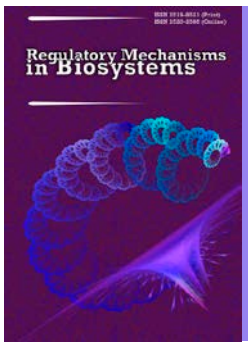

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Morphological features of development of *Strongyloides westeri* (Nematoda, Rhabditida) *in vitro*

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Strongyloides westeri (Ihle, 1917), a parasitic horse nematode, has an unusual lifecycle, which allows it to exist for a long time in the environment. Morphometric features of eggs, larvae and free-living *S. westeri* were studied *in vitro* under different temperature regimes. The optimal temperature for their embryonic development is 25 °C, under which 90% of the first stage rhabditiform larvae are formed and released within 7 hours of cultivation. The temperatures of 20 and 30 °C are less favorable for their development. Embryonic development of *Strongyloides* has four stages that differ in morphology and size. The eggs of a parthenogenetic female are 3.7% longer and 19.6% wider than eggs isolated from free-living females of *S. westeri*. In embryogenesis, the eggs shorten by 4.4 μm (6.5%) and widen by 5.35 μm (8.3%). New data were obtained on postembryonic development of *S. westeri*. The differential morphometric features of stage 1 and 2 rhabditiform larvae which grow both in length and width (33.7% and 30.4% respectively) are established. The development of filariform larvae is associated with loss of bulbous thickening and formation of cylindrical oesophagus. Simultaneously, the body elongates, and the gut becomes shorter. Differential morphometric features of free-living males and females of *S. westeri* are the length and width of body, length of oesophagus, gut, tail end, and size of spicules. Postembryonic development of the free-living and parasitic generations from rhabditiform larvae is temperature-dependent. Most of the free-living generations of *Strongyloides* (54.0%) are formed at 20 °C, and filariform larvae mostly (70.0%) develop at 30 °C. The obtained results of morphological studies improve differential diagnostics of the nematode at various stages of development and further advance the study of its intraspecific variability.

Keywords: *Strongyloides*; horses; nematode eggs; larvae; biological properties; morphometry

Introduction

Among the world parasitic fauna, parasitic worms are a most impressive group (Levine, 1980; Anderson, 2000; Kennedy & Harnett, 2013). Wild and domestic animals are well-known reservoirs of helminths, and prevalence of infection depends on a number of factors, such as the species composition and population sizes of the hosts, environmental conditions, anthropogenic impact, and biological properties of the helminth (Lee et al., 2002; John et al., 2011; Goater et al., 2014; Boyko & Brygadyrenko, 2016, 2017; Carlson et al., 2017).

Nematodes *Strongyloides westeri* Ihle, 1917 are widely distributed equine helminths. According to the literature, levels of equine infection depend on the animals' age, living conditions, prophylactics, climatic conditions. Prevalence can reach 90% (Lyons et al., 2007; Araujo et al., 2012; Ricardo et al., 2012; Lyons and Tolliver, 2014, 2015; Miller et al., 2017).

Nematodes of the genus *Strongyloides* (Grassi, 1879) are of specific interest because of their development cycle, which has alternative parasitic and free-living generations. The parasitic stage is represented only by parthenogenetic females living in the upper sections of the equine small intestine. Free-living nematodes are not parasitic and represented by both males and females living outside the animal host. There is evidence that depending on environmental factors, in particular the air temperature and humidity, eggs in faeces of sick animals or laid by a free-living female can develop differently. In case of direct development, the egg releases a rhabditiform larva that further transforms into a filariform one, which upon maturing can infect the host. Under indirect development, the rhabditiform larvae develop into either males or females. Postembryonic development of *Strongiloides* has distinct morphological traits by which its stages are identified (Lyons et al., 1973; Dewes and Townsend, 1990; Grant et al., 2006; Viney, 2006; Santos et al., 2010; Thamsborg et al., 2016).

Such specific biological properties of *Strongyloides* nematodes indicate the appearance of parasitism in non-parasitic species, followed by the evolution of relevant adaptations. The regressive morphological and biological changes lead to parthenogeny in the parasitic female. Meanwhile, the free-living larvae have the possibility of variable biological adaptations (Blaxter et al., 1998; Dorris et al., 2002; Thompson et al., 2006; Eberhardt et al., 2007).

The establishment of a helminth faunistic complex in certain environmental conditions is also heavily influenced by a number of factors. The most important are the biological properties of parasites that are so far not sufficiently studied in *Strongyloides* species of equines. Hence, investigating the morphological properties of embryonic and postembryonic stages of *S. westeri* outside its host will allow us to complement the already known facts of its biology and to better understand its parasitic adaptations. The aim of present work is to investigate the specifics of morphometric structure and biological properties of the embryonic and postembryonic stages of *S. westeri* nematode *in vitro*.

Materials and methods

Research was carried out in 2016–2017 in the laboratories of Parasitology and Veterinary-Sanitary Expertise of the Department of Veterinary Medicine of Poltava State Agrarian Academy and Dnipro State Agrarian and Economic University. Morphological and size parameters of the eggs of *S. westeri* were obtained from different substrates: gonads of free-living females and faeces of infected horses. The shape and shell features, including thickness, length and width of eggs were studied.

The development of *S. westeri* was investigated by culturing eggs, isolated from the faeces of infected horses and from free-living females, in a thermostat at 20, 25 and 30 °C for 10 hours. The culture was examined hourly under a microscope to count the percentage of released larvae and study the egg morphometry.

Postembryonic stages of *S. westeri* were measured in experimental culture *in vitro* at 25 °C for 10 days. The parameters of rhabditiform larvae L_1 and L_2, filariform larvae, and free-living adult males and females were investigated.

The percentage of rhabditiform larvae developing into filariform larvae (directly) and into free-living males and females (indirectly) was established at different temperatures (20, 25 and 30 °C).

Morphometric parameters of embryonic, postembryonic and adult stages of *S. westeri* were measured using ImageJ for Windows® (version 2.00) in interactive mode using ×10 and ×40 objective, and ×10 photo eyepiece. To calibrate the image analyzer, ruled scale of ocular micrometer was coincided with the scale of stage micrometer included in MikroMed microscope kit. Microphotographs were taken using a 5 Mpix digital camera of MikroMed microscope. The material and significance levels were analyzed using standard methods of statistical processing. All the data are reported as the sample mean ± the standard deviation (SD).

Results

Differences in the formation of rhabditiform larvae (L_1) were found at different temperatures. Most of the larvae (more than 56%) are released within 3–6 hours. Meanwhile, embryonic development can be divided into four stages: blastomere cleavage, larval formation, formation of mobile larvae, and release. The optimal temperature for development of rhabditiform larvae and their release from eggs was 25 °C (Table 1).

Table 1
Embryonic development of *S. westeri* eggs at different temperatures (%, n = 100)

Developmental stage	T °C	Culture time, hours												
		before culture	1	2	3	4	5	6	7	8	9	10	11	12
Blastomere cleavage	20	100	23	15	9	8	8	8	8	8	8	8	8	8
	25	100	20	11	8	6	6	6	6	6	6	6	6	6
	30	100	19	10	9	8	8	8	8	8	8	8	8	8
Larva formation	20	–	77	8	6	1	–	–	–	–	–	–	–	–
	25	–	80	9	3	2	–	–	–	–	–	–	–	–
	30	–	81	9	1	1	–	–	–	–	–	–	–	–
Mobile larva formation	20	–	–	57	28	6	1	–	–	–	–	–	–	–
	25	–	–	63	22	6	2	1	–	–	–	–	–	–
	30	–	–	66	13	5	3	2	2	2	2	2	2	2
Release of larvae from eggs	20	–	–	–	56	19	8	6	3	3	3	3	3	3
	25	–	–	–	62	20	6	6	4	4	4	4	4	4
	30	–	–	–	59	18	8	5	4	4	4	4	4	2
End of development	20	–	–	–	–	8	8	8	13	13	13	13	13	13
	25	–	–	–	–	6	6	6	10	10	10	10	10	10
	30	–	–	–	–	8	8	10	14	14	14	14	14	14

At the start of the experiment, 100% of eggs were at the blastomere cleavage stage (Fig. 1a). Later, the percentage of eggs at this stage decreased and after a 4-hour-long exposure more than 90% of them started the next stage of development (Fig. 1b). The percentage of eggs that stopped developing at this stage was 8% at 20 and 30 °C, and 6% at 25 °C. Larval formation begins quite early, within the first hour of the experiment more than 77% of *Strongyloides* eggs contained an immobile larva. The next stage of embryogenesis was characterized by the formation of a mobile rhabditiform larva (Fig. 1c). It peaked at the sixth hour of culturing. The percentage of eggs that stopped developing was 11 at 20 °C, 8 at 25 °C, and 10 at 30 °C.

Larval release from eggs was first registered at the third hour of culturing (Fig. 1d), and at the sixth hour it peaked (to 90%). Meanwhile the percentage of arrested larvae and undeveloped eggs was 13 at 20 °C, 10 at 25 °C and 14 at 30 °C. Thus the lowest mortality of *S. westeri* eggs was at 25 °C.

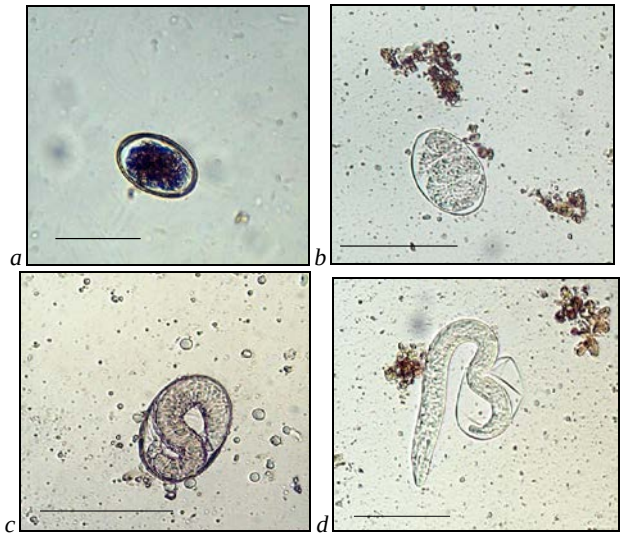

Fig. 1. Embryonic development of *S. westeri in vitro*: *a* – blastomere cleavage; *b* – larval formation; *c* – mobile larva formation; *d* – release of rhabditiform larva (L_1) from egg; bar – 50 μm

The eggs were oval with wide flat poles and thin shells, grey and half-transparent (Fig. 1). The size parameters of the eggs isolated from the gonads of free-living females and from the faeces of infected horses were significantly different (Table 2).

Table 2
Size parameters of *S. westeri* eggs, isolated out of various substrates, n = 10

Parameters, μm		Min	Max	x ± SD
Length	from free-living female gonads	39.76	52.71	47.71 ± 4.61
	from faeces of infected horses	41.88	52.33	49.21 ± 2.90
Width	from free-living female gonads	23.64	31.65	27.50 ± 2.44
	from faeces of infected horses	29.95	39.13	34.24 ± 3.77***
Shell thickness	from free-living female gonads	0.87	1.30	1.07 ± 0.13
	from faeces of infected horses	0.98	1.32	1.16 ± 0.09

Note: *** – P < 0.001 compared to values of eggs isolated from free-living female gonads.

The eggs isolated from the faeces of infected horses, were slightly longer – by 3.1% than the ones isolated from the gonads of the females (47.7 ± 4.6 and 49.2 ± 2.9 μm). The most pronounced differences were in the width dimension. The eggs isolated from faeces were wider by 19.6% (P < 0.001), and their shells thicker by 7.7% compared to the same parameters in eggs isolated from gonads (27.5 ± 2.4 and 1.1 ± 0.1 μm, respectively).

During *in vitro* embryogenesis of *S. westeri* larva, there were changes not only in their internal structure, but in the parameters of the egg length, width and shell thickness (Table 3).

In embryogenesis the egg length and shell thickness significantly decreased, while width increased. Thus, during blastomere cleavage the egg length decreased significantly by 4.4%, during larva formation by 6.1% (P < 0.05), during mobile larva formation by 6.5% (P < 0.01). Egg width increased by 5.3% during blastomere cleavage, by 7.6% during larva formation, by 8.3% during mobile larva formation (P < 0.05). Eggshell thickness also changed and at the mobile larva formation stage was the least (thinner by 19.4% compared to before culture, P < 0.001).

Thus, eggs of *S. westeri* differ by morphology and size parameters depending on the embryogenesis stage and the substrate they were isolated from. Such features should be taken into consideration in species identification.

In the first day of observations, stage 1 rhabditiform larvae (L_1) of *S. westeri* developed, with subsequent transition into stage 2 on the second to third day of development (L_2). Rhabditiform larvae had their own specific features: bulbous thickening of oesophagus, gut filled by pigmented grainy mass in two rows (Fig. 2a, b). On the fourth day of culture, we found filariform larvae with long cylindrical oesophagus and thinner tail end. Morphometrically, the rhabditiform and filariform larvae of *S. westeri* are distinctly different (Table 4).

Table 3
Size parameters of embryonic development of *S. westeri in vitro* (n = 10)

Parameters, μm	Before culture		Developmental stage					
			blastomere cleavage		larval formation		formation of mobile larva	
	x ± SD	Min – Max	x ± SD	Min – Max	x ± SD	Min – Max	x ± SD	Min – Max
Length	48.25 ± 1.93	45.12 – 51.23	46.12 ± 2.52	41.35 – 50.15	45.29 ± 3.12*	41.13 – 49.36	45.11 ± 2.02**	41.35 – 47.21
Width	34.33 ± 3.75	29.05 – 40.19	36.27 ± 2.60	32.14 – 40.42	37.17 ± 2.33	32.02 – 39.85	37.46 ± 1.59*	35.02 – 40.12
Shell thickness	1.13 ± 0.11	0.94 – 1.32	1.06 ± 0.11	0.83 – 1.21	0.99 ± 0.08**	0.85 – 1.14	0.91 ± 0.09***	0.74 – 1.03

Note: * – P < 0.05, ** – P < 0.01, *** – P < 0.001 compared to pre-cultivation values.

Fig. 2. Larvae of *S. westeri*: *a* – rhabditiform (L_1), *b* – rhabditiform (L_2), *c* – filariform; bar – 100 μm

Table 4
Size parameters of rhabditiform and filariform larvae of *S. westeri in vitro* (n = 10)

Parameters, μm	x ± SD	Min	Max
First stage rhabditiform larva (L_1)			
Length	313.48 ± 28.54	264.19	347.25
Width	15.52 ± 2.21	12.03	18.75
Oesophagus length	115.71 ± 8.32	104.01	130.12
Gut length	167.63 ± 26.09	134.10	211.15
Tail end length	30.95 ± 2.05	26.95	34.57
Second stage rhabditiform larva (L_2)			
Length	473.23 ± 28.37	421.21	521.10
Width	22.3 ± 5.46	16.40	31.35
Oesophagus length	119.22 ± 9.91	102.16	134.75
Gut length	310.21 ± 30.64	257.02	354.16
Tail end length	44.52 ± 6.85	35.14	58.13
Filariform larva			
Length	516.42 ± 19.38	484.26	541.43
Width	15.08 ± 1.38	12.46	17.01
Oesophagus length	261.80 ± 11.59	241.26	284.25
Gut length	160.11 ± 8.97	144.35	173.22
Tail end length	95.24 ± 6.53	81.03	102.41

The average body length of L_2 was 473.23 ± 28.37 μm, which is 33.7% more than length of L_1 (313.48 ± 28.54 μm). Body width of L_2 was also 30.4% greater than in L_1. Comparing L_2 and filariform larvae we found the latter to be slightly longer (by 8.3%) and thinner (by 32.3%). The most typical trait of developing filariform larvae was oesophagus formation and loss of the bulbous tip. The process was accompanied by oesophagus growth by 54.4% and gut shortening by 48.3%, which is evidently linked to larvae becoming parasitic.

Postembryonic development of L_2 was followed by their transformation either into filariform larvae or into free-living males and females. In culture, free-living generations appeared from Day 4. They have distinct morphological features; male *S. westeri* has weakly delineated buccal capsule, and on the tail end two spicules of the same size, gubernaculum and pre- and postnatal papillae (Fig. 3a, b, c). The female has a thinner anterior end, straight tail end, vulva in the middle of the body, eggs in the uterus (usually 2–4, sometimes 5) (Fig. 3). The oesophagus had two thickenings, the frontal one elongated and the tail-end one a bulb with a valve apparatus (Fig. 4a, b, c).

Morphometric studies found sex dimorphism in free-living generations of *Strongyloides* (Table 5). Average female length was 934.84 ± 59.37 μm, which is 18.9% longer than average male (757.72 ± 60.04 μm). Females were also 18.5% wider.

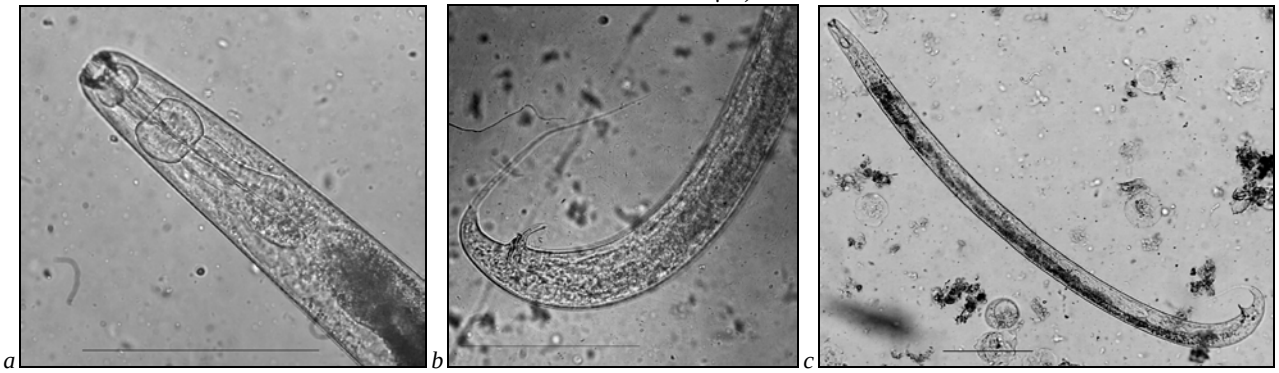

Fig. 3. *S. westeri* (♂): *a* – anterior end, *b* – tail end, *c* – whole specimen; bar – 100 μm

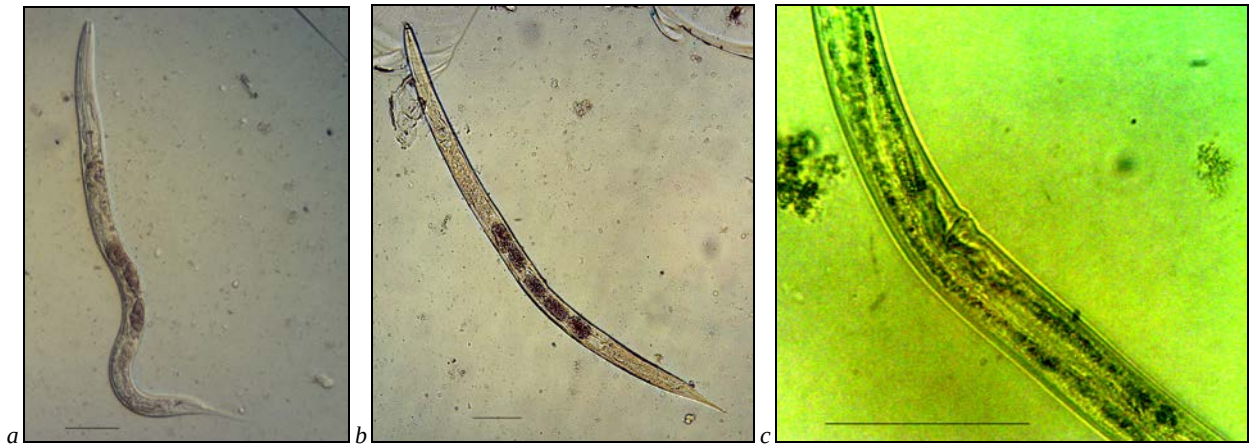

Fig. 4. *S. westeri* (♀): *a*, *b* – general appearance of females with different numbers of eggs; *c* – vulval region; bar – 100 μm

Table 5
Size parameters of *S. westeri* free-living generations *in vitro* (n = 10)

Parameters, μm	♂			♀		
	x ± SD	Min	Max	x ± SD	Min	Max
Body length	757.72 ± 60.04	645.9	834.5	934.84 ± 59.37	846.0	1007.3
Body width	28.31 ± 3.75	22.7	34.7	37.9 ± 5.23	28.3	45.8
Oesophagus length	126.34 ± 10.81	104.5	137.5	148.43 ± 8.71	138.4	165.5
Gut length	577.69 ± 63.68	472.8	654.4	689.36 ± 49.28	611.3	751.0
Tail end length	54.67 ± 11.62	34.7	72.5	98.19 ± 11.32	80.8	122.9
Spicule length	3.61 ± 0.65	2.89	4.73	–	–	–
Eggs in uterus length	–	–	–	39.86 ± 2.09	36.45	42.54
Eggs in uterus width	–	–	–	22.83 ± 1.93	20.25	26.75
Number of eggs	–	–	–	2.80 ± 1.08	1	5

In free-living males, the tail end length was less than 44.3% shorter than in females (98.19 ± 11.94 μm). The ratio of oesophagus to gut in males and females were almost the same (1 : 4.5 and 1 : 4.6, respectively). In culture and postembryonic development of *S. westeri* rhabditiform larvae, the majority of filariform larvae were formed at 30 °C (70 %), fewer larvae were observed at 25 °C (63%) (Table 6).

Table 6
Postembryonic development of rhabditiform larvae of *S. westeri in vitro* (n = 100)

Developmental stage	T, °C	Day of culture										Total
		0	1	2	3	4	5	6	7	8	9	10	
Rhabditiform larvae	20	100	100	100	95	72	41	12	3	3	3	3	3
	25	100	100	100	89	63	29	9	3	2	2	2	2
	30	100	100	98	81	52	20	6	3	3	3	3	3
Filariform larvae	20	–	–	–	5	10	11	16	1	–	–	–	43
	25	–	–	–	8	16	22	12	4	1	–	–	63
	30	–	–	2	15	20	21	10	2	–	–	–	70
Free-living males	20	–	–	–	–	8	12	7	4	–	–	–	31
	25	–	–	–	1	4	6	3	2	–	–	–	16
	30	–	–	–	1	5	6	2	–	–	–	–	10
Free-living females	20	–	–	–	–	5	8	6	4	–	–	–	23
	25	–	–	–	2	6	6	5	–	–	–	–	19
	30	–	–	–	1	4	5	2	1	–	–	–	17

The filariform larvae developed faster in cultures at 30 °C and were found from Day 2, while the percentage of free-living generations was the least observed (27%). Cultures at 25 °C had 63% of filariform larvae and 35% free-living males and females. The highest percentage of free-living generations (54%) was found at 20 °C. Thus, our research supports the dependence of the alternation of *Strongyloides* generations on temperature regimes.

Discussion

Analyzing the obtained data, we should note that abiotic factors greatly affect the development and morphometric parameters of embryonic and postembryonic stages of *S. westeri*. We established that the optimal temperature for culturing eggs of equine *Strongyloides* is 25 °C. It was found that the embryogenesis of *S. westeri* takes 4 to 6 hours at 20 to 30 °C. We also obtained novel data on the morphometric structure of eggs isolated from different substrates during their embryonic development. Our morphometric results are insignificantly different from those previously published (Ivashkin & Dvojnos, 1984), according to which the egg length of equine *Strongyloides* is 39 to 60 μm, and width 39 to 42 μm (compared to the 41.9–52.3 μm and 29.9–39.1 μm, respectively in the present study). Such data are in agreement with the findings of Ihle (1918) and others. Also, morphometric changes during embryogenesis were found, in particular the decrease in length (by 4.4 μm or 6.5%, $P < 0.01$) and thickening (by 5.3 μm or 8.3%, $P < 0.05$), and the thinning of eggshells (by 19.4%, $P < 0.001$).

Rhabditiform and filariform larvae and free-living generations of *S. westeri* were described quite a while ago (Ihle, 1918; Blieck & Baudet, 1920; Schuurmans-Stekhoven, 1930), yet there are no detailed descriptions of these helminths and their variability in Ukraine. We found morphometric parameters of rhabditiform larvae of the first and second stages. In the available literature we found general descriptions of *S. westeri* rhabditiform larvae regardless of developmental stages. Our research allows one to identify separate morphometric parameters of L_1 and L_2: mean length of L_1 was 313.5 ± 28.5 μm, width 15.5 ± 2.2 μm, and those of L_2 473.2 ± 28.4 and 22.3 ± 5.5 μm, respectively. We also measured larval oesophagus, gut and tail end. During the development of filariform larvae they grow slightly in length, with the most typical changes occurring in the structure of the oesophagus and its ratio to gut length. In L_2 the ratio was 1 : 2.60, and in filariform larvae it was 1.63 : 1. Thus, the larval ontogenesis is characterized by important morphometric changes that should be taken into account when identifying *Strongyloides* species.

We obtained new differential data on the morphometry of the free-living *S. westeri* generations. The free-living female mean length was 934.8 ± 59.4 μm, width 37.9 ± 5.2 μm. Males were smaller by 18.9–18.5% (length – 757.7 ± 60.0 μm, width – 28.3 ± 3.8 μm). The parameters are in accord with most of the previous findings, which in its turn indicates adaptability of the helminths. However, one should note that most authors report seeing 5 to 7 eggs in the gonads of free-living females, which is more than what we observed in most cases (2.8 ± 1.1 eggs).

The developmental biology of the helminths is characterized by their high adaptability and survival rates in unfavorable conditions. Tsuji & Fujisaki (1993) in their studies on culturing *S. venezuelens in vitro* prove that changing temperature from 25 to 37 °C is the main factor influencing the development of invasive larvae. Also, filariform larvae were found in extreme temperatures, as high as as 30 °C in the cultures of *S. stercoralis* (Shiwaku et al., 1988). The same was found for *Strongyloides* species in culture (Minato et al., 2008). In a study of the effect of temperature on L_1 stage of *S. ratti*, the larvae kept at 4 or 10 °C for 120 hours could not develop due to the arrested or delayed growth. However, L_1 could develop after transfer to the culture at 25 °C during

48 hours. The larvae stimulated by cold (4 or 10 °C) developed directly into invasive L_3 stages and it took as little as one minute of exposure to the low temperatures to induce direct development. Correspondingly, *Strongyloides* sp. can survive growth arrest or delay (Sakamoto & Uga, 2013).

Our studies showed that culturing rhabditiform larvae at 20 °C favored the formation of a greater number of free-living generations, and at 25–30 °C that of filariform larvae. It is in accordance with the findings of field biology of *S. westeri* (Malygin, 1957; Vislobokov, 2008). Our research proves that males and females develop in different quantities at different temperatures, yet the overall numbers are practically the same – 57 males and 59 females. Thus, our data, as well as the literature, show the significant effect of the environment on the development of different generations of *Strongyloides* sp.

Conclusions

Size parameters of the embryonic development stages of *S. westeri* have significant differences and depend on the substrate and the developmental stage. The process of embryogenesis of *S. westeri* in vitro has four stages: blastomere cleavage, larval formation, mobile larva formation, and release from egg; the stages have morphometric and significant size changes. Embryonic development of *S. westeri* occurs at 20 to 30 °C in 4–6 hours, and average survival rates is 87.7%.

Postembryonic development of *Strongyloides* is characterized by the formation of rhabditiform larvae (L_1 and L_2), filariform larvae, free-living generations of males and females, whose development is accompanied by morphometric changes. The main differential features of *S. westeri* at the discussed developmental stages are body length and width, structure and size of oesophagus and gut and their ratio, length of the tail end. It is possible to regulate the formation of filariform larvae and free-living generations of males and females by adjusting the temperature regime of the culture.

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