THE TRANSMISSION OF BLUETONGUE VIRUS TO EMBRYONATING CHICKEN EGGS BY *CULICOIDES VARIIPENNIS* (DIPTERA:CERATOPOGONIDAE) INFECTED BY INTRATHORACIC INOCULATION

ROBERT HENRY JONES [1] AND NEIL M. FOSTER [1]

Preliminary research (Foster *et al.*, 1963) has demonstrated in the laboratory that the biting midge, *Culicoides variipennis* (Coquillett), is capable of biological transmission of bluetongue (BT) disease of sheep. Five transmissions were obtained when susceptible sheep were bitten (18, 26, 37, 67, and 122 bites) by flies that had been incubated from 10 to 13 days after their first blood meal on artificially infected sheep. In this earlier work, procedures were not available to determine what percentage of the flies that fed on an infected animal became infected, and subsequently what percentage of these transmitted the disease. The small number of transmissions is believed to have occurred because the majority of the flies were not infected by the methods used. It is possible that each of the 5 transmissions resulted from the bites of only one or two infected flies.

It was evident from this early work that more precise information was needed to determine the role of *C. variipennis* in the transmission of bluetongue disease. To obtain data in a more economical and convenient way than was possible with sheep, we initiated transmission experiments with egg-adapted BT virus and with embryonating chicken eggs as both the recipient host and the assay system for the detection of virus. In an effort to insure that all insects would be infected, thereby avoiding the resistance to infection that might normally be encountered in the gut upon oral ingestion of the virus, we used the technique of inoculating the virus directly into the hemocoel of each insect (see review by Chamberlain and Sudia, 1961).

MATERIALS AND METHODS. The virus used was an egg-adapted (fifth-passage) bluetongue virus. This virus was originally isolated (BT–262) in experimental sheep at the Animal Disease Research Laboratory at Denver from blood that was collected from sheep during an epizootic of bluetongue near Gillette, Wyoming, in 1962.

The virus inoculum for infection of insects was prepared by infecting 9-day embryonating chicken eggs via yolk sac and processing those embryos that died from the second through the fourth day. The embryos were blended with Genesolv-D (Trifluorotrichlorethane, obtained from General Chemical Division, Allied Chemical Company), the emulsion was centrifuged, and the supernatant fluid was partially purified by two more extractions with Genesolv-D. The inoculum had a minimum virus titer of 10^5/0.1 ml. when inoculated intravascularly into 10-day embryonating eggs, and $10^{3.5}$/0.1 ml. when inoculated via yolk sac into 7-day eggs. To determine that the inoculum contained BT virus, two groups of insects were inoculated with the virus, incubated for 10 and 8 days, and then allowed to take a blood meal on two susceptible sheep (7 and 14 bites respectively); a third sheep was inoculated with 4 ml. of the inoculum. The three sheep developed clinical signs of bluetongue and were found to be immune on homologous challenge with known BT virus at 21 days post-trial.

All eggs used in these experiments were obtained from a single source that provided

[1] U. S. Department of Agriculture, Agricultural Research Service, Animal Disease Research Laboratory, Denver, Colorado 80225. R. H. Jones is with the Entomology Research Division and N. M. Foster with the Animal Disease and Parasite Research Division.

fertile chicken eggs uniformly susceptible to the virus. Normal eggs were incubated at 37.5° C; eggs that had been inoculated with BT virus or exposed to infection by fly bite were incubated at 33–34° C.

The majority of the flies used were from a colony (SONORA strain) maintained at Denver (Jones, 1964). Wild flies (HUDSON strain) were collected as larvae and pupae from a breeding site near Hudson, Colorado.

All operations with flies were performed inside a plexiglas isolation chamber (Jones, 1966) located in an insect-proofed room. Flies 24 to 48 hours old (maintained on 5 percent sugar solution the previous day) were inoculated (Jones, 1966) with undiluted virus suspension following their anesthetization with carbon dioxide gas. Each fly was observed under the microscope as it was inoculated to insure that at least the minimum amount of fluid needed to distinctly swell the abdomen was injected. The flies inoculated on any one day were combined to form a pool of infected flies. On subsequent days, members of the pool were withdrawn for transmission experiments. Transmissions were attempted with flies from each pool as long as live flies remained. Pools were established on the following dates (1964): colony flies on May 18, May 31, July 13, and August 27; both colony and wild flies, as paired pools, on August 13.

Inoculated flies were incubated in the isolation chamber, which was maintained at 24±2° C. They were kept in half-pint cardboard cages and fed an alternating diet of water and 5 percent sugar solution. Sugar solution was not provided the day before flies were to be used in an experiment, since the sugar meal would have lessened their feeding response. Since a blood meal was not given except on the recipient host, egg deposition by the females was not provided for before the transmission experiments.

Embryonating chicken eggs were prepared for use in transmission experiments by removing the shell over the air cell, wetting the membrane thoroughly with saline, and blotting off excess fluid. The eggs were used while the membrane was still moist and transparent.

In a typical transmission experiment, a single infected fly was fed on a single 9-day embryonating egg (for procedures and equipment, Jones, 1966). (However, in earlier work, the procedures were not fully refined and sometimes resulted in more than one fly feeding on a single transmission egg.) After a fly engorged, it was examined microscopically to determine the type (blood, allantoic fluid, or both) and the extent of the meal it had taken. The fly was then placed in a vial of phosphate-buffered antibiotic solution. For every transmission experiment, a corresponding test was conducted to determine whether the fly was infected. The fly was ground in a TenBroeck grinder in 1 ml. of phosphate-buffered solution containing 10,000 units penicillin, 20 mg. streptomycin, and 1,000 units mycostatin. This suspension was then inoculated in equal amounts via yolk sac into three 7-day eggs that were then incubated and tested for the presence of virus in a manner similar to that used for the transmission eggs.

As each egg was removed from a transmission experiment it was temporarily incubated with the open end loosely covered with a shell cap obtained from a clean egg. Subsequently (in later tests, after treatment of the air-cell area with a small amount of antibiotic), the cap was sealed in place with collodion. The egg was incubated until the embryo died, or if it survived, for 7 days; the embryo was then collected for assay to determine whether BT virus was present.

Each embryo collected from both transmission experiments and from tests to determine the infection rate was ground and centrifuged to obtain the supernatant fluid. This material was serially subpassed into sets of ten 7-day eggs until a conclusion could be made as to the presence or absence of BT virus; the criteria were the death pattern in each set of eggs and the results of gross examination of all dead embryos for evidence of viral pathology

(extensive subcutaneous edema and hemorrhage). Usually one subpassage was sufficient to give a definite result. Eggs that died within 24 hours after inoculation or their exposure to infection by fly bite were routinely discarded because their death was usually nonspecific.

RESULTS. The infection rate was 100 percent for flies incubated at least 1 day after the artificial introduction of BT virus into the insect. (With 0 to 2 hours of incubation, virus was detected in only 2 of the 14 flies tested.) Table 1 gives the transmission rates obtained for all experiments in which flies took detectable meals. This rate was zero percent for 1 and 2 days of incubation of the fly. The transmission rate became 100 percent at 6 days of incubation, and, with the exception of two negative experiments with colony flies incubated 21 days, continued at 100 percent throughout the remainder of the 28 days of incubation.

In the course of the experiments, three biological transmissions were also obtained from the probes of flies that had been incubated 3, 4, and 6 days after their inoculation with virus, while three tests with probes of flies incubated 7, 21, and 28 days were negative.

The data of Table 2, comparing the transmission rates for concurrently tested pools of wild and colony flies incubated 1–7 days, indicate that the colonized strain

TABLE 1.—Transmission rates of *Culicoides variipennis*, from all 6 pools of colony and wild flies, at various periods of incubation following intrathoracic inoculation of BT virus. The infection rate was 100 percent.

Number of days fly incubated	Number of individuals taking the listed type of meal from recipient host eggs [a, b]				Transmission rate	
	Blood	Blood and allantoic fluid	Allantoic fluid and blood trace	Allantoic fluid	No. of flies that transmitted/ no. tested	%
1	(11)	..	(1)	(3) [c]	0/15	0
2	(10)	(2)	(1) [d]	(8) [c]	0/21	0
3	6 (4)	1	..	1 (5)	8/17	47
4	9 [c] (2)	3	..	2	14/16	88
5	9	..	1	2 (1)	12/13	92
6	7 [d]	..	1	3	11/11	100
7	10 [c]	3	1	2	16/16	100
8	1	..	1	1	3/3	100
10	1	..	1	..	2/2	100
14	..	..	..	1	1/1	100
15	..	..	..	2	2/2	100
21	3	2	(1)	1 (1)	6/8	75
28	2	2	..	1	5/5	100

[a] Unless otherwise specified each test consisted of one fly per recipient host egg.
[b] Parentheses indicate negative results.
[c] One recipient host egg received two bites; both flies took same type of meal.
[d] One recipient host egg received two bites; second fly took meal of allantoic fluid.

TABLE 2.—A comparison of the transmission rates of colony and wild flies (paired pools—August 13, 1964) for 1–7 days of incubation following intrathoracic inoculation of BT virus.

Day of incubation	Transmission rates: No. flies that transmitted/no. tested (%)			
	Colony flies[a]		Wild flies	
1	0/3	(0)	0/3	(0)
2	0/5	(0)	0/5	(0)
3	2/3	(67)	3/4	(75)
4	4/4	(100)	2/2	(100)
5	1/1	(100)	5/5	(100)
6	3/3	(100)	4/4	(100)
7	5/5	(100)	4/4	(100)

[a] In addition, two more transmission experiments were attempted with flies from these pools, resulting in two transmissions with wild flies at 21 days of incubation.

of *C. variipennis* is representative for the species in our studies with BT virus at this laboratory, inasmuch as equivalent results were obtained with the two strains.

DISCUSSION. Summarization of the test results was complicated by the fact that these flies were not selective in taking a meal; they often fed on allantoic fluid alone, as well as on blood or some combination of the two fluids. The source of the meal was easy to determine with flies that obviously engorged; flies that took only a trace of a blood meal, or that distinctly inserted their proboscis without taking a meal (probe), could also easily be identified. When it was not certain that the fly fed, or at least distinctly probed, the data were excluded.

If one considers only those transmission experiments in which flies took a meal containing more than a trace of blood, it can be stated that, with at least 5 days of incubation after infection, 100 percent of the flies that took a definite blood meal transmitted virus to the host. However, a closer scrutiny of the data is warranted to inquire why two transmission experiments with flies having a 21-day incubation period were negative. Upon scanning the data "graphed" in Table 3, it is apparent that transmission eggs took longer to die (in general, 2–3 days versus 2 days, a difference significant at the 1-percent level of probability) when the fly's meal was allantoic fluid rather than blood, or that is, when the virus reached the circulatory system at a dilute rate. This observation indicates that the amount of virus initially available to the embryo is important, and that the route of infection influences the sensitivity of the assay system. Typically, for the transmission experiments listed in Table 1, a higher percentage of negative results occurred when flies took allantoic meals rather than blood meals, e.g., at 3 days of incubation, 83 versus 40 percent; and at 5 days, 33 versus 0 percent. It is possible that the two negative transmission experiments with colony flies incubated 21 days resulted from the insensitivity of the transmission egg assay system to a small amount of BT virus when that virus was placed in the allantoic cavity rather than inoculated directly into the blood stream.

We have attributed some of the negative transmission results, at least in part, to the injection of a small amount of virus by the bite of the fly. One should also take into account that the injection of a small amount of virus could have resulted from the use of older flies. The occurrence of two negative transmission experiments with flies incubated 21 days, together with the occurrence of three negative transmission experiments from probes of flies incubated 7, 21, and 28 days (especially when the probes of three flies incubated 3–6 days yielded transmissions), may indicate that older flies are less efficient as vectors.

It is likely, judging by the life span of the fly in the laboratory (Jones, 1964), that the 28-day incubation period obtained in these experiments is representative of

TABLE 3.—Time required for recipient host eggs to die after transmission of BT virus to each egg by the bite of an infected fly. (Only host eggs that received single bites by flies taking meals of blood or allantoic fluid are included; eggs that died within 24 hours or tests in which the flies were incubated for 2 days or less are not included.)

Number of days fly incubated	Number of transmission eggs that died on given day after trial or that survived (meals of blood=B, of allantoic fluid=A)						
	2	3	4	5	6	7	Survived
			Period of intermediate transmission rate				
3	BBB	E	B				B A
4	BBBB	BBB	BB A	B			
5	BBBBBB A	A					A
			Period of high transmission rate				
6	BBBBBB AA	A					
7	BBBBBBBBB		A				
8	B A						
15		A					
21	BB A	A	B				
	BB	A	—				
Numerical totals for days 6–28 B:	20		1				
A:	4	4	1				

the normal life span of the fly in the field. Even though the fly may lose some of its ability to transmit as it ages, the data suggest that *C. variipennis* is probably capable of remaining a vector throughout its lifetime.

ACKNOWLEDGMENTS. We wish to thank R. W. Chamberlain and W. D. Sudia, Department of Health, Education and Welfare, Public Health Service, Communicable Disease Center, Atlanta, Georgia, for their many suggestions for the improvement of this manuscript. We are also indebted to R. P. Lehmann, U. S. Department of Agriculture, Biometrical Services, Agricultural Research Center, Beltsville, Maryland, who confirmed the statistical significance of some of our data.

Literature Cited

CHAMBERLAIN, R. W., and SUDIA, W. D. 1961. Mechanism of transmission of viruses by mosquitoes. Ann. Rev. Entomol. 6:371–390.

FOSTER, N. M., JONES, R. H. and MCCRORY, B. R. 1963. Preliminary investigations on insect transmission of bluetongue virus in sheep. Amer. J. Vet. Res. 24(103):1195–1200.

JONES, R. H. 1964. Mass-production methods in rearing *Culicoides variipennis* (Coquillett). Bull. Wld. Hlth. Org. 31:571–572.

———. 1966. Some procedures and related equipment for disease-transmission research with *Culicoides*. Mosquito News 26(2):179–184.