5	1-3	1.53±0.8	0.09±0.4

N - sample size; Ninf. - number of snails infected; P% - occurrence; I - intensity of invasion; Iav - average intensity;
A - average abundance

Discussion

The average body length at L_3 of *M. capillaris* is 0.624 mm and these values are close to those reported by Cabaret (1981) and Gerichter (1951). The length of the tail matches the one described by Samnaliev (1968). The length of the esophagus turned out to be a variable. Each author has reported different values for it but there is noticeable similarity with the data of Samnaliev (1968).

The collected data for *N. linearis* is similar to the results of Zdarska (1960) and to some extent to those of Samnaliev (1968). Our results about the length of the esophagus are very similar to those of Pohl (1960).

The possible reasons for the morphometric differences in the results of the researches already conducted on third-stage larvae of protostrongylids could be the lack of generally accepted identification key, as well as geographically determined differences in the morphology of this stage larvae. In addition, the development of protostrongylid larvae in gastropods is affected by a variety of factors including temperature, humidity, intensity of infection, and the age and physiological condition of the intermediate hosts (Cabaret, 1987; Solomon et al., 1996). In this regard, the present study showed compliance with the commonly accepted parameters. Our results are similar to these of Zdarska (1960) and Samnaliev (1968).

For even better differentiation of the larvae we propose the use of an additional parameter – "body width", which was also applied by Pohl (1960) in order to describe the third-stage larvae of *Neostrongulys linearis*. We propose this parameter as critical in distinguishing the third-stage larvae from the second-stage larvae. Very often both stages of larvae are extracted from the intermediate hosts. According to our observations the body width correlates with the length of the tail of the Protostrongylids and can be used as an important diagnostic parameter. Additional analyses are needed to confirm the observed correlation.

Conclusion

Considering the analysis of the results and the comments made we can deduce that our results are closest to the fundamental average values for *M. capillaris* and *N. linearis* reported for Bulgaria. In regards to *C. ocreatus* this resemblance refers to their maximum dimensions. Results from this study enriched the existing knowledge on diagnosis and diversity of protostrongylid fauna in the ruminants in Central South Bulgaria and provide tools for ongoing biodiversity assessment or to anticipate shifts in species ecology and distribution.

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