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PECULIARITIES OF EMBRYONIC AND POST-EMBRYONIC DEVELOPMENT OF *OESOPHAGOSTOMUM DENTATUM* (NEMATODA, STRONGYLIDAE) LARVAE CULTURED IN VITRO

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Peculiarities of Embryonic and Post-Embryonic Development of *Oesophagostomum dentatum* (Nematoda, Strongylidae) Larvae Cultured in Vitro. Yevstafieva, V. A., Panikar, I. I., Melnychuk, V. V., Korchan, L. N., Perederii, N. A. — Morphometric peculiarities of the development of *Oesophagostomum dentatum* Rudolphi, 1803 from egg to infective larva were studied under laboratory conditions at various temperatures. The determined optimum temperature for embryonic and post-embryonic development of *O. dentatum* larvae from domestic pig (*Sus scrofa domesticus* Linnaeus, 1758) is 22 °C. At this temperature, 81 % of larvae develop to the third stage (L3) on the 10th day. Temperatures of 24 °C and 20 °C are less favorable for the development of the nematode, at those temperatures only 67 and 63 % of larvae, respectively, reached infective stage by the 10th day of cultivation. Embryonic development of *O. dentatum* eggs is characterized by their lengthening (by 8.87–9.50 %, $p < 0.01$) and widening (by 6.77–9.35 %, $p < 0.05$–0.01), and post-embryonic larval development is associated with lengthening (by 4.59–17.33 %, $p < 0.01$–0.001).

K e y w o r d s: *Oesophagostomum dentatum*, embryonic development, post-embryonic development, temperature factor, morphometric characteristics.

Introduction

Oesophagostomum dentatum Rudolphi, 1803(Nematoda, Strongylidae) is among the most common intestinal porcine nematodes. It is registered in many countries of the world regardless of the level of development of the livestock industry, and oesophagostomosis is known to cause significant economic losses (Murrell, 1986; Arinkin et al., 2006; Ponomar, Antipov, 1998; Yevstafieva, 2007; Kotkov, 2009).

According to literature (Mozgovoy, 1967; Yamschikov, 2003; Kotelnikov, 1984), *O. dentatum* is wide-spread all over the world including Ukraine, and parasitizes both pigs (*Sus scrofa domestica*) and wild boars (*Sus scrofa scrofa* Linnaeus, 1758). Other species of the genus *Oesophagostomum* were reported in Europe, New Guinea (*O. quadrispinulatum* Marcone, 1901), North America (*O. brevicaudum* Schwartz et Alicata, 1930; *O. georgianum* Schwartz et Alicata, 1930), Northern Africa, Russia, Belarus (*O. longicaudatum* Goodey, 1924), and India (*O. maplestonei* Schwartz 1931; *O. brevicaudatum*) (Scherbovitch, 1940; Várady et al., 1996; Christensen et al., 1997).

Scientists have been studying the development of eggs and larvae of *O. dentatum* in nature since 1908. For example, T. Goodey (Goodey, 1924) described morphological features of separate larval stages of the species, including their size: the length of first-stage larvae was 435 μm, and of the infective larvae were 660–720 μm long. He also found the infective larvae fairly resistant to drying for 1–2 days, and "very active" at 37 °C.

Temperature was proved to influence egg development of *O. dentatum* in the environment (Miasnikova, 1937; Paulikas, 1990; Petrukhin, 2003). Thus, at 20–24 °C larvae develop in the eggs in 42 hours, at higher temperatures (35–40° C) their development is delayed, and at 45–50 °C the eggs are killed. Meanwhile, if the temperature drops to 3 °C, the development is delayed, however, they retain viability (Miasnikova, 1937).

There are reports suggesting that the optimum temperature of egg development in the environment is 25–30 °C. Under such conditions, first-stage larvae develop in 10–24 hours (Popova et al., 1963).

Kaarma's research (Kaarma, 1970, 1977) shows that at 1 to 11°C the eggs stop developing, while at 15–18 °C and 25–26 °C the first-stage larvae are observed in the eggs in 24 hours and hatch in 48 hours. The development of infective larvae occurs in 7 days at 25–26 °C, in 10 days at 15–18 °C, and at – 5 to – 10 °C most of the larvae die.

Thus, despite the efforts of many scientists to study morpho-biological characteristics of *O. dentatum*, the results of their research are sometimes inconsistent, mostly dating back to 1930–1970s, and do not consider the climatic conditions of different regions and the adaptive ability of helminthes to change the duration of embryonic and post-embryonic development. Hence studying influence of temperature on *O. dentatum* development ab ovo to invasive larvae allows supplying new data on its life and optimal development conditions.

Material and methods

The research was conducted in 2015–2016 at the Laboratory of Parasitology and Veterinary-Sanitary Expertise of the Department of Veterinary Medicine of Poltava State Agrarian Academy.

Eggs of *O. dentatum* were sampled in a laboratory study of pigs from farms of Poltava Region using the Kotelnikov's copro-ovoscopy method (Kotelnikov, 1984).

The obtained eggs were then cultured in Petri dishes according to A. M. Petrov and V. G. Gagarin (Dakhno et al., 2010) in thermostat under different temperatures (20 °C, 22 °C, and 24 °C) for 10 days until the development of third-stage larvae (L3). Each day the samples were examined using copro-ovoscopy flotation (Kotelnikov, 1984) or larvoscopy according to Berman and Orlov (Dakhno, 2010). The obtained material was examined under the light microscope (×100, ×400), and the shape, structure, length and width of embryonic and post-embryonic helminth stages were studied. Altogether 619 eggs and 2381 larvae of *O. dentatum* were studied.

Morphometric parameters of eggs and larvae of *O. dentatum* in culture were analyzed using ImageJ for Windows® (version 2.00) in interactive mode using 16× objective and 10× photo eyepiece. Photomicrographs were taken using a 3Mpix digital camera mounted on the MikroMed (China) microscope. Statistical processing of the experimental results was carried out using the Student t-test (Lapach et al., 2001).

Results and discussion

The third-stage larvae (L3) in experimental cultures developed in 10 days, their development occurred at different rates and, in a certain way, depended on the temperature factor. Embryonic and post-embryonic development of *O. dentatum* includes six tentatively identified stages: fragmentation of blastomeres in the egg; formation of an embryo in the egg; formation of a mobile larva in the egg (embryonic); the L1, L2, and L3 developmental stages (postembryonic). The optimum temperature for the development of third-stage larvae in *O. dentatum* appeared to be 22 °C (table 1).

At such a temperature, 81 % of eggs developed into L3 and only 19 % of eggs died, of them 2 % stopped their development at the blastomere stage, and 17 % at the L1 stage. At

Table 1. Influence of temperature on embryonic and postembryonic development of *Oesophagostomum dentatum* in laboratory culture, % (n = 100)

	Stage of development	T °C	Day of culture										
			before cultivation	1	2	3	4	5	6	7	8	9	10
Embryonic	Fragmentation of blastomeres	20	100	82	5	5	5	5	5	5	5	5	5
		22	100	49	2	2	2	2	2	2	2	2	2
		24	100	45	4	4	4	4	4	4	4	4	4
	Embryo development	20	–	18	27	9	2	1	–	–	–	–	–
		22	–	42	9	1	–	–	–	–	–	–	–
		24	–	44	7	–	–	–	–	–	–	–	–
	Mobile larva development	20	–	–	47	19	9	3	1	–	–	–	–
		22	–	9	36	7	1	1	–	–	–	–	–
		24	–	11	35	5	–	–	–	–	–	–	–
Post-embryonic	First-stage larva (L1)	20	–	–	21	56	54	5	5	1	–	–	–
		22	–	–	53	44	30	28	–	–	–	–	–
		24	–	–	54	39	24	21	–	–	–	–	–
	Number of dead larvae	20	–	–	–	11	18	20	22	27	28	31	32
		22	–	–	–	7	12	14	14	16	17	17	17
		24	–	–	–	12	18	22	23	25	29	29	29
	Second-stage larva (L2)	20	–	–	–	–	12	66	67	67	17	6	–
		22	–	–	–	39	55	55	49	8	–	–	–
		24	–	–	–	40	54	53	45	7	–	–	–
	Third-stage larva (L3)	20	–	–	–	–	–	–	–	–	50	58	63
		22	–	–	–	–	–	–	35	74	81	81	81
		24	–	–	–	–	–	–	28	64	67	67	67

22 °C in the first day of culture, fragmentation of blastomeres occurred in 49 % (fig. 1, *a*), formation of larvae in 42 % (fig. 1, *b*), and mobile larvae development in 9 % of eggs (fig. 1, *c*).

On the second day of cultivation, 2 % of eggs remained at the fragmentation of blastomeres stage, 9 % eggs were at the formation of embryo stage, 36 % were at the development of mobile larvae stage, and 53 % were hatching L1 (fig. 2, *a*). On the third to fifth days the ratio of L1 gradually decreased from 44 to 28 % due to their transition to L2 (fig. 2, *b*) which numbered 39–55 % at that time. Typically, on the fifth day of cultivation L2 had a distinct intestine (fig. 2, *c*). At that time, 14 % of larvae died and 1–7 % of embryos remained in the eggs.

On the sixth day of experiment, 35 % of all helminthes were L3, and on the tenth day their ratio was 81 % (fig. 3, *a, b*).

The temperatures 24 °C and 20 °C were less favorable for the development: in ten days, 67 and 63 % of larvae reached the L3 stage, respectively (table 1). At these conditions, 33 and 37 % of embryonic and post-embryonic stages of *O. dentatum* died, respectively (4 and 5 % as eggs, 29 and 32 % as larvae).

At 24 °C on the first day of cultivation 45 % of eggs were at the stage of blastomere fragmentation, 44 % at the embryo formation, and 11 % at the mobile larvae formation. On the second day, 4 % of eggs were still at the blastomere fragmentation stage, 7 % at the embryo formation, and 35 % at the mobile larvae formation stage. Meanwhile, 54 % of L3 developed. On the third to fifth days, the numbers of L1 gradually decreased (from 39 to 21 %), and the numbers of L2 conversely increased (from 40 to 53 %). During that period, 22 % of larvae died, and 4 % of eggs stopped developing. During sixth to tenth days, the numbers of L3 increased as follows: on the sixth day 28 %; on the seventh day 64 %; on the eight to tenth days 67 %. At the same time, 29 % of larvae died, and 4 % of eggs stopped developing.

At 20 °C, embryonic and post-embryonic development of *O. dentatum* was faster but a significant amount of individuals died in culture. For example, on the first day of cultivation, 82 % of eggs were at the blastomere fragmentation stage, and 18 % were at the embryo formation stage. On the second day, 5 % of eggs remained at the blastomere fragmentation stage, 27 % at embryo formation stage, 47 % at the mobile larva formation stage, 21 % at

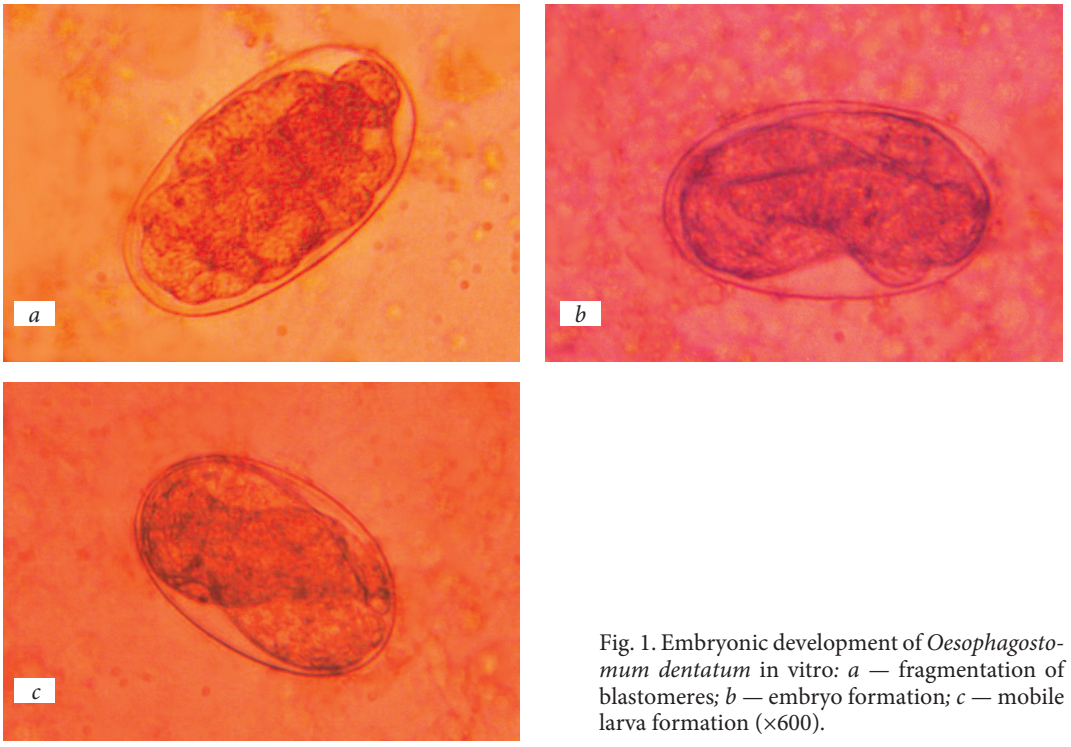

Fig. 1. Embryonic development of *Oesophagostomum dentatum* in vitro*: a* — fragmentation of blastomeres*; b* — embryo formation*; c* — mobile larva formation (×600).

Fig. 2. Post-embryonic development of *Oesophagostomum dentatum* in vitro: *a* — L1 (×400); *b* — L2; *c* — development of intestine cells in L2 (×100).

the development and hatching of L1. On the third to seventh days, intense formation of L2 (12–67 %) was detected. At that time, 27 % of larvae died, 5 % of eggs stopped developing. On the eighth to tenth day, L2 formation was observed (50–63 %); 5 % of embryonic and 32 % of post-embryonic *O. dentatum* died simultaneously.

Analysis of the morphometry of *O. dentatum* during its embryonic and post-embryonic development showed that at different temperatures, changes in the parasite's length and width evidencing its growth were observed. For example, at 22 °C and 24 °C in the cultures, the length of *O. dentatum* eggs began to grow significantly: in the first day by 8.87–9.26 % (76.90 ± 1.39–77.23 ± 1.23 μm, $p < 0.01$), in the second day by 9.22–9.43 % (77.20 ± 1.30–77.38 ± 1.17 μm, $p < 0.01$), in the third day by 9.49–9.50 % (77.20 ± 1.30–77.38 ± 1.17 μm, $p < 0.01$) compared with the pre-culture values (70.08 ± 1.84 μm) (table 2).

Simultaneously, the width of *O. dentatum* eggs also changed. At 20 °C it significantly grew in the first and second days of experiment by 6.77 μm (8.55 %): 44.18 ± 0.17 and 45.04 ± 0.75 μm, $p < 0.05$ compared with pre-culture values (41.19 ± 1.22 μm). At 22 °C, egg width increased in the first day by 8.85 % (45.19 ± 0.57 μm, $p < 0.01$), in the second

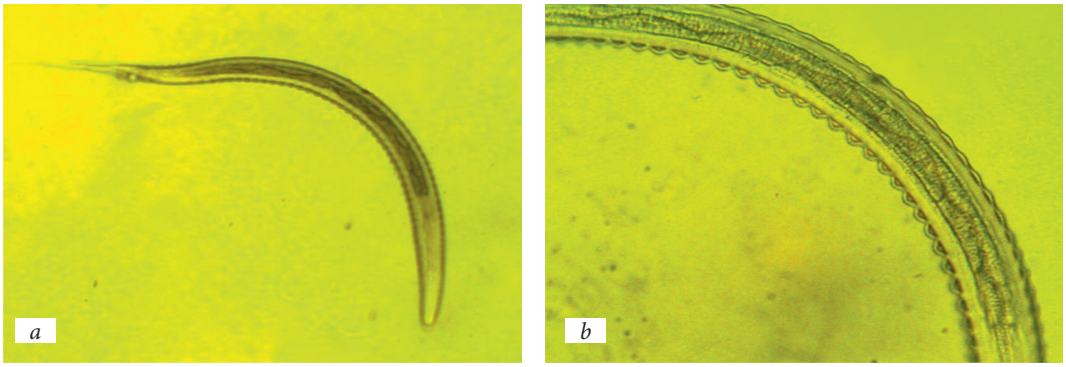

Fig. 3. Formation of *Oesophagostomum dentatum* L3 in vitro: *a (×100); b (×400).*

Table 2. Morphometric characteristics of *Oesophagostomum dentatum* eggs in embryonic development in vitro (M ± m, n = 10)

Characteristics, μm	Day of experiment	Before culture	Temperature		
			20 °C	22 °C	24 °C
Egg length	first	70,08 ± 1,84	73.47 ± 1.01	76.90 ± 1.39**	77.23 ± 1.23**
	second		74.33 ± 2.04	77.20 ± 1.30**	77.38 ± 1.17**
	third		75.06 ± 1.67	77.44 ± 1.29**	77.43 ± 1.26**
Egg width	first	41,19 ± 1,22	44.18 ± 0.17*	45.19 ± 0.57**	45.41 ± 0.57**
	second		44.19 ± 0.86	45.36 ± 0.42**	45.41 ± 0.55**
	third		45.04 ± 0.75*	45.44 ± 0.47**	45.43 ± 0.58**

* $p < 0.05$; ** $p < 0.01$ — compared with values before culture.

Table 3. Morphometric characteristics of *Oesophagostomum dentatum* larvae in post-embryonic development in laboratory culture (M ± m, n = 10)

Larval stages	Characters, μm	Temperature		
		20 °C	22 °C	24 °C
First-stage larva (L1)	Length	471.93 ± 14.23	551.72 ± 7.03	545.04 ± 6.64
	Width	27.16 ± 0.85	29.91 ± 0.75	28.46 ± 0.54
Second-stage larva (L2)	Length	541.61 ± 2.00***	566.17 ± 2.49	545.12 ± 10.40
	Width	27.04 ± 0.89	30.36 ± 0.50	29.08 ± 0.50
Third-stage larva (L3)	Length	570.87 ± 2.24***▪▪▪	578.29 ± 3.21** ▪▪	576.79 ± 3.18***▪▪
	Width	27.04 ± 0.59	30.52 ± 0.31	29.14 ± 0.50

** $p < 0.01$; *** $p < 0.001$ — compared with L1 values;
▪▪ $p < 0.01$; ▪▪▪ $p < 0.001$ — compared with L2 values.

day by 9.19 % (45.36 ± 0.42 μm, $p < 0.01$), in the third day by 9.35 % (45.44 ± 0.47 μm, $p < 0.01$). At the same time, at 24 °C egg width increased during the first and second days of development by 9.29 % (45.41 ± 0.57 and 45.41 ± 0.55 μm, $p < 0.01$), at the third day by 9.33 % (45.43 ± 0.58 μm, $p < 0.01$).

Various temperatures unequally influenced the development and morphometric characteristics of *O. dentatum* larvae (table 3).

At 20 °C, the length of L2 and L3 increased by 12.86 % (541.61 ± 2.00 μm, $p < 0.001$) and 17.33 % (570.87 ± 2.24 μm, $p < 0.001$), respectively, compared with L1, and by 5.13 % ($p < 0.001$) compared with L2. At 22 °C, the L3 length significantly increased by 4.59 % (578.29 ± 3.21 μm, $p < 0.01$ compared with L1 values) and by 2.09 % ($p < 0.01$ compared with L2 values).

At 24 °C the L3 length increased by 5.50 % (576.79 ± 3.18 μm, $p < 0.001$ compared with L1 length) and by 5.49 % ($p < 0.01$ compared with L2).

Summarizing the results of the research, we conclude that the temperature factor significantly affects the rates of embryonic and post-embryonic development of *O. dentatum* larvae and is one of the factors limiting their survival. It was proved that the most favorable temperature for laboratory cultivation of *Oesophagostomum* eggs from pigs, allowing for formation of the highest amount of third-stage larvae (L3) in ten days, is 22 °C. Our data are consistent with the results of research of Miasnikova (1937) that showed larval formation in *O. dentatum* eggs at 20–24 °C in 42 hours. At the same time, most authors pointed out that the optimal and most favorable for release of larvae from the eggs temperature ranges from 23 to 25 °C (Popovaet al., 1963, 1967).

We also present new data on morphometric characteristics of *O. dentatum* in its embryonic and post-embryonic development. The embryonic development occurs within three days and is characterized by increase in egg length by 9.50 % and egg width by 9.35 %. In the post-embryonic development, larvae grow by 17.33 % with minor fluctuations in their width.

Conclusion

The temperature factor is one of the indicators affecting the development and survival of embryonic and larval stages of *O. dentatum* in the environment and, therefore, it is crucial to its survival. Optimum temperature for cultivation of *O. dentatum* eggs to the third-stage infective larva is 22 °C, the larvae develop in ten days with 81 % survival. The embryonic development of these helminthes is characterized by increasing egg sizes (length by 8.87–9.50 %, $p < 0.01$, and width by 6.77–9.35 %, $p < 0.05$–0.01), and post-embryonic development by increasing larval length (by 4.59–17.33 %, $p < 0.01$–0.001).

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