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# OBSERVATIONS ON THE EMBRYONIC DEVELOPMENT OF *TRICHURIS SYLVILAGI* (NEMATODA, TRICHURIDAE) UNDER LABORATORY CONDITIONS

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**Observations on the Embryonic Development of *Trichuris sylvilagi* (Nematoda, Trichuridae) under Laboratory Conditions. Yevstafieva, V., Melnychuk, V., Nagorna, L., Stybel, V., Gutyj, B., Yatsenko, I., Petrenko, M., Nikiforova, O., Filonenko, S., Savenkova, O., Tahiltseva, Ya.** — In the present study, we observed *in vitro* the rate and stages of exogenous development of the nematode *Trichuris sylvilagi* Tiner, 1950 isolated from the European hare, *Lepus europaeus*. The viability of eggs and embryos at different temperatures was investigated. It was found that the most favorable temperature for embryonic development of the species was 30 °C, with the formation of 88.67 % of viable eggs with a motile larva in 20 days at laboratory conditions. With a decrease in temperature to 20 °C, the embryogenesis occurred in 32 days. The viability of eggs cultured at 20 °C decreased: 68.0 % of eggs reached larval stage, and 32.0 % of eggs died. At 10 °C eggs did not develop to infective stage. On the 32nd day of cultivation at 10 °C, 27.33 % of eggs remained at the zygote stage, 52.0 % at the stage of blastomere cleavage and formation, and 20.67 % died. Embryogenesis was characterized by metric changes in egg parameters. At optimal temperature, the growth and development of eggs was accompanied by an increase in the egg and plugs' width with a simultaneous decrease in their length, as well as with thinning of the shell.
Key words: *Trichuris sylvilagi*, nematode, eggs, exogenous development, temperature, viability.

**Introduction**

Parasitic nematodes of the genus *Trichuris* Röderer, 1761 are intestinal parasites of a number of mammalian hosts, including domestic animals and humans (Callejón et al., 2016; Adriko et al., 2018; Susana et al., 2019).

Two species of the genus, *T. sylvilagi* Tiner, 1950 and *T. leporis* Zeder, 1803 parasitize hares of the genus *Lepus* Linnaeus, 1758 and are among the most common helminths of wild hares. For example, 39.8 % of the studied European hares (*Lepus europaeus*) in Czech Republic harbored *T. leporis* (Lukešová et al., 2012). In Belarus, the prevalence of *T. leporis* infection was 13.2 %, and in Germany it reached 66.7 % (Haupt & Hartung, 1976; Shimalov, 2001). In northern Alberta (Canada), the prevalence of infection of snowshoe hares (*Lepus americanus*) with *T. leporis* ranged from 13 to 77 % during the year, and in Poland the prevalence of *T. syluilagi* in the European hare population ranged from 47 to 85 % (Czaplińska et al., 1965; Keith et al., 1986). In northern California, 4 % of the studied black-tailed jackrabbits (*Lepus californicus*) were infected with *T. sylvilagi* (Clemons et al., 2000). The same species was found in eastern coattails (*Sylvilagus floridanus*) in Italy, with a prevalence of 3.2 % (Tizzani et al., 2020). At the same time, *Trichuris* sp. was found in only 1.82 % of domestic rabbits kept on Ankara farms (Turkey) (Gürler & Doğanay, 2007). In Iraq, *T. leporis* has been identified in domestic rabbits, where its infection prevalence was 30.4 % (Al-Moula, 2005).

Among the factors favoring the wide distribution of parasitic nematodes of the genus *Trichuris* is their viability at various stages of development. The parasites develop in the host's organism and in the external environment. *Trichuris* spp. are geohelminths that do not require intermediate hosts, and a significant part of their life cycle at the egg stage takes place in the external environment (Beer, 1973; Hurst & Else, 2013). There, the eggs embryonate and become infective for the host when the larvae are fully formed (Anderson, 2000). The viability rate of *Trichuris* spp. in the external environment depends on different factors such as humidity, temperature, the presence of vegetation, the degree of insolation, as well as on the level of protection of the embryo from unfavorable environmental factors (Beer, 1976; Bundy & Cooper, 1989; Manz et al., 2017). The egg shell of *Trichuris* spp. is quite strong, consists of several layers which have different structure and, in addition to mechanical protection, provides the resistance to chemicals (Meng et al., 1986; Mahmoud, 2002).

The embryogenesis of *T. sylvilagi* parasitic in lagomorphs is insufficiently studied. There is very little information on the terms of the formation of infective eggs and the influence of abiotic factors on their viability during the embryo development. Therefore, the purpose of our work was to establish the rate and stages of the development of eggs of *T. sylvilagi* under laboratory conditions, as well as the effect of temperature on the degree of their viability during embryogenesis.

**Material and methods**

During 2020, the large intestines of 8 gray hares (*Lepus europaeus* Pallas, 1778) were examined for the presence of intestinal parasites. Wild animals were obtained by hunters during the hunting season in the Sumy and Poltava regions of Ukraine. The nematodes isolated from the intestines were identified as *T. syluilagi* using the identification key of Gvozdev et al. (1970). Mature female worms collected from the intestine were dissected under a stereoscope in Petri dishes with distilled water to isolate eggs from their gonads. Females were cut lengthwise with a tip of dissecting needle in the vulvar area to obtain gonads filled with eggs. Then the gonads were transferred to saline solution and destroyed to release the eggs. The eggs were transferred to Petri dishes (5 cm in diameter) with 0.9 % saline solution (minimum 50 eggs per Petri dish) for further development. Cultivation of eggs was performed in a thermostat at various temperatures: 10 °C, 20 °C, and 30 °C. Egg cultures were examined under a microscope every four days. The degree of development of eggs was determined morphologically. The stages of egg development and the terms of the formation of larvae in eggs were identified; the number of mature and dead eggs was counted. Each experiment was carried out in triplicate.

The metric parameters of *T. syluilagi* eggs (length and width; length and width of plug; shell thickness) were studied at the zygote and larval stage using ToupView software version × 64, 4.10.17015.20200426 (Hangzhou ToupTek Photonics Co., Ltd, China) and AxioVision, Release SPS 4.8.2 (Carl Zeiss MicroImaging GmbH, Germany). Microphotography was performed using a digital camera attached to a MICROmed 5 Mpix microscope (China) and SIGETA M3CMOS 14000 14.0 MP (China).

Standard deviation (SD) and average values (M) were calculated. Significance of the difference between average values in the studied *T. syluilagi* eggs was calculated using one-way analysis of variance and F-test for 95 % confidence level.

## Results and discussion

The development of *T. syluilagi* eggs obtained from *L. europaeus* in experimental culture occurred in separate stages characterized by certain morphological changes. The zygote stage was characterized by the evenly distributed contents without segmentation in the egg. The presence of pronucleus was also noted (fig. 1, *a*). The stage of blastomere cleavage was characterized initially by the formation of two, almost equal in size, blastomeres (fig. 1, *b*). Subsequently, the cleavage process continued, the number and densification of blastomeres increased while their

size decreased (fig. 1, *c*). At the next stage, the blastomeres were no longer visible, the embryo was bean-shaped and denser at the egg poles (fig. 1, *d*). Then it stretched unevenly in a tadpole shape (fig. 1, *e*). The growth and development of the embryo continued, it stretched even more, thinned, elongated and transformed into a larva, which actively moved when heated (fig. 1, *f*).

The terms of embryogenesis and the viability of *T. syluilagi* eggs depended on the temperature. At a temperature of 10 °C, eggs stopped developing at the stage of zygote cleavage and the formation of blastomeres and did not reach the infective stage during 32 days of cultivation. At this temperature, 20.67 ± 6.11 % of the eggs died (table 1).

On the 4th day of cultivation, 95.33 % of the eggs were at the zygote stage, and 4.67 % of the eggs already contained two blastomeres. On 8th day, 2.67 % of the eggs contained more than two blastomeres, 10.67 % were still at the stage of two blastomeres, and 86.67 % were at the zygote stage. Subsequently, the number of eggs with zygote gradually decreased, and on the 32nd day of cultivation, 27.33 % of such eggs remained. At the same time, the number of eggs at the stage of two or more blastomeres increased up to 32 days, when 39.33

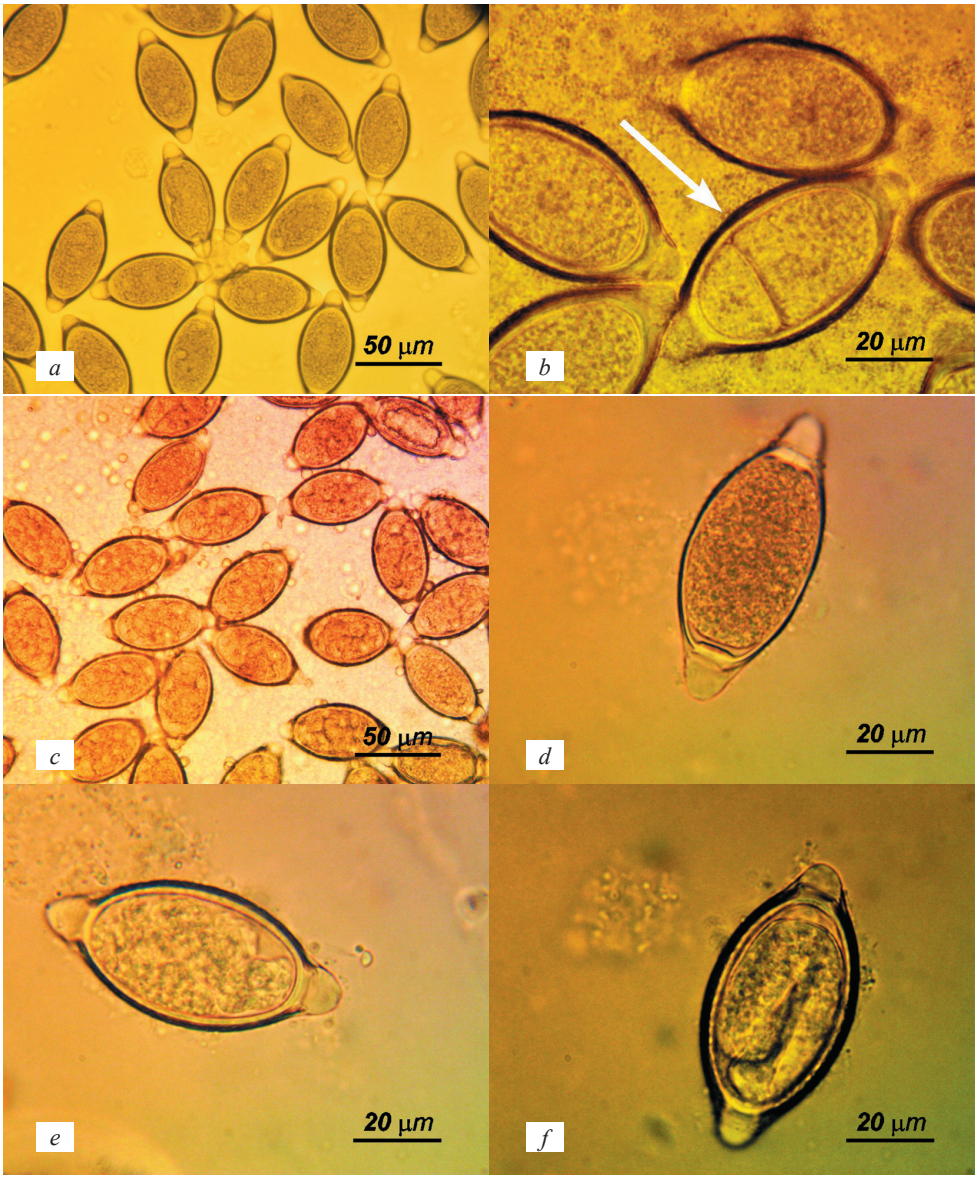


Fig. 1. Stages of embryonic development *Trichuris sylvilagi*: *a* — zygote; *b* — formation of two blastomeres; c — blastomere cleavage; *d* — bean-shaped embryo; *e* — tadpole-shaped embryo; *f* — larva.

**Table 1. Embryonic development of eggs of *Trichuris sylvilagi* nematodes at 10 °C, M ± SD**

| Day of cultivation | Stage of egg development, % | | | | | | Egg death, % |
|---|---|---|---|---|---|---|---|
| | Zygote | Cleavage and blastomere formation | | Beansha-ped embryo | Tadpole-shaped embryo | Forma-tion of larva | |
| | | two | more than two | | | | |
| Before cultivation | 100.00 | – | – | – | – | – | – |
| 4 | 95.33 ± 3.06 | 4.67 ± 3.06 | – | – | – | – | – |
| 8 | 86.67 ± 4.16 | 10.67 ± 5.03 | 2.67 ± 1.15 | – | – | – | – |
| 12 | 72.00 ± 8.00 | 22.00 ± 6.00 | 6.00 ± 2.00 | – | – | – | – |
| 16 | 61.33 ± 15.53 | 32.00 ± 12.49 | 6.67 ± 3.06 | – | – | – | – |
| 20 | 38.67 ± 9.24 | 39.33 ± 6.11 | 6.67 ± 3.06 | – | – | – | 15.33 ± 3.06 |
| 24 | 35.33 ± 7.02 | 38.00 ± 4.00 | 8.67 ± 3.06 | – | – | – | 18.00 ± 4.00 |
| 28 | 27.33 ± 6.11 | 40.67 ± 2.31 | 11.33 ± 2.31 | – | – | – | 20.67 ± 6.11 |
| 32 | 27.33 ± 6.11 | 39.33 ± 1.15 | 12.67 ± 3.06 | – | – | – | 20.67 ± 6.11 |

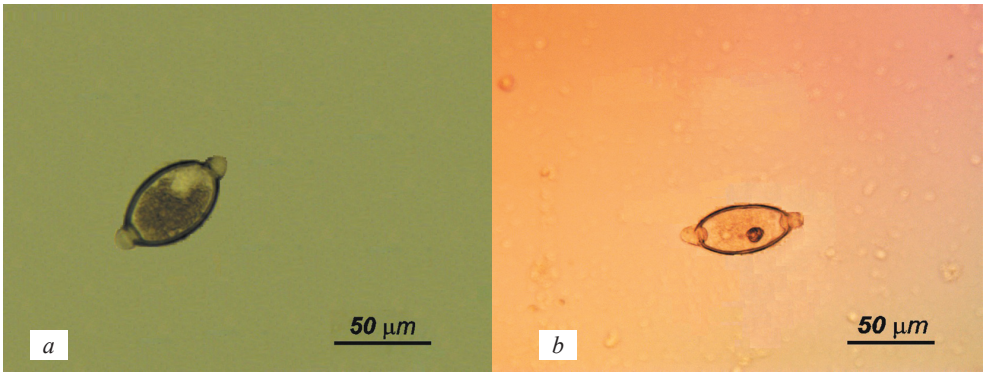


Fig. 2. Morphological indicators of death of *Trichuris sylvilagi* eggs during cultivation.

and 12.67 % of such eggs were detected. From the 20th day of cultivation, dead eggs were detected in the amount of 15.33 %, where their number increased and on the 28th day it was 20.67 %. The dead eggs were morphologically distinct by the loosening and melting of the embryo (fig. 2, *a*), wrinkling and lysis of the embryo (fig. 2, *b*).

At a temperature of 20 °C, embryogenesis of T. syluilagi ended in 32 days, when 68.0 % of the eggs reached the infective stage with the formation of larva, while 32.0 % of the eggs died during cultivation (table 2).

**Table 2. Embryonic development of eggs of *Trichuris sylvilagi* nematodes at 20 °C, M ± SD**

| Day of cul-tivation | Stage of egg development, % | | | | | | Death of eggs, % |
|---|---|---|---|---|---|---|---|
| | Zygote | Cleavage and blastomere formation | | Bean-shaped embryo | Tadpole-shaped embryo | Formation of larva | |
| | | two | more than two | | | | |
| Before cultivation | 100.00 | – | – | – | – | – | – |
| 4 | 88.00 ± 3.46 | 8.67 ± 1.15 | 3.33 ± 2.31 | – | – | – | – |
| 8 | 48.00 ± 4.00 | 24.67 ± 3.06 | 27.33 ± 7.02 | – | – | – | – |
| 12 | 36.00 ± 5.29 | 27.33 ± 3.06 | 28.67 ± 9.87 | 8.00 ± 2.00 | – | – | – |
| 16 | 32.00 ± 5.29 | 9.33 ± 3.06 | 30.67 ± 5.03 | 20.67 ± 3.06 | 7.33 ± 3.06 | | |
| 20 | – | – | 10.00 ± 4.00 | 34.00 ± 4.00 | 24.00 ± 5.29 | – | 32.00 ± 5.29 |
| 24 | – | – | – | 5.33 ± 3.06 | 32.00 ± 4.00 | 30.67 ± 6.11 | 32.00 ± 5.29 |
| 28 | – | – | – | – | 8.67 ± 3.06 | 59.33 ± 6.11 | 32.00 ± 5.29 |
| 32 | – | – | – | – | – | 68.00 ± 5.29 | 32.00 ± 5.29 |

Thus, on the 4th day of cultivation, 88.0 % of the eggs were at the zygote stage, 8.67 % were at the stage of formation of two blastomeres, and 3.33 % were at the stage of cleavage and formation of more than two blastomeres. Subsequently, the number of eggs at the zygote stage gradually decreased and on 20th day such eggs were not detected in culture. The stage of formation of two blastomeres was recorded from 4th to 16th days, their maximum number (24.67–27.33 %) was detected within 8th–12th days. The stage of further cleavage lasted from 4th to 20th days, and their maximum number (28.67–30.67 %) was detected within 12th–16th days.

The tadpole stage lasted from 16th to 28th days of cultivation, and the largest number of eggs with tadpole-shaped embryo (24.0–32.0 %) was detected during the 20th–24th days. The formation of infective eggs with a mobile larva was recorded from 24th to 32nd days, when their number gradually increased from 30.67 to 68.0 %. Egg death was observed from the 20th day of cultivation and did not exceed 32.0 %.

At a temperature of 30 °C, the whole embryogenesis occurred in the shortest time and lasted up to 20 days. The viability rate of was the highest, 88.67 % of eggs contained motile larvae. The rate of egg death during cultivation was just 11.33 % (table 3).

On the 4th day of cultivation, 44.67 % of the eggs were at the zygote stage, 42.67 % were at the stage of two blastomere formation, and 12.67 % were at the stage of cleavage and the formation of more than two blastomeres. The zygote stage lasted only 4–8 days of cultivation. The stage of formation of two blastomeres was recorded from 4th to 12th days; their maximum number was detected on the 4th day. The stage of cleavage and formation of more than two blastomeres lasted from 4th to 12th days; the maximum number of eggs at that stage (24.00–24.67 %) was detected within 8th–12th days. The formation of a bean-shaped embryo was detected from 8th to 16th days of cultivation. The largest number of such eggs (43.33 %) was recorded on the 12th day. The tadpole stage lasted from 12th to 16th days of cultivation, their rate varied from 18.0 to 11.33 %. The formation of infectious eggs with a mobile larva was recorded on the 16th–20th days. Their number gradually increased from 72.0 to 88.67 %. The death of eggs was observed beginning from the 12th day of cultivation, with the rate not exceeding 11.33 %.

The growth and development of eggs was accompanied by a change in their metric parameters. Thus, 30 °C was the optimal temperature for egg development, at which significant decrease was seen in the length of eggs, by 2.61 % (72.09 ± 1.79 μm, $P < 0.01$) (fig. 3, *a*), and in the length of plug decreased by 7.05 % (8.70 ± 0.86 μm, $P < 0.05$) (fig. 3, *c*) in eggs at the larval stage relative to eggs at the zygote stage (74.02 ± 1.44 and 9.36 ± 0.84 μm, respectively). At the same time, an increase was observed in several metric parameters of mature eggs: egg width grew by 5.04 % (32.97 ± 1.53 μm, $P < 0.001$) (fig. 3, *b*) and the egg plug width increased by 12.55 % (11.78 ± 0.45, $P < 0.001$) (fig. 3, *d*) compared with corresponding parameters of

**Table 3. Embryonic development of eggs of *Trichuris sylvilagi* nematodes at 30 °C, M ± SD**

| Day of cultivation | Stage of egg development, % | | | | | | Death of eggs, % |
|---|---|---|---|---|---|---|---|
| | Zygote | Cleavage and blastomere formation | | Bean-shaped embryo | Tadpole-shaped embryo | Formation of larva | |
| | | two | more than two | | | | |
| Before cultivation | 100.00 | – | – | – | – | – | – |
| 4 | 44.67 ± 17.01 | 42.67 ± 14.47 | 12.67 ± 3.06 | – | – | – | – |
| 8 | 15.33 ± 3.06 | 22.00 ± 4.00 | 24.67 ± 5.03 | 38.00 ± 7.21 | – | – | – |
| 12 | – | 3.33 ± 2.31 | 24.00 ± 8.00 | 43.33 ± 1.15 | 18.00 ± 4.00 | – | 11.33 ± 4.16 |
| 16 | – | – | – | 5.33 ± 4.16 | 11.33 ± 2.31 | 72.00 ± 5.29 | 11.33 ± 4.16 |
| 20 | – | – | – | – | – | 88.67 ± 4.16 | 11.33 ± 4.16 |

eggs at the zygote stage (34.72 ± 1.05 and 13.47 ± 0.66 μm, respectively). Also, in the process of embryogenesis, the membrane thinned by 3.25 % (1.23 ± 0.03 μm, P < 0.001) (fig. 3, *e*).

Thus, the present study has showed the features of the embryonic development of *T. sylvilagi* isolated from *Lepus europaeus*, identified the stages of embryogenesis and the effect of temperature on the terms of the formation and viability of infective eggs containing larvae. The developmental cycle of this nematode species has not been studied previously. However, there are reports describing the development of a related species *T. leporis* Zeder, 1803, ac-

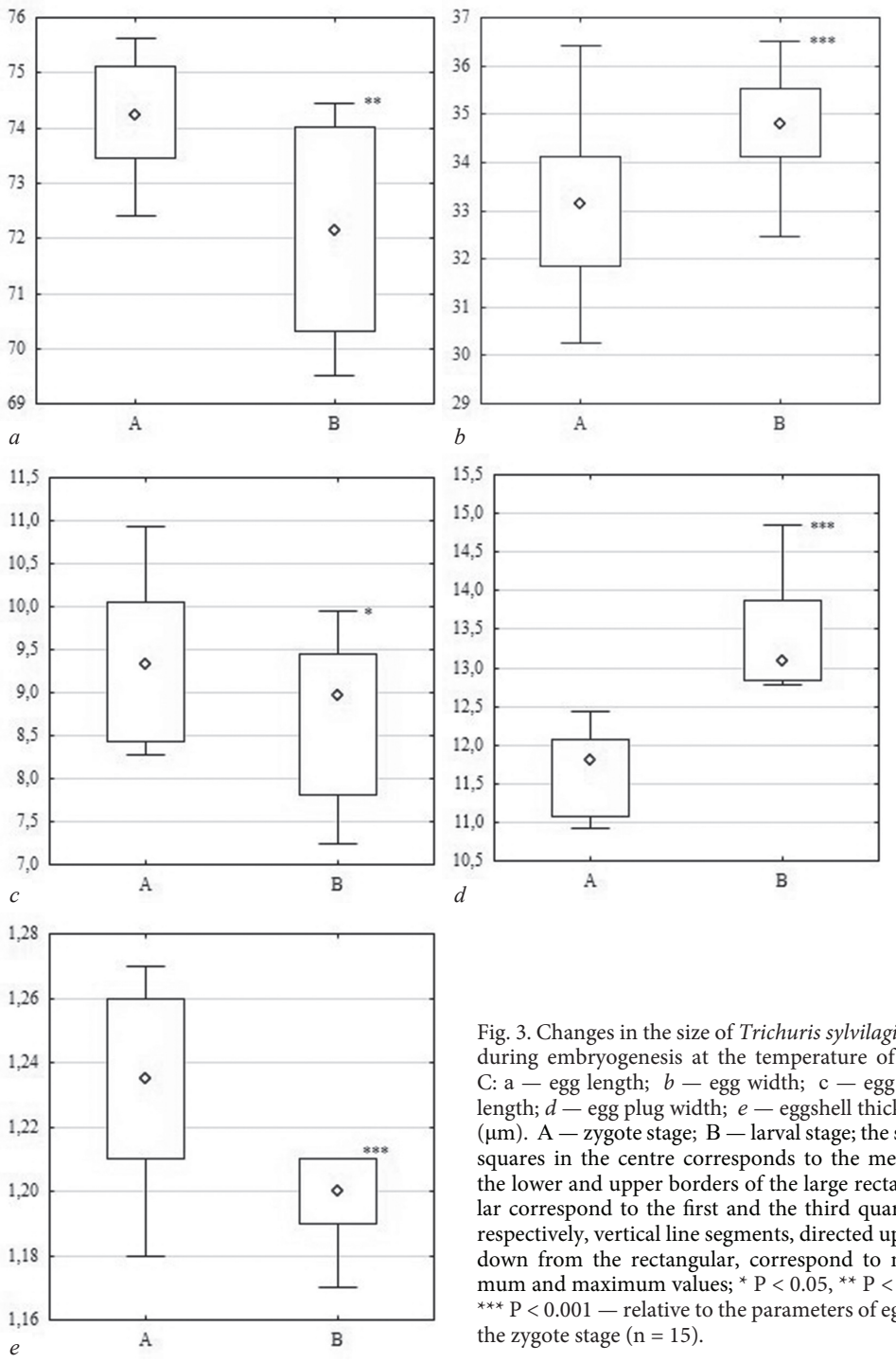


Fig. 3. Changes in the size of *Trichuris sylvilagi* eggs during embryogenesis at the temperature of 30 °C: a — egg length; *b* — egg width; c — egg plug length; *d* — egg plug width; *e* — eggshell thickness (μm). A — zygote stage; B — larval stage; the small squares in the centre corresponds to the median, the lower and upper borders of the large rectangular correspond to the first and the third quartiles, respectively, vertical line segments, directed up and down from the rectangular, correspond to minimum and maximum values; * P < 0.05, ** P < 0.01, *** P < 0.001 — relative to the parameters of eggs at the zygote stage (n = 15).

cording to which the formation of infective larva occurs in situ at 25 °C in 49 days (Gvozdev et al., 1970). Also, many authors point to the influence of abiotic factors, including temperature, on the timing of the development of eggs of parasitic nematodes and their viability in the environment (Bundy & Cooper, 1989; Pullan et al., 2011; Manz et al., 2017).

We found that the temperature affects the duration of embryogenesis and the viability rate of eggs in *T. sylvilagi*. Under laboratory conditions, 30 °C was found to be the optimal temperature for the development and formation of the maximum number of infective eggs (up to 88.67 %). Development occurred in 20 days. When the temperature decreased to 20 °C, the duration of development slowed down to 32 days, and the number of formed infective eggs decreases to 68.0 %. At 10 °C, the eggs of the *T. sylvilagi* stopped developing at the stage of blastomere formation and did not develop to the infective stage. Similar findings were obtained in the studies of the embryogenesis of *T. globulosa* Linstow, 1901 from cattle (Yevstafieva et al., 2020). The authors note that the optimal temperature for the development of eggs was 25 °C, with 76.3 % of infective eggs formed by 48 days. A decrease in temperature to 20 °C and an increase to 30 °C led to a decrease in the viability of eggs.

We also identified six stages of embryonic development in *T. sylvilagi*, each with certain morphological features: zygote, formation of two blastomeres, blastomere cleavage, formation of a bean-shaped embryo, formation of a tadpole-shaped embryo, and formation of a larva. At the same time, the developmental stages of other species of Trichurida are slightly different, which, in our opinion, indicates the specificity and uniqueness of each individual parasite species (Fataliev, 2013; Melnychuk & Berezovsky, 2018; Yevstafieva et al., 2019). The degree of development of *T. sylvilagi* eggs is evidenced not only by their morphological changes, but also by changes in their metric parameters. Thus, the infective eggs during embryogenesis became shorter and wider with shorter and wider plugs, and with a thinner shell, which, most likely, was a necessary factor for the normal development of the larva. Similar data on metric changes in eggs of nematodes of the genus *Trichuris* isolated from sheep, pigs, and cattle have already been described in our previous works, but those changes were specific for each particular species (Yevstafieva et al., 2015; Melnychuk & Berezovsky, 2018; Yevstafieva et al., 2019). The obtained findings expand the existing data on the biological characteristics of *T. sylvilagi*. This is the first such study conducted in Ukraine.

## Conclusion

The embryonic development of the nematode *T. sylvilagi* from *Lepus europaeus* is characterized by morphological and metric changes, and the terms of the formation and viability of infective eggs depend on temperature. Under laboratory conditions, the temperature of 30 °C was found to be the optimal for the development of nematode eggs of the studied species. At that temperature, the embryogenesis occurred in 20 days with the formation of 88.67 % of viable eggs containing larvae. At lower temperature, the egg development slowed down, and the number of viable mature eggs decreased. At the temperature of 10 °C, the development of eggs stopped at the stage of cleavage and formation of blastomeres and the eggs did not reach the infective stage. The growth and development of eggs is accompanied by a decrease of egg length and plug length, along with simultaneous increase of egg width and plug width, and with a thinning of the shell.

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