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TRANSOVARIAN TRANSMISSION OF BLUETONGUE VIRUS UNLIKELY FOR *CULICOIDES VARIIPENNIS*

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ABSTRACT. Transovarian transmission of bluetongue virus was not demonstrated for colony *Culicoides variipennis* (Coquillett) (Diptera: Ceratopogonidae) when the first to the fifth egg batches of infected females were reared and progeny females were given a normal blood meal, incubated, and then assayed for the presence of virus.

The ability of a virus to persist in an area after an epizootic is important to the epidemiology of a viral disease. The possibility of persistence of the virus would be increased by transovarian transmission in the vector, that is, if the infective agent passes through the egg to the next generation. If activity of the vector were seasonal, transovarian transmission would allow the virus to overwinter in a sheltered, immature form of the insect, such as the overwintering larval form of *Culicoides variipennis* (Coquillett) in Colorado (Jones, 1967a).

Burgdorfer and Varma stated in their review (1967) that, although transovarial development of arboviruses takes place in ticks, there is no conclusive evidence for this in mosquitoes and the evidence for it in phlebotomines has not been settled. Because transovarian transmission of arboviruses has not been conclusively demonstrated for any species of biting fly, we have not considered it likely for *C. variipennis*, the probable primary vector of bluetongue (BT) disease of sheep and cattle in the United States, and we have not found it expedient to test for it until recently.

Because the flies used in our research program with the transmission of BT virus needed further standardization, we developed insect rearing facilities to conduct genetic studies with infected flies. A desirable first step was to determine the feasibility of dividing the parent colony of *C. variipennis*, established in 1957 and maintained without the addition of wild flies (Jones *et al*, 1969), through genetic selection into divergent lines resistant and susceptible to the transmission of BT virus. The selection process involved the individual mating and subsequent rearing of progeny of single females that were killed and assayed for virus after they had deposited one or more egg batches. Because normally a second, and occasionally a third, egg batch was also collected, the possibility of the infection of some progeny by transovarian transmission was greater than if we had used only the first egg batch. This statement assumes that the reproductive system, or some part of it, might be resistant to infection, or at least not become infected within the first few days needed to deposit a first egg batch after ingestion of the infective blood meal.

Clearly, we had to show that the positive assay results in our genetic program were not caused by transovarian transmis-

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sion, but rather were the result of the normal biological route of infection after ingestion of an infective blood meal.

Our experimental design and methods of handling flies were governed by the reproductive behavior of the fly (Jones, 1967b). Both sexes mate repeatedly, and mating is common within 12 hours after eclosion. The female stores sperm in a single spermatheca so that there is no appreciable reduction in fertility from one mating through the first four egg batches. A female requires a blood meal for each batch of eggs. She can deposit seven or more batches, but the number of eggs per batch gradually diminishes. The female will take its first blood meal within 24 hours after eclosion and take subsequent blood meals on the same day as egg deposition, which can occur as soon as 3 days after a blood meal.

Females were given blood meals artificially through chick-skin membranes in a six-position membrane feeder (Jones and H. W. Potter, Jr., unpublished data). The blood used was normal sheep blood that had been defibrinated by agitating with glass beads and refrigerated for 0 to 4 days. An infective meal consisted of a 9:1 mixture of blood and BT virus suspension. The virus used was the embryo supernate of an egg-adapted strain, 62–45S (previously designated Wyoming BT 262), 34th passage in embryonating chicken eggs; its concentration was 3×10^7 egg median lethal doses per ml. After blood feeding, the engorged females were separated from the other flies and incubated at 21° C and 30 percent RH with a 16 light : 8 dark photoperiod until they were used in virus-isolation tests.

Embryonating chicken eggs were used to assay flies for BT virus. Each pool of flies was ground in a TenBroeck grinder in 1 ml of phosphate-buffered saline solution, pH 7.3, containing 10,000 units penicillin, 10 mg streptomycin, and 1000 units nystatin. This suspension was inoculated into seven 7-day chicken eggs. Bacterial cultures were made as necessary to insure that embryo deaths were the result of virus rather than bacterial contamination. Occasionally, additional subpassages were made when results were in doubt. The criteria for the presence of BT virus were the death pattern for each set of eggs and pathological changes in dead embryos indicative of virus presence, such as extensive subcutaneous edema, hemorrhage, and a cherry-red appearance. Fluorescent-antibody tests were used for final verification of BT virus from dead embryos.

Our procedure was based on our feeling that second or later egg batches of infected females would be more likely to be infected transovarially than the first egg batch. So that any virus passed transovarially could multiply to detectable levels, it was necessary to give progeny females a normal noninfective blood meal and then incubate them at least 10 days before assay for BT virus.

In the primary test for transovarian transmission (Table 1), we used F_2 flies from a susceptible colony (code no. 301) that was established from the F_1 of a selection schematic for a susceptible line. A pool of mixed sexes was offered an infective blood meal, and the engorged females were removed and incubated. These flies were allowed to deposit eggs at 3 and 4 days of incubation and given a second (noninfective) blood meal at 4 days of incubation. The flies that engorged at the second feeding were removed, allowed to deposit eggs at 3 and 4 days of additional incubation, and given a third (noninfective) blood meal at 4 days after the second blood meal. This procedure was continued until five pools of flies were established that had had up to five blood meals—a first infective blood meal and from 0 to 4 additional noninfective blood meals at 4-day intervals. Blood feeding was managed so that females taking only a partial, or a "clear fluid," engorgement were discarded. The test was designed so that at any intermediate blood-feeding level, the females that did not feed were incubated for virus assay of the previous blood feeding, while the fed females were incubated for egg deposition and for the next blood feeding. The incubation periods for all pools of flies appear in Table

TABLE 1.—Primary test for transovarian transmission. All progeny were negative for BT virus.

Parent females						Progeny females		
				Virus assay				
No. of blood meals		No. of days flies incubated *	Days lapse from infective meal to oviposition	No. flies infected /no. used	Infection rate (%)	From parent egg batch	No. of days flies incubated **	Total no. of flies assayed
Infective	Noninfective							
1	0	10	4	7/14	50	first	11	46
1	1	10	8	14/25	56	second	15–36	52
1	2	24	12	7/8	88	third	16	50
1	3	24	16	9/10	90	fourth	15–29	67
1	4	24	20	4/7	56	fifth	11–16	8
			Total or mean	41/64	64			223

* Duration of incubation from infective meal to removal from test for assay.
** Duration of incubation from noninfective meal to removal from test for assay.

1. Eggs were collected from each of the five pools and reared as previously described (Jones *et al.*, 1969). Pools of mixed sexes of the progeny from each of the five infected parent pools were offered non-infective blood meals, and the engorged females were removed, incubated 11–36 days, and then assayed for BT virus.

The secondary test was to make sure that the progeny of individual infected females of the genetic study were not infected unless they themselves were given an infective meal. Single females, which had been given infective blood meals and individually mated, were incubated and their egg batches collected and reared. Pools of mixed sexes of the progeny from known infected females were offered non-infective blood meals, and the engorged females were removed, incubated 10 or more days, and then assayed as pools for BT virus.

In both tests, the results were negative for all progeny that could have acquired BT virus by transovarian transmission. In the primary test, 223 female progeny were tested; these were from the first to fifth egg batches of a pool of females that were 64 percent infected (Table 1). In the secondary test, a total of 224 female progeny were tested as occasional checks during the genetic study, 171 from the first and 53 from the second egg batches of known infected females that represented 8 different sibling matings in the F_1 and F_2 of both the susceptible and resistant lines of the genetic selection schematic. The numbers of flies tested were large enough and the females were from sufficiently diversified sources to show that transovarian transmission of BT virus had not occurred during our test procedures and that it was not likely to occur under normal conditions for *C. variipennis*. The conditions under which this research was conducted were standard and quite fairly represent all our transmission research.

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