FOLIA PARASITOLOGICA (PRAHA) 15: 183–187, 1968.

EXPERIMENTAL SURVIVAL OF THE VIRUS ŤAHYŇA IN HIBERNATING MOSQUITOES *THEOBALDIA ANNULATA* (SCHRK.)

V. DANIELOVÁ, J. MINÁŘ, B. ROSICKÝ

Institute of Parasitology, Czechoslovak Academy of Sciences, Prague

Abstract. The paper deals with the experimental infection of mosquitoes *Theobaldia annulata* (Schrk.) with the virus Ťahyňa and its persistence in mosquitoes during hibernation. It was established by two repeated experiments that the virus harboured by the mosquitoes which were kept at 25° or 27 °C, multiplied and was maintained in them under conditions of hibernation. The virus was detected in the hibernating mosquitoes on 47th, 60th and 82nd days after infection. Immediately after the hibernation was interrupted, a distinctly smaller amount of virus was demonstrated in the mosquitoes than the amount in the same mosquitoes kept at 27 °C seven days before the isolation test.

The mode of hibernation of the virus Ťahyňa is still unknown, because its main vector, the mosquito *Aëdes vexans* hibernates in the egg stage and the transovarial transmission is still problematical (CHAMBERLAIN, SUDIA, GOGEL 1964). It possibly survives in females of the hibernating mosquitoes. On grounds of negative isolation tests from a large number of mosquitoes captured in localities where the virus Ťahyňa occurs (BÁRDOŠ, DANIELOVÁ 1961, DANIELOVÁ 1964b, DANIELOVÁ et al. 1966) and on the grounds of negative laboratory experiments (DANIELOVÁ 1966) the widely spread species *Culex pipiens* and *Anopheles maculipennis* s.l. are excluded. The species *Theobaldia annulata*, which is relatively sparsely distributed nearly throughout our territory, including the localities where the virus Ťahyňa was isolated, has not been sufficiently studied as yet both in isolation tests and other experiments. We have therefore selected it as the object of our studies.

MATERIAL AND METHODS

The mosquitoes *Theobaldia annulata* (Schrk.) were collected in November and January in karst caves near Srbsko, central Bohemia, where they were hibernating. On the one hand, they were kept in silon cages in a thermostat at 90–90% humidity and at 25 °C during the first and at 27 °C during the second experiment, on the other they were kept in a cellar at 5.7.–9.6 °C during the first and at 7.9–10.2 °C during the second experiment. Diurnal temperature changes were 0.1 to 0.4, exceptionally 0.5 °C.

Virus. The infectious suspension was prepared by using 46th and 47th mouse i.c. passage of the strain 181 of the Ťahyňa virus. The mosquitoes became infected by feeding on the suspension prepared from 1 part of 20% brain suspension in saline solution pH 7.4 with 10% horse serum, 3 parts of defibrinized guinea pig blood and 5% of glucose.

Isolation tests. The mosquitoes were triturated and suspended in 1 ml 10% horse serum in buffered saline with 1 000 units PNC and 1 000 units STM per 1 ml. The suspension was centrifuged for 5 min. at 2,000—3,000 rev/min and inoculated to suckling mice, 0.01 ml i.c. and 0.03 ml s.c. per mouse. The titration was performed by inoculating 0.02 ml i.c. to 8—10 g young mice each.

Suspensions prepared irrespective of weight and number of mosquitoes, were regarded as concentrated, i.e. dilution 10^0, and were further diluted 10 times and inoculated to 8—10 g mice, 0.02 ml i.c. per mouse. The virus level was expressed as the total average amount LD_{50} of virus in the body of one mosquito in the following way: the amount of virus determined by titration in 0.02 ml (by method of Reed and Muench 1938) was multiplied by 50, the result corresponding to the amount of virus in 1 ml, and was divided by the number of mosquitoes used.

RESULTS

We conducted two subsequent experiments. The first one took place between November 18, 1966 and January 17, 1967, the second one between January 20 and April 25, 1967.

Experiment No 1. The mosquitoes, already settled for winter, were disturbed in hibernation and infected by allowing them to feed on infectious suspension with the titre $10^{-3,84}$ LD_{50}. After feeding they were kept for 6 days in a thermostat, then deposited in a cellar to hibernate. Some mosquitoes, however, were left in the thermostat in order to find out whether it is possible for the virus Ťahyňa to multiply in the body of the mosquito *Theobaldia annulata*. Isolation

Table 1.

Exp. No.	Date of infection	Titration of virus source	mosquitoes at 25 °C		Titre of virus ×	Hibernating mosquitoes		
			number of days	isolation test +		number of days after infection	isolation test +	titre of virus ×
1.	18.11.	3.84	20	7/7	—			
			20	8/8	—			
			20	9/9	—			
			20	9/9	3.44			
						60	5/10	
2.	20.1.	5.0	0		≧4.37			
						47	4/9	0
						47*	10/10	2.25
						82	10/10	—
						82	10/10	—
						95**	10/10	2.34

\+ — number of sick and dead (number of inoculated) suckling mice
× — in log LD_{50}
* — mosquitoes in the last 8 days before isolation test at 27 °C
** — termination of hibernation, mosquitoes at 27 °C in the last 14 days

test was performed from these mosquitoes 20 days after infection. The virus was demonstrated in all 4 suspensions prepared from 10 mosquitoes each. By titration of the suspension stored for 6 days on dry ice it was established that the body of one mosquito contained $10^{3.44}$ LD_{50} of virus.

Two months later another isolation test was performed from the hibernating mosquitoes, but a relatively small amount of virus was isolated from the suspension made from 5 mosquitoes.

Experiment No 2. The mosquitoes were disturbed in hibernation and became infected by feeding on a tampon with a virus titre $10^{-5.0}$. Immediately after the infectious meal titration was performed of the suspension prepared from 5 mosquitoes and it was established that one mosquito ingested on an average $\geqq 10^{4.37}$ LD_{50} of virus. After the incubation period of 7 days the mosquitoes were transferred from the thermostat to cellar. Seven weeks after infection two isolation tests were conducted, each from 15 mosquitoes. The first group contained hibernating specimens, the second group consisted of mosquitoes whose hibernation had been interrupted a week before. Until the isolation test these mosquitoes were kept in the thermostat and fed on glucose. The isolation test as well as titration were performed simultaneously from both groups of mosquitoes under same conditions. The difference in the amount of virus was considerable, because in the first isolation test 4 out of 9 inoculated suckling mice fell ill or died and no virus was detected in young mice by titration. In the isolation test from the second group of mosquitoes all 10 inoculated suckling mice fell ill or perished. By titration on young mice the average amount of virus was found to be $10^{2.25}$ LD_{50}. The virus was even detected in the mosquitoes after 12 weeks of hibernation when they started to wake up. The remaining specimens were therefore transferred to the thermostat and fed on glucose. After another 14 days an isolation test from 15 mosquitoes was conducted and the average amount of $10^{2.34}$ LD_{50} of virus was detected in the body of one mosquito by titration.

During the experimental hibernation at the temperature not higher than 9 °C the mosquitoes were attached to the walls of cages in their hibernating posture and moved about only when disturbed. At the temperature above 9 °C they flew more frequently. In the first experiment 10 % and in the second experiment 77.3 % of mosquitoes survived in the cages after 3 months. In the first experiment, when the temperature rose above 9 °C after one month, moist tampons were applied, while in the second experiment a higher humidity was maintained throughout the whole experimental period.

DISCUSSION

The possible winter survival of virus in mosquitoes has been studied in California by REEVES et al. (1958) and BELLAMY et al. (1958). These authors arrive at the conclusion, that *Culex tarsalis* may maintain the WEE virus during the winter season by means of gradual transmissions; nevertheless they do not exclude the

possible winter survival of the virus in chronically infected birds. On the other hand, RUSH et al. (1963a and 1963b) dismiss the possible maintenance of the WEE virus in mosquitoes *Culex tarsalis* during the winter season under conditions of North-western U.S. (In this paper we do not intend to verify the correctness of these authors' arguments but wish to discuss only the different opinions of this problem, although the climatic differences in both studied regions must not be overlooked.)

The authors BELLAMY et al. (1958) presume that for the successful maintenance of the WEE virus in the mosquitoes *Culex tarsalis* in California the transmission to vertebrates and the following infection of mosquitoes during the winter period is necessary, in other words a "two-step" and not "one-step" transmission as asserted by previous hypothesis. In our experiments we observed, likewise as the above mentioned authors, a decrease of the amount of virus following a certain period of hibernation, but we succeeded in demonstrating an increase after transferring the mosquitoes to a higher temperature. On the basis of this fact we do not consider the transmission during hibernation to be necessary and under our conditions even possible. More detailed experiments, however, will have to be conducted to find out, whether the amount of virus during the long-term hibernation decreases below the verifiable limit and whether in this case also the amount of virus would increase after the transfer of mosquitoes to higher temperature, or after the conclusion of hibernation and whether this would not be the case of attenuation of strain, as assumed by BELLAMY et al. (1958).

Although we cannot yet draw final conclusions on the circulation of the virus in nature, we consider our results to be interesting mainly in view of ecology of the mosquitoes *Theobaldia annulata*. This species feeds both on cattle in sheds and on free-living animals in nature, similarly as *Aëdes vexans*, which is considered to be the main vector of the Ťahyňa virus in the summer season. There is a direct connection between these species through the ascertained hosts of the virus Ťahyňa, i.e. hares in nature and cattle in sheds.