Vestnik zoologii, **39**(6): 19—24, 2005

UDC 595.132:595.771

EXPERIMENTAL INFECTION OF MOSQUITOES WITH *DIROFILARIA REPENS* (NEMATODA, FILARIOIDEA) LARVAE

Yu. Kuzmin[1], E. Varodi[1], N. Vasylyk[2], G. Kononko[3]

[1] *Schmalhausen Institute of Zoology of NAS of Ukraine, vul. B. Khmelnits'kogo, 15, Kyiv, 01601 Ukraine*
E-mail: yurkuzmin@yandex.ru
[2] *National Agricultural University, Kyiv, Ukraine*
[3] *Taras Shevchenko University, Kyiv, Ukraine*

Accepted 21 December 2004

Experimental Infection of Mosquitoes with *Dirofilaria repens* (Nematoda, Filarioidea) Larvae. Kuzmin Yu., Varodi E., Vasylyk N., Kononko G. — Gravid females of three mosquito species: *Anopheles maculipennis atroparvus*, *Culex pipiens molestus* and *Aedes aegypti*, all from laboratory cultures, were experimentally infected with microfilaria of *Dirofilaria* repens from blood of a domestic dog from vicinity of Kyiv (Ukraine). Larval stages of the nematode successfully developed in all three vector species at room temperature 18—29°C. Microfilaria migrated from the stomach and intestine to the Malpighian tubules of mosquitoes during first three days post infection (pi). Quiescent first-stage larvae ("sausage stage") were first observed on day 4 pi; in some specimens of *Ae. aegypti* this stage remained in Malpighian tubules till day 21 pi (end of observations). Second stage larvae were observed from day 6 till day 19 pi. Third stage larvae were first observed in Malpighian tubules of all three mosquito species on day 10 pi; on days 14 and 16 pi they were first found in body cavity, salivary glands and proboscis. The following values of vector efficiency index were calculated for each mosquito species: 11.9% in *A. maculipennis*, 7.9% in *Ae. aegypti* and 1.9% in *C. pipiens*. General morphology of experimentally obtained *D. repens* larval stages is described. *A. maculipennis atroparvus* and *C. pipiens molestus* are supposed to participate in the *D. repens* transmission under natural conditions in Ukraine.

Key words: *Dirofilaria repens*, Filarioidea, development, vector, *Anopheles maculipennis atroparvus, Culex pipiens molestus, Aedes aegypti*, domestic dog.

Экспериментальное заражение кровососущих комаров личинками *Dirofilaria repens* (Nematoda, Filarioidea). Кузьмин Ю., Вароди Э., Василик Н., Кононко А. — Гоноактивные самки комаров *Anopheles maculipennis atroparvus, Culex pipiens molestus* и *Aedes aegypti* из лабораторных культур были экспериментально заражены микрофиляриями *Dirofilaria repens* из крови собаки из окр. Киева (Украина). Во всех трех видах переносчиков происходило развитие личиночных стадий нематоды при комнатной температуре 18—29°C. Микрофилярии мигрировали из желудка и кишечника в мальпигиевы сосуды комаров в первые трое суток после заражения. Покоящиеся личинки первой стадии наблюдались в мальпигиевых сосудах начиная с 4-х сут эксперимента; в некоторых особях *Ae. aegypti* эти личинки оставались до 21-х сут эксперимента (окончание наблюдений). Личинки второй стадии обнаруживались с 6-х по 19-е сут. Первые личинки третьей стадии наблюдались в мальпигиевых сосудах на 10-е сут; на 14-е и 16-е сут они проникали в полость тела, слюнные железы и хоботок комаров. Индекс эффективности переносчиков был равен 11,9% у *A. maculipennis*, 7,9% у *Ae. aegypti* и 1,9% у *C. pipiens*. Даны описания личиночных стадий *D. repens* из промежуточных хозяев. Предполагается, что *A. maculipennis atroparvusi* и *C. pipiens molestus* участвуют в циркуляции *D. repens* в естественных условиях в Украине.

Ключевые слова: *Dirofilaria repens*, Filarioidea, развитие, переносчик, *Anopheles maculipennis atroparvus, Culex pipiens molestus, Aedes aegypti*, собака.

Introduction

Dirofilaria (*Nochtiella*) *repens* Railliet et Henry, 1911 is a common subcutaneous parasite of Carnivora, mainly dogs, in South Europe. The species was also reported from Eastern Europe, Middle East, Central and South-Eastern Asia, both Americas, Africa and Australia. *D. repens* can infect human but was not found to mature in this host.

About 20 species of mosquito species were specified as intermediate hosts and vectors of this nematode under experimental conditions (Anderson, 2000). Few of them were proved to be the vectors under natural

conditions. Apparently, different mosquito species participate in *D. repens* transmission in separate parts of its distribution area. In South Europe and some other regions, mosquitoes from the genus *Aedes* were reported as the main vectors of *D. repens* (Mantovani, Restani, 1965; Cancrini et al., 1995; Anyanwu et al., 2000). Khudaverdiev (1976) reported natural infection of *Aedes caspius* and *Culex pipiens* with *D. repens* larvae in Nakhichevan' Republic (Transcaucasia). The development of *D. repens* larvae in the intermediate hosts was experimentally investigated in Germany (Fülleborn, 1908; after Anderson, 2000), Italy (Coluzzi, 1964), Azerbaydzhan (Khudaverdiev, 1976), France (Bain, 1978), Nigeria (Anyanwu et al., 2000) and the USA (Webber, Hawkins, 1955). In Eastern Europe, the biology of the nematode has not been studied.

During the last years, *D. repens* has been often found, both in dogs and in human, in Ukraine. According to the information of Kyiv Municipal Department of State Veterinary Medicine, 15 cases of dog dirofilariasis were registered in 1999, and 254 cases were registered in 2002. Apparently, *D. repens* is successfully transmitted by some local species of mosquito vectors.

In our experimental studies, we successfully infected three mosquito species: *Anopheles maculipennis atroparvusi, Culex pipiens molestus* (both are common in northern part of Ukraine) and *Aedes aegypti* with *D. repens* microfilariae from a dog naturally infected with *D. repens*. The latter mosquito species is absent in the region but is known as appropriate intermediate host of *D. repens* and is widely used as a model object for *Dirofilaria* infection studies. The rate and peculiarities of the nematode larval development in experimentally infected mosquitoes is described herein.

Material and methods

Females of *C. pipiens molestus* (140 specimens), *A. maculipennis atroparvus* (60 specimens) and *Ae. aegypti* (120 specimens) were selected for experimental infections from laboratory cultures. All mosquitoes were free from metazoan parasites prior to experiment. A dog from homeless animals' shelter from Kyiv vicinities was used for mosquitoes' infection. In the dog's blood samples, the density of microfilariae reached 5300 in 1 ml of blood. Gauze-covered cages with mosquitoes were placed on the surface of dog's body and kept for 30—40 minutes. During this period, almost all female mosquitoes have fed on dog's blood *ad libitum*. Those individuals which had not fed (66 — *C. pipiens*, 8 — *A. maculipennis*, and 46 — *A. aegypti*) where removed from cages and thus separated from experimental group. Mosquitoes were kept in gauze-covered cages 15 x 15 x 15 cm, from 50 to 80 specimens in each cage, at mean temperature 25°C (from 18°C to 29°C), under natural lighting. During 21 days of experiment mosquitoes have been fed with 5% sugar solution.

Mosquitoes were dissected daily and examined for the presence of nematode larvae under low-magnification microscope. The larvae were observed *in vivo* in saline and then fixed with 70% alcohol. Live specimens were used for micro-photographs. Fixed and clarified in glycerine specimens were measured and figured. All measurements given below are in micrometers.

Vector efficiency index (Kartman, 1954) was calculated for each mosquito species by dividing the number of third-stage infective larvae by the mean number of microfilariae ingested and converting it to a percentage.

Results

Microfilariae (mf) of *D. repens* were observed in the midgut of *A. maculipennis* and *C. pipiens* till day 3 post infection (pi). In the latter species, only dead mf were found in midgut on the third day pi. In *Ae. aegypti*, mf were observed in midgut during first 2 days pi, and on day 3 pi all mf were found in Malpighian tubules only. Maximum number of mf in midgut was 656 in *A. maculipennis*, 885 in *C. pipiens* and 355 in *Ae. aegypti*. A number of mf was eliminated during their migration from the midgut to Malpighian tubules of all three mosquito species. In the Malpighian tubules, mf were observed from day 3 to day 10 in *C. pipiens* and to day 13 in *A. maculipennis*. In *Ae. aegypti* live and motile mf were observed in Malpighian tubules till the end of experiment (day 21 pi). The decline in mf numbers in infected mosquitoes is shown on figure 1.

Mf observed possessed shape and structure characteristic for this stage, with general body length 310—350 and maximum width 5—7 (n = 15).

During the first three days pi, sufficient mortality of individuals of all three mosquito species was observed. By day 3 pi, 23% of *A. maculipennis*, 13% of *C. pipiens* and 28% of *Ae. aegypti* have died. During the days 4—21, mortality of *A. maculipennis* and *Ae. aegypti* was comparatively much lower (16% and 14%, correspondingly), whereas 45% of *C. pipiens* died during this period.

Quiescent first-stage larvae ("sausage stage") were observed from day 4 to day 11 pi in *A. maculipennis* and from day 6 to day 10 pi in *C. pipiens* (fig. 2, *A*, *B*). In

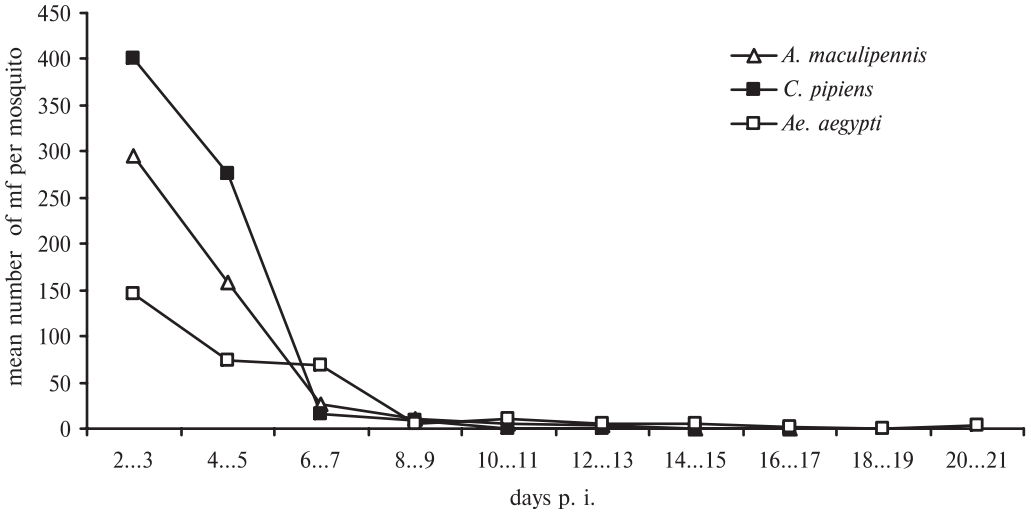

Fig. 1. Changes in number of *D. repens* microfilaria (mf) in experimentally infected mosquitoes.

Рис. 1. Изменение количества микрофилярий (mf) *D. repens* в экспериментально зараженных комарах.

Ae. aegypti, this stage was observed from day 4 pi till the end of experiment (fig. 2, *C*). Number of "sausage stage" larvae and their proportion among all larval stages gradually decreased due to mortality and development. In *Ae. aegypti*, however, number and proportion of "sausage stage" larvae slightly increased on day 16 pi (fig. 2, *C*). Maximum number of the "sausage stage" larvae was 128 in *A. maculipennis*, 108 in *C. pipiens* and 38 in *Ae. aegypti*. The average number of L1 compared to that of mf equalled 14.4% in *A. maculipennis*, 6.4% *C. pipiens* and 7.3% in *Ae. aegypti*.

The "sausage stage" larvae (fig. 3, *A*) had characteristic short and stout body, 150—202 long and 17—20 wide (n = 8). Tail was 62—75 long, including 37—45 of terminal spine. Anal cork was obvious.

The second-stage larvae (L2) were infrequently observed in the mosquitoes examined, due to the comparatively short duration of this stage (fig. 2). L2 were found from day 6 to day 12 pi in *A. maculipennis* and from day 9 to day 11 in *C. pipiens*. In *Ae. aegypti*, the second-stage nematode larvae, similarly to earlier stages, have been observed for longer period, from day 8 to day 19 pi.

L2 (fig. 3, *B*) differed from the previous stage by elongated body, 387—452 long and 25—27 wide (n = 7), shorter tail terminal spine, 32—37 long. Tail length was 62—70, terminal spine included. Anal cork was present.

The third-stage larvae (L3) were first observed in Malpighian tubules of all three mosquito species on day 10 pi (fig. 2). Maximum number of L3 in tubules was 96 in *A. maculipennis*, 19 in *C. pipiens* and 38 in *Ae. aegypti*. From day 14 to day 21 pi the third-stage larvae were observed in Malpighian tubules of *Ae. aegypti* together with earlier stages: mf and L1. L3 were first found in the body cavity, salivary glands and proboscis of mosquitoes on day 13 pi in *A. maculipennis* and on day 14 pi in *C. pipiens* and *Ae. aegypti*. In these sites, the maximum number of larvae was 6 in *A. maculipennis*, 7 in *C. pipiens* and 11 in *Ae. aegypti*. L3 were observed twice in thoracial muscles of mosquitoes. Beginning from day 16 pi no L3 were found in Malpighian tubules of *C. pipiens*, whereas in two other species some L3 remained there till the end of observation (day 21 pi). In the mosquito proboscis, as many as 6 or 7 L3 were usually observed, excluding one specimen of *Ae. aegypti* containing 9 larvae in the proboscis. According to our observation, the infective larvae did not leave the proboscis during mosquitoes' feeding with sugar solution. On the other hand, it was possible to stimulate emission of larvae by heating the temporary preparations of dissected mosquitoes (fig. 3, *C*).

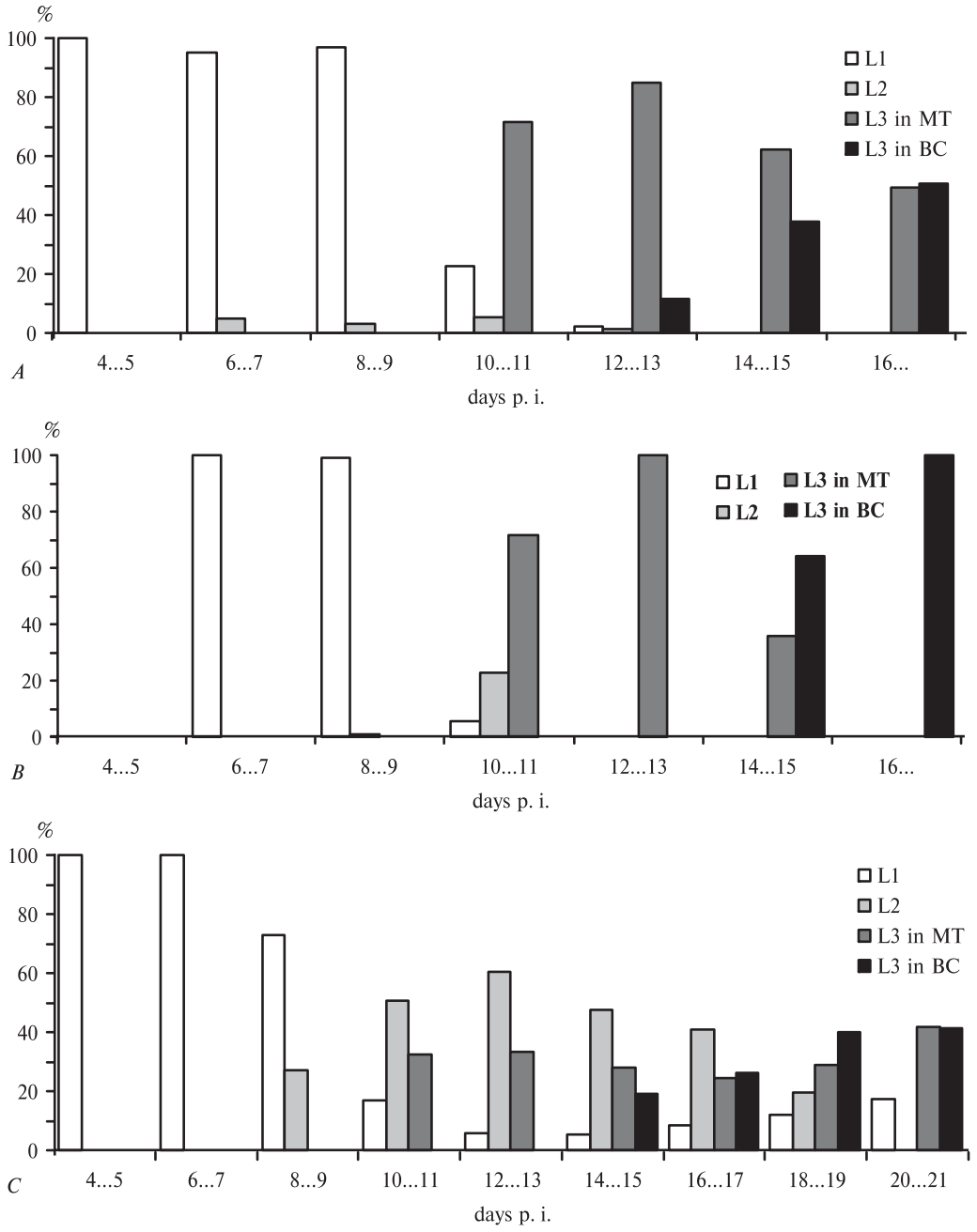

Fig. 2. Percentage of different larval stages of *D. repens* in mosquitoes: *A* – in *A. maculipennis*; *B* – in *C. pipiens*; *C* – in *Ae. aegypti*. L1 – first-stage larvae ("sausage stage"), L2 – second-stage larvae, L3 – third-stage larvae in Malpighian tubules (MT) and in body cavity (BC).

Рис. 2. Доля отдельных личиночных стадий *D. repens* в зараженных комарах: *A* – *A. maculipennis*; *B* – *C. pipiens*; *C* – *Ae. aegypti*. L1 – покоящиеся личинки первой стадии ("sausage stage"), L2 – личинки второй стадии, L3 – личинки третьей стадии в мальпигиевых сосудах (MT) и полости тела (BC).

The body of larvae was elongated, 667–1005 long and 22–25 wide (n = 15), with rounded anterior and posterior extremities. Small terminal cuticular tuberculum was observed on the tail end.

The following values of vector efficiency index were calculated for each mosquito species: 11.9% in *A. maculipennis*, 7.9% in *Ae. aegypti* and 1.9% in *C. pipiens*.

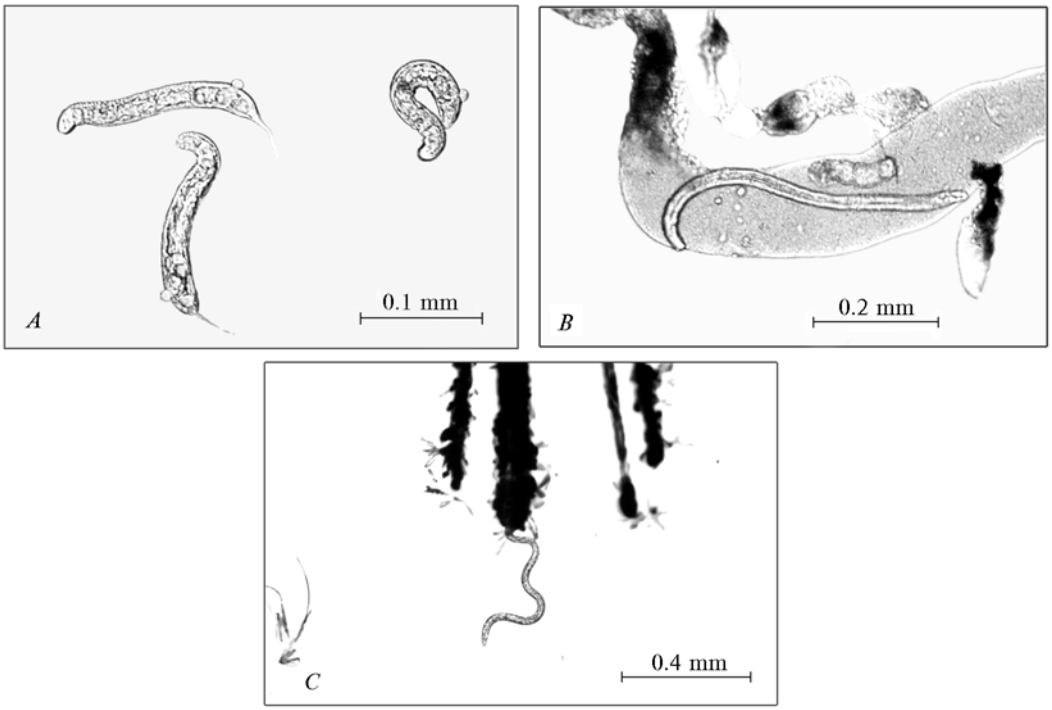

Fig. 3. Larval stages of *D. repens* from experimentally infected mosquitoes: *A* – first-stage larvae ("sausage stage") from Malpighian tubules; *B* – second-stage larva in Malpighian tubule; *C* – third-stage larvae leaving mosquito's proboscis.

Рис. 3. Личиночные стадии *D. repens* из экспериментально зараженных комаров: *A* – личинки первой стадии ("sausage stage") из мальпигиевых сосудов; *B* – личинка второй стадии в мальпигиевом сосуде; *C* – личинка третьей стадии, покидающая хоботок комара.

Discussion

Of three mosquito species used for experimental infection, none was refractory to *D. repens* infection. *A. maculipennis atroparvus* appeared to be the most suitable host for the nematode larvae development, since comparatively more larvae entered the Malpighian tubules and reached the "sausage" stage and, thereafter, the infective L3 stage in this species. These values were the lowest in *C. pipiens molestus*, resulting in the lowest vector efficiency index. Moreover, both the nematode larvae mortality and the infected mosquitoes mortality were comparatively the highest in *C. pipiens molestus*.

Despite the low value of vector efficiency index observed in *C. pipiens molestus* we believe the species may support *D. repens* transmission, especially in urban localities, where both the density of *C. pipiens molestus* and the prevalence of *D. repens* in dogs are rather high. *A. maculipennis atroparvus*, on the other hand, has scattered distribution within the large area and is absent in cities. Thus, this species is supposed to be important in *D. repens* transmission only within the small suburban, rural or natural localities.

In our experiments, the development of *D. repens* larval stages occurred under the conditions of hyperinfection of the intermediate hosts. T. P. Khudaverdiev (1976) reported the development of *D. repens* in naturally infected *Ae. caspius* and *C. pipiens* occurred under infection intensity not higher than 80 larvae per mosquito. In the present study, however, the initial intensity of mosquitoes' infection with microfilariae reached several hundreds of specimens and the further development of larvae was successful.

Despite the high initial intensity of infection, the number of the third-stage larvae developed in each of three mosquito species was comparatively small. The most dramatic decline in larva burden occurred during the first days of development, when

microfilariae were migrating from the midgut to the Malpighian tubules. Similar observations were presented by R. C. Russell and M. J. Geary (1996), who studied the development of *D. immitis* under various initial intensity of mosquitoes' infection. According to G. Cancrini et al. (1992), a number of *D. repens* microfilariae may be destroyed while passing through the cibarial pump in the foregut of *C. pipiens*. These data were indirectly confirmed by the present study, since numerous dead microfilariae were observed in the stomach of *C. pipiens* but not in two other species. On the other hand, according to McGreevy et al. (1978), the cibarial and pharyngeal armature is well developed in *Anopheles* species, poorly developed in *Culex* spp. and absent in *Aedes* species.

R. C. Russell and M. J. Geary (1996) reported faster clotting of blood meal in *C. annulirostris* than that in *Ae. notoscriptus*. The authors beleived this difference explained the larger mortality of *D. immitis* microfilariae in the former species. However, L. A. Kuznetsova (1987) stated that the clotting process in *Culex* species is longer than in *Aedes* spp., and the formation of peritrophical membrane and its structure is identical in both genera. Thus, we believe the influence of alimentary channel structures and alimentary physiology in separate mosquito species on the mortality of microfilaria is still to be investigated.

High mosquitoes' mortality during the first days of experiment apparently was caused by their hyperinfection with larval *D. repens*. Moreover, the "crowding effect" might have caused the arrest of nematode larval development in *Ae. aegypti*. In our experiments, microfilariae resumed their development on day 15 pi, possibly due to the death of a part of larvae and migration of L3 from Malpighian tubules to body cavity.

We would like to express our sincere thanks to Iryna Vazhenina from Zoological Department of Taras Shevchenko University (Kyiv) for her advisory help in mosquito cultures' maintaining.

Anderson R. C. Nematode Parasites of Vertebrates: Their Development and Transmission. – 2nd Edition. – CAB Publishing, 2000. – 672 p.

Anyanwu I. N., Agbede R. I. S., Ajanusi O. J. et al. The incrimination of Aedes (Stegomyia) aegypti as the vector of Dirofilaria repens in Nigeria // Veterinary Parasitology. – 2000. – **92** (4). – P. 319–327.

Bain O. Developpement en Camargue de la filaire du chien, Dirofilaria repens Railliet et Henry, 1911, chez les Aedes halophiles // Bull. Mus. Nation. d'Hist. Natur., Zoologie. – 1978. – **510** (351). – P. 19–27.

Cancrini G., Della Torre A., Coluzzi M. Different probabilities of transmission of Dirofilaria immitis and D. repens by Culex pipiens: Proceedings of EMOP VI, Parma, Italy // Parassitologia. – 1992. – **38** (1–2). – P. 90.

Cancrini G., Pietrobelli M., di Regalbono A. F. et al. Development of Dirofilaria and Setaria nematodes in Aedes albopictus // Parassitologia (Roma), 1995. – **37** (2/3). – P. 141–145.

Coluzzi M. Osservazioni sperimentali sul comportamento di Dirofilaria repens in diversi gruppi di artropodi ematofagi // Parassitologia. – 1964. – **6**. – P. 57–63.

Fülleborn F. Ueber Versuche an Hundefilarien und deren Uebertragung durch Mücken // Archiv für Schiffs- und Tropen-Hygiene, Pathologie und Therapie Exotischer Krankheiten. NV. – 1908. – **12**. – P. 313–351.

Kartman L. Suggestions concerning an index of experimental filaria infection in mosquitoes // Amer. J. Tropic. Medic. and Hygiene. – 1954. – **3**. – P. 329–337.

Khudaverdiev T. P. The biology of Dirofilaria repens Raillet & Henry, 1911, the causal agent of dirofilariasis of dogs in the Nakhichevan' Republic, USSR // Materialy Nauchnoi Konferentsii Vsesoyuznogo Obshchestva Gel'mintologov. – 1976. – **28**. – P. 163–169. – Russian.

Kuznetsova L. A. Formation of the peritrophic membrane in the stomach of female Culex pipiens molestus Forsk mosquitoes (Diptera, Culicidae) // Meditsinskaya parazitologiya i parazitarniye bolezni. – 1987. – **3**. – P. 20–23. – Russian.

Mantovani A., Restani R. Riserche sui possibili artropodi vettori di Dirofilaria repens in alcune provincie dell'Italia centrale // Parassitologia. – 1965. – **7**. – P. 109–116.

McGreevy P. B., Bryan J. H., Athuman P., Kolstrup N. The lethal effects of the cibarial and pharyngeal armatures of mosquitoes on microfilaria // Transactions of the Royal Society of Tropical Medicine and Hygiene. – 1978. – **72**. – P. 361–368.

Russell R. C., Geary M. J. The influence of microfilarial density of dog heartworm Dirofilaria immitis on infection rate and survival of Aedes notoscriptus and Culex annulirostris from Australia // Medical and Veterinary Entomology. – 1996. – **10** (1). – P. 29–34.

Webber W. A. F., Hawking F. Experimental maintenance of Dirofilaria repens and D. immitis in dogs // Experimental Parasitology. – 1955. – **4**. – P. 143–164.