# Part 2. Biotechnology

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## PROLONGED HYPOXIA INDUCED MELANOTIC PSEUDOTUMORS IN THE LARVAE OF BLOOD-SUCKING MOSQUITOES

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**Summary.** It was found that the presence of mosquito eggs in artificially created conditions of prolonged hypoxia causes the appearance of numerous melanotic pseudotumors in the larvae hatching from such eggs. In the cells of melanotic pseudotumors multilayer concentric membrane-like structures were found in the cytoplasm. In the immediate vicinity of such membranes, small spherical virus-like particles (VLP) with a diameter of about 30 nm were observed. The possible role of hypoxia in the development of melanotic pseudotumors of mosquito larvae is discussed

**Keywords:** electron microscopy, virus-like particles, *Aedes*, Culicidae, Diptera, Ukraine

**Introduction.** There are a large number of both benign and malignant insect tumors (Harshbarger and Taylor, 1968; Tascedda and Ottavifni, 2014). These tumors have many features in common with human and animal tumors. Modern molecular genetic research methods have established, for example, that more than 50% of the proteins that are involved in the processes of tumor formation in humans and animals have analogues in the fruit fly *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) (Gonzalez, 2013).

The oncology of insects is mainly based on the results of studies of melanotic tumors of the fruit fly *D. melanogaster.* This fly has a phenomenon called 'melanotic encapsulation', which is formed as a result of the deposition of melanin grains in the form of pigmented masses on or near the surface of an embedded pathogen. In addition to melanin grains, these pigmented masses, as a rule, consist of clusters of adherent hemocytes, or various endogenous tissues encapsulated by these cells (Christensen et al., 2005). Since there is no full correspondence in the definitions between oncology of vertebrate and invertebrate animals, some researchers call such insect tumors 'pseudotumors' (Harshbarger and Taylor, 1968).

In Europe, some mosquitoes can transmit several diseases and parasites that dogs and horses are very susceptible (Hubálek, Rudolf and Nowotny, 2014; Pagès and Cohnstaedt, 2018). Despite the important role of mosquitoes in the transmission of pathogens of infectious diseases of humans and animals, neoplasms of blood-sucking mosquitoes have not been studied previously.

**The aim of the work** was describes the method of inducing pseudotumors in mosquito larvae by hypoxia in the laboratory conditions, and also presents the results of electron microscopic studies of affected cells obtained from this melanotic pseudotumors.

**Materials and methods.** Field studies of mosquito larvae were carried out in Kyiv Region (the villages of Kruhlyk, Vita-Poshtova, Feofaniia, and Zahaltsi).

To study the effect of hypoxia, soil samples collected in the habitats of mosquito larvae (family Culicidae) along with egg laying mosquitoes were stored for 6 months in dense plastic bags without air with temperature fluctuations from 0°C in the winter months to 25°C in the spring. Control soil samples with egg laying were kept at the same temperatures, but with air access.

Hatching after 6 months, mosquito larvae with melanotic pseudotumors were examined under a light microscope and then processed for electron microscopy. For this, the larva was cut into several fragments, fixed in 2.5% solution of glutaraldehyde on cacodylate buffer, and then in 1.5% solution of osmium tetroxide on phosphate buffer. Further, the material was dehydrated in alcohols of increasing concentration, acetone and placed into EPON-812. Ultrathin sections were made on LKB microtome, contrasted with 2% solution of uranyl acetate and 0.5% solution of lead citrate, and then examined under JEM-7A electron microscope.

**Results and discussion.** Numerous small melanotic pseudotumors of a dark color were revealed in the larvae of blood-sucking mosquitoes spawning from ovipositors kept under prolonged hypoxia, which were visible through a transparent cuticle (Fig. 1).

After storing of mosquitoes ovipositor under artificially created hypoxic conditions, melanotic pseudotumors formed in most mosquito larvae (Table 1). In control larvae, such tumors were very rare. The formation of melanotic pseudotumors and the accompanying symptoms of the disease in larvae of various mosquito species were similar in all cases. Most often, symptoms were manifested in stage III–IV larvae, less often in younger stage larvae and pupae.

**Figure 1.** Larva of the blood-sucking mosquito *Aedes communis* (De Geer, 1776) with numerous melanotic pseudotumors in the form of dark spots throughout the body

**Table 1** — Manifestation of melanotic pseudotumors in blood-sucking mosquito larvae after the maintenance of their ovipositions under prolonged hypoxia

| Indexes | Package No. | | | | | | Mean |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| Total number of larvae, sp. | 68 | 56 | 68 | 67 | 186 | 42 | 81.17 ± 21.37 |
| Number of larvae with pseudotumors, sp. | 44 | 32 | 56 | 34 | 132 | 36 | 55.67 ± 15.68 |
| Percentage of larvae with pseudotumors, % | 64.7 | 57.1 | 82.4 | 50.7 | 71.0 | 85.7 | 68.58 ± 5.65* |
| Percentage of larvae with pseudotumors in control groups (without hypoxia), % | 0 | 0 | 0 | 0 | 0.53 | 0 | 0.09 ± 0.09 |

Note. * — $p < 0.001$ compared to control.

Affected larvae became inactive, convulsively twitching when irritated. Sometimes their body was deformed, distorted, the segments contracted, because of which the larva became like an accordion. At the first stages of the disease, the fatty body and the dorsal parts of the segments of the chest and abdomen were weakly pigmented, then separate spots of irregular shape appeared. They compacted, darkened and subsequently acquired a spherical or oval shape, reaching sizes of 50–80 μm.

An electron microscopic study of ultrathin sections of adipose body cells of affected larvae showed that a large number of peculiar multilayer concentric membrane-like structures were found in the cytoplasm (Fig. 2), which were never found in healthy mosquito larvae. In the immediate vicinity of such membranes, small spherical virus-like particles (VLP) with a diameter of about 30 nm were observed.

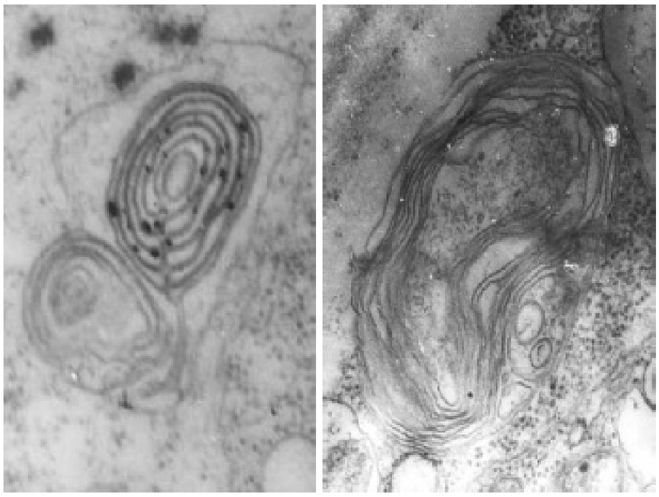

**Figure 2.** Concentric membrane structures in the cytoplasm of cells of the blood-sucking mosquito *Aedes cantans* (Meigen, 1818) larvae affected by a melanotic pseudotumors

In natural habitats, larvae of blood-sucking mosquitoes with melanotic pseudotumors are very rare, the extent of damage in water pools is only a fraction of a percent. As a result of many years of field studies, we found such melanotic pseudotumors in the larvae of 11 species of blood-sucking mosquitoes from natural populations: *Aedes dorsalis* (Meigen, 1830), *Ae. cantans* (Meigen, 1818), *Ae. annulipes* (Meigen, 1830), *Ae. excrucians* (Walker, 1856), *Ae. cyprius* Ludlow, 1919, *Ae. communis* (De Geer, 1776), *Ae. cataphylla* Dyar, 1916, *Ae. leucomelas* (Meigen, 1804), *Ae. vexans* (Meigen, 1830), *Ae. cinereus* Wiedemann, 1818, *Culex territans* Walker, 1856 (Buchatskyi and Sheremet, 1978).

As a result of the studies in laboratory conditions, it was found that the presence of mosquito eggs in artificially created conditions of prolonged hypoxia causes the appearance of numerous melanotic pseudotumors in the larvae hatching from such eggs. A characteristic feature of these pseudotumors is the presence of a large number of membrane structures in the affected cells. In *Drosophila*, similar melanotic pseudotumors occur during experimental infection with Rous sarcoma virus (Burdette and Yoon, 1967).

It is known that in cancer cells of humans and animals, as well as during viral reproduction, often observed are complexes of membrane structures formed by a modified endoplasmic reticulum, as well as a variety of concentric membrane formations, which were called 'X-structures' (Solov'ev, Khesin and Bykovskiy, 1979). In kalyptorhynch flatworm *Gyratrix hermaphroditus* Ehrenberg, 1831 (Turbellaria: Polycystididae), similar membrane structures were seen in the cytoplasm of cells along with small virus-like particles. Sometimes these concentric membrane formations were surrounded by several VLP, but more often they were empty. Researchers called them 'concentric multilayer membrane structures' and believe that their appearance is associated with the reproduction of the virus (Reuter, 1975). Recently, Norwegian researchers (Bjørgen et al., 2015) found that in melanotic deposits, in a large number of Atlantic salmon (*Salmo salar* Linnaeus, 1758) present in skeletal and cardiac muscles, there are a large number of spherical virus-like particles with a diameter of about 30 nm. The authors suggest that these melanin deposits are produced by the fish organism in response to various viral infections often found in salmon.

As a result of our research, small spherical VLP of the same diameter as in salmon muscles (about 30 nm) were found in the cytoplasm of cells obtained from melanotic pseudotumors of blood-sucking mosquitoes of various species. In our opinion, all these small VLP under conditions of prolonged hypoxia can be induced by the transcription factor HIF-1α.

The role of hypoxia in the malignant transformation of cells is well known (Osinsky, Zavelevich and Vaupel, 2009; Minchenko et al., 2016; Zhang et al., 2020). The main factor of hypoxia is the transcription factor HIF-1α, the content of which is regulated at the level of protein synthesis (Centanin et al., 2008; Minchenko et al., 2015). In insects, the HIF-1α factor plays a large role in the process of postembryonic development, since a sharp increase in the body size of larvae during metamorphosis requires an increased level of oxygen supply (Lundquist, 2016). Under hypoxic conditions, this factor is able to activate the expression of many genes that affect the ontogenesis of insects and activate the LTR-retrotransposons of the *Tu-1-copia* family located in their genomes (Rachidi et al., 2005). The latter, as we know, form spherical VLP with a diameter of 30–40 nm in the cells (Yoshioka et al., 1990; Semin and Il'in, 1994). Such VLP by means of metagenomics method have recently been detected in flies and mosquitoes. All of them are united in the genus *Hemivirus* within the Pseudoviridae family (Buchatskyi, 2020). Thus, the induction of melanotic pseudotumors in blood-sucking mosquito larvae and the excessive formation of intracellular membranes under prolonged hypoxia can be the result of activation of endogenous viral elements. These endogenous viral elements, close to viruses of the families Retroviridae, Parvoviridae, Filoviridae, Bornaviridae, and Circoviridae are found in the genomes of various domestic and wild animals (Yudin, Aitnazarov and Ermolaev, 2011). According to some estimates, endogenous retroviruses, for example, can account for 7 to 9% of the vertebrate genome (Yu et al., 2019). Therefore, the study of the role of hypoxia in the activation of endogenous viral elements is an important task.

Currently, in many scientific laboratories, mosquitoes and flies are used to test on them insecticides, viral and bacterial preparations, as well as for various biotechnological experiments. The facts obtained in our experiments indicate that when breeding insects, the oxygen regime of their maintenance should be taken into account.

**Conclusions**. 1. In natural habitats, larvae of blood-sucking mosquitoes with melanotic pseudotumors are very rare, their incendence in water pools is only a fraction of a percent.

2. It was found that the presence of mosquito eggs in artificially created conditions of prolonged hypoxia causes the appearance of numerous melanotic pseudotumors in the larvae hatching from such eggs.

3. It is established that in the cells of melanotic pseudotumors multilayer concentric membrane-like structures were found in the cytoplasm. In the immediate vicinity of such membranes, small spherical virus-like particles with a diameter of about 30 nm were observed.

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