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

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**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; Goutet 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 Toffiver, 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 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 *Strongyloides* 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*.

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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

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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 Dniprop 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 L1 and L2, 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 isolated from the faeces of infected horses and from free-living females, Embryonic development of S. westeri 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.

Results

Differences in the formation of rhabditiform larvae (L1) were found at different temperatures. Most of the larvae (more than 56%) are decreased and after a 4-hour-long exposure more than 90% of them 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.

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 1

| Developmental stage | Temperature (°C) | Before culture | Culture time, hours | Length | Width | Shell thickness |
|---------------------|-----------------|----------------|---------------------|--------|-------|----------------|
| Blastomere cleavage | 20              | 100            | 8                   | 39.76  | 9.68 | 3.41           |
|                     | 25              | 100            | 8                   | 41.88  | 9.23 | 3.64           |
|                     | 30              | 100            | 8                   | 43.64  | 8.60 | 3.87           |
| Larva formation     | 20              | 77             | 8                   | 9.34   | 9.23 | 8.60           |
|                     | 25              | 80             | 3                   | 34.24  | 9.13 | 8.88           |
|                     | 30              | 81             | 1                   | 35.13  | 8.88 | 8.88           |
| Mobile larva formation | 20              | 57             | 6                   | 39.76  | 9.68 | 3.41           |
|                     | 25              | 63             | 2                   | 41.88  | 9.23 | 3.64           |
|                     | 30              | 66             | 3                   | 43.64  | 8.60 | 3.87           |
| Release of larvae from eggs | 20              | 56             | 19                  | 39.76  | 9.68 | 3.41           |
|                     | 25              | 62             | 20                  | 41.88  | 9.23 | 3.64           |
|                     | 30              | 59             | 18                  | 43.64  | 8.60 | 3.87           |
| End of development  | 20              | 8              | 8                   | 39.76  | 9.68 | 3.41           |
|                     | 25              | 6              | 6                   | 41.88  | 9.23 | 3.64           |
|                     | 30              | 8              | 8                   | 43.64  | 8.60 | 3.87           |

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.
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 | formation of mobile larva |
|---------------|----------------|---------------------|--------------------------|
|               | 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.

### Table 4

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

| Parameters, µm | Before culture | Developmental stage | formation of mobile larva |
|---------------|----------------|---------------------|--------------------------|
|               | x ± SD | Min – Max | x ± SD | Min – Max | x ± SD | Min – Max |
| Length        | 313.48 ± 28.37 | 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 |

First stage rhabditiform larva (L_1)

| Length        | 473.23 ± 38.27 | 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 |

Second stage rhabditiform larva (L_2)

| 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 |

Filariform larva

The average body length of L_2 was 473.23 ± 38.27 µm, which is 33.7% more than length of L_1 (313.48 ± 28.34 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. 3). 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.
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 filament 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 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Total |
|---------------------|------|---|---|---|---|---|---|---|---|---|---|----|------|
| 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    | 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  |
|                     | 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 (2%). 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 embryos 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; Blicke & 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 L1 and L2: mean length of L1 was 313.5 ± 28.5 μm, width 15.5 ± 2.2 μm, and those of L2 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 L1 the ratio was 1:1.63:1. 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 morphology 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 18.9–18.8% (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. venezuelensis* in vitro prove that changing temperature from 25 to 37 °C is the main factor influencing the development of invasive larvae. Also, filament larvae were found in extreme temperatures, as high as as 30 °C (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 L1 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, L1 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 L₃ 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, 1984).

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 filamentiform larvae. It is in accordance with the findings of field biology of S. westeri (Malysin, 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 larvae, 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 (L₁ and L₂), filamentiform larvae, free-living generations of males and females, whose development is accompanied by morphometric changes. The main differential features 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 filamentiform larvae and free-living generations of males and females by adjusting the temperature regime of the culture.

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