ISOLATION OF EASTERN ENCEPHALITIS VIRUS FROM DIPTERA IN WISCONSIN[1]

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INTRODUCTION. The isolation of eastern encephalitis virus from a turkey and the serological evidence of widespread infection among turkeys in southwestern Wisconsin in 1959 (Spalatin, *et al.*, 1960) stimulated a study of EEV transmission among turkeys in 1960. In 1959, attempts were not made to isolate virus from arthropods until after serological results were known. Therefore, only 137 Diptera of 9 species were tested, all with negative results.

Turkey sera were again examined for neutralizing antibodies to both EEV and WEV in 1960, and increased emphasis was given to collecting and processing hematophagous Diptera from areas of known virus activity in the previous year. In the 1960 search for naturally infected blood-sucking flies, 402 pools of 10,635 specimens representing 32 species were processed in laboratory hosts.

MATERIALS AND METHODS. Throughout May and June, flies were collected from the windows of brooder houses containing young poults, and some were occasionally aspirated directly from birds. Field collections were made from April through September by exposing caged birds and capturing all insects attracted to and feeding on them as described by Anderson and DeFoliart (1961).

On most occasions living flies were transported to the laboratory where they either were anesthetized or killed by exposure to a temperature of —20 C. for 5 to 30 minutes. On a few occasions flies were immobilized by chloroform in the field, rapidly transferred to screw cap vials, placed in a thermos bottle containing dry ice, and transported to the laboratory. All flies were identified in the laboratory and pooled by species for virus isolation attempts. Depending on the number of insects collected on a specific date and the availability of laboratory hosts, the insect pools varied in size from one specimen to 116 of a species. Engorged and non-engorged specimens of a species were usually processed in separate pools.

Insects were triturated in 1 to 3 ml of tryptone broth, normal calf sera, or 20% antibody-free egg yolk (pH 7.7) containing 5000 units of penicillin and 5 mg of dihydrostreptomycin per ml. After large particulate matter had settled out, the supernatant fluid was filtered through sterile cotton and 0.10 ml inoculated into the allantoic chamber of 10-day-old embryonated eggs or subcutaneously into ½-day-old chicks. The brains of chicks which died and fluids from dead embryos were tested for bacterial contamination on nutrient agar and in thioglycollate broth. Bacteria-free harvests were tested for Newcastle disease virus and then repassed in either the above hosts or 3-day-old suckling mice. Mice received 0.02 ml of the inoculum intracerebrally and 0.03 ml intraperitoneally. Isolates were identified by standard virus neutralization tests in 10-day-old chick embryos.

Blood was collected from the experimental hosts at weekly or bi-weekly intervals prior to and after their exposure in the field, and examined for the presence of *Leucocytozoon* parasites and neutralizing antibodies to both EEV and WEV. As

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TABLE 1.—Summary of virus isolations, 1960

	Modified passage history[a]								
	First			Final				Log Neut. index	
	Host[b]	Mortality[c] (No.)	Incubation (Hrs.)	Host	Mortality (No.)	Incubation (Hrs.)	Titration[d]	EEV	WEV
Eusimulium johannseni (Hart)	CE	2/12	72–120	SM	9/9	24–72	7.0	1.8	0
Aedes sticticus (Mg.) [e]	CE	5/12	48–120	SM	11/11	24–56	7.2	2.3	0
Simulium meridionale Riley	CE	5/12	98–144	WC	4/6	24–48	5.5	2.7	0
Aedes sticticus (Mg.)	WC	2/6	24–96	CE	6/6	24	3.7	1.7	0

[a] Different isolates underwent from 2–5 passages before identification.
[b] CE=10-day-old chicken embryo; WC=one-half-day-old chicks; and SM=3-day-old suckling mice.
[c] Number dead over total number inoculated.
[d] Titered from final passage material in 10-day-old chicken embryos.
[e] Collected in turkey brooder house on June 8.

in 1959, blood was also obtained from several turkey flocks in the study area. Serum for antibody detection was collected on absorbent paper discs and tested as an eluate in the manner described by Adams and Hanson (1956) and Karstad, *et al.*, (1957).

RESULTS. Four isolations of EEV were made from the 402 pools of flies processed in 1960. These isolates were from a pool of 46 engorged *Eusimulium johannseni* (Hart) which had fed on an experimental turkey poult and a ring-necked pheasant exposed on May 2; a pool of 15 engorged *Aedes sticticus* (Mg.) and a pool of 100 non-engorged *Simulium meridionale* Riley, both pools collected in a turkey brooder house on June 8; and a pool of 5 engorged and 2 non-engorged *Ae. sticticus* collected from pheasants exposed as experimental field hosts on August 4. The isolation histories are summarized in Table 1. This is believed to be the first report of EEV being isolated from these species in nature.

Hematophagous Diptera processed for chicken embryo or wet-chick lethal agents included 8,464 Simuliidae, 1,122 Culicidae, and 1,049 Ceratopogonidae, classified as follows. The figures are the total number of pools inoculated, over the total number of individuals processed, per species.

SIMULIIDAE: *Cnephia taeniatifrons* (End.)—9/82; *Eusimulium aureum* (Fries)—25/255; *E. johannseni* (Hart)—57/750; *Simulium rugglesi* N. & M—2/146; *S. meridionale* Riley—158/7,236; *S. venustum* Say—1/10, and *S. decorum* Walk.—1/15. CULICIDAE: *Aedes cinereus* (Mg.)—3/5; *Ae. excrucians* (Walk.)—4/26; *Ae. sticticus* (Mg.)—14/169; *Ae. stimulans* (Walk.)—8/78; *Ae. triseriatus* (Say)—2/5; *Ae. trivittatus* (Coq.)—7/133; *Ae. vexans* (Mg.)—23/473; *Anopheles punctipennis* Say—6/79; *An. quadrimaculatus* Say—3/14; *An. walkeri* Theobald—5/51; *Culex erraticus* (D. & K.)—1/3; *C. pipiens* L.—4/8; *C. salinarius* Coq.—1/2; *C. tarsalis* Coq.—1/2; *C. territans* Walk.—1/1; *Mansonia perturbans* (Walk.)—7/41; Mixed species—4/35. CERATOPOGONIDAE: *Culicoides arboricola* R. & H.—4/10; *C. biguttatus* (Coq.)—5/240; *C. crepuscularis* Mall.—18/513; *C. guttipennis* (Coq.)—1/4; *C. haematopotus* (Mall.)—14/173; *C. stellifer* (Coq.)—2/15; *C. variipennis* (Coq.)—1/12; *C. venustus* Hoff.—2/71; *C. villosipennis* (R. & H.)—1/3; Mixed species—1/8.

In Table 2, insect genera are tabulated in the monthly sequence in which they were captured and processed. The April total represents blackflies collected from 4 experimental hosts exposed in a woodland habitat near a turkey farm during the last week of April. At this time the principal blood-sucking fly collected was *Cnephia*

TABLE 2.—Genera of Diptera captured and inoculated into laboratory hosts by month, 1960. (Figures are the total number of pools inoculated per genus over the total numbers of the several species.)

Genera	April	May	June	July	August	September	No. pools	No. individuals
Cnephia	5/50	4/32	..	..	..	..	9	82
Eusimulium	1/1	56/749	1/7	4/44	16/133	4/41	82	975
Simulium	..	40/2099	22/1661	12/420	74/2705	14/522	162	7407
Culicoides	..	..	3/124	7/168	34/720	5/37	49	1049
Aedes	..	8/109	18/170	10/64	33/515	2/76	71	934
Anopheles	..	..	1/3	..	9/73	3/62	13	138
Mansonia	..	..	..	1/6	6/26	..	7	32
Culex	..	..	..	1/1	7/15	..	8	16
No. pools	6	108	45	35	179	28	401[a]	..
No. individuals	51	2989	1965	703	4187	738	..	10,633[a]

[a] A mixed pool of one *An. walkeri* and one *C. pipiens* are excluded from the totals. Grand total= 402 pools and 10,635 individuals.

taeniatifrons. This fly was not found in brooder houses, but it was attracted to and fed on turkeys exposed in the field (Anderson and DeFoliart, 1961).

The May and June totals include both brooder house collections and flies captured after being attracted to experimental host birds in the field. All July, August, and September totals are from experimentally exposed hosts. Brooder house visits were made on April 23 and 29, May 2, 7, 14, 21, and 31, and on June 8. After the latter date all farm turkey flocks were on open ranges. No hematophagous insects were observed in the houses prior to May 7.

The following species were represented in the various genera collected during May: *C. taeniatifrons, E. johannseni, S. meridionale, Ae. cinereus, Ae. vexans, Ae. sticticus,* and *Ae. stimulans*. All mosquitoes and the 1,953 *S. meridionale* collected in May were obtained from brooder houses on May 31. The only blood-sucking insect captured in turkey shelters prior to this date was *E. johannseni*.

Except for the genera *Eusimulium* and *Culicoides,* all insects captured and processed in June were from brooder house collections on June 8. With the exception of 2 pools of 146 *S. rugglesi* collected in May and 1 of 10 *S. venustum* and 1 of 15 *S. decorum* collected in September, all *Simulium* captured and processed throughout the year were *S. meridionale*. All *Eusimulium* collected and processed from July through September were *E. aureum*. The remaining species are listed below according to the months in which they were collected: JUNE—*Culicoides biguttatus, C. crepuscularis, Ae. sticticus, Ae. vexans,* and *An. punctipennis*. JULY—*Culicoides biguttatus, C. crepuscularis, C. haematopotus, C. guttipennis, Ae. cinereus, Ae. vexans, Ae. excrucians, Ae. trivittatus, Ae. stimulans, M. perturbans,* and *Culex pipiens*. AUGUST—*Culicoides crepuscularis, C. haematopotus, C. stellifer, C. arboricola, C. guttipennis, C. venustus, C. variipennis, C. villosipennis, Ae. trivittatus, Ae. vexans, Ae. stimulans, Ae. excrucians, Ae. triseriatus, Ae. sticticus, M. perturbans, Culex pipiens, C. tarsalis, C. erraticus, C. territans, C. salinarius, An. punctipennis, An. quadrimaculatus,* and *An. walkeri*. SEPTEMBER—*Culicoides haematopotus, C. crepuscularis, Ae. vexans, Ae. trivittatus, An. walkeri,* and *An. punctipennis*.

Of the four experimental hosts exposed on May 2, two (a turkey poult and pheasant hen) died on May 4 and 10 respectively. No chicken embryo lethal agents were isolated from either bird. Serum neutralizing antibodies to EEV and WEV were not detected in any birds used as experimental field hosts.

DISCUSSION. Spalatin, *et al.* (1960), found that turkeys of various ages usually responded with the appearance of virus neutralizing antibodies when challenged with 5 to 1,000 LD_{50} units of virus; therefore, serological results obtained by bleeding farm turkey flocks were used to measure flock infection rates under natural field conditions. The number of antibody positive birds showed very little virus activity in 1960 as compared with 1959, but greater emphasis on collecting and processing hematophagous insects during 1960 resulted in the isolation of EEV from 2 pools of *Ae. sticticus,* 1 pool of *E. johannseni,* and 1 pool of *S. meridionale*.

The detected arthropod infection rate paralleled the low incidence of infection serologically determined in turkeys. However, since most simuliids collected and processed for virus isolation attempts were obtained in a related study on their feeding habits and host preferences (Anderson and DeFoliart, 1961), it was estimated that at least half the total number of black flies processed were captured when seeking their initial blood meals following emergence. Similar observations were not made on mosquitoes or ceratopogonids.

Although the significance of isolations from engorged specimens is usually difficult to interpret, 2 of our 3 pools (the 46 *E. johannseni* collected on May 2, and the 15 *Ae. sticticus* captured on August 4) containing engorged flies were captured

after feeding on experimental hosts from which no evidence of infection was obtained. As the third pool of engorged specimens (15 *Ae. sticticus*) was collected in a turkey brooder house, it is not known whether they were infected prior to or after feeding.

The isolation of virus from a pool of 100 non-engorged *S. meridionale* collected in a brooder house on June 8, indicated that this species may serve as a biological vector when exposed to the virus. This fly takes at least two separate blood meals and is one of the known vectors of the protozoan blood parasite, *Leucocytozoon smithi* L. & L. of turkeys (Skidmore, 1932; and Anderson and DeFoliart, 1961). Since field studies revealed two major feeding peaks (May 31 and June 8) of the first generation females, it appears that at least one of the non-engorged specimens collected on June 8 may have served as a biological vector of EEV.

Both blackfly species from which the virus was isolated are ornithophilic. All previous investigations on the potential of simuliids as vectors of viruses in North America have, apparently, involved mammalophilic species (Knowlton, 1934; Knowlton and Rowe, 1934; and Hammon, *et al.*, 1942).

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NOTES ON A MALE *AEDES AEGYPTI* WHOSE TERMINALIUM FAILED TO ROTATE [1]

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In a series of studies on the physiology of the reproductive system of *Aedes aegypti* (Linnaeus) in which many males of the Bangkok strain were being routinely force-copulated with females using a slight modification of the McDaniel-Horsfall technique (McDaniel and Horsfall, 1957), a single, six-day-old male was discovered whose terminalium had failed to undergo the normal 180° rotation (Christophers, 1915). This male was presented successively for five minutes to each of five virgin females of suitable age, but he made no attempt either to

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