# AN EVALUATION OF THE ROLE OF PHORMIA REGINA IN TRANSMISSION OF EASTERN ENCEPHALITIS

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DURING epizootics of eastern encephalitis in Connecticut pheasant populations, *Phormia regina* (blow flies) were consistently observed in the vicinity of pheasant pens and were frequently responsible for the larval infestations of carcasses. Because the ecology of eastern encephalitis is poorly understood, the possibility that the flies played a role in the dissemination of the infection was immediately considered. Five experiments designed to test this hypothesis were performed in this study.

## Methods

Extracts of *P. regina*, used for the detection of EE virus in tissue culture, were prepared in Hanks' lactalbumin hydrolysate solution containing 2 percent by volume of inactivated (60° C. for 30 minutes) calf serum, 10 percent by volume of a saturated aqueous solution of 1-phenyl-2-thiourea, 1,000 units of penicillin per milliliter, and 1,000 micrograms of streptomycin per milliliter (*1*). (1-phenyl-2-thiourea, which inhibits the action of tyrosinase, was used to prevent toxic effects of insect pools in cell cultures; preliminary studies indicate that it does not interfere with the detection and replication of EE.) The extracts were clarified at 4° C. by centrifugation at 650 g for 5 minutes and at 9,000 g for 30 minutes.

Sources of EE virus were prepared from 20 percent suspensions of infected suckling mouse brains in phosphate-buffered saline (pH 7.0) containing 5 percent of bovine albumin. These suspensions were clarified in a manner similar to the preparation of the *P. regina* extracts and stored in the fluid-frozen and lyophilized states at −40° C. The virus sources came from a plaque-purified laboratory stock. This stock originated through numerous mouse passages from the brain of a fatal human case (*2*). Dilutions of infected mouse brain extracts were prepared in the same phosphate-buffered saline containing 5 percent bovine albumin.

Chick embryo cell cultures, used for the detection and quantitation of EE virus, were prepared from trypsinized chick embryo fibroblasts. Culture tubes were each seeded with 1 ml. of a 1:500 suspension of cells in Hanks' lactalbumin hydrolysate solution containing 2 percent by volume of inactivated calf serum, 100 units of penicillin per milliliter, and 100 μg. of streptomycin per milliliter. The tubes were incubated at 37° C. in a stationary position. Fifty percent tissue culture dose ($TCD_{50}$) endpoints were calculated by the Reed-Muench method (*3*). Agar overlaid monolayers of chick embryo fibroblasts were prepared in 3-ounce prescription bottles after the method originally described by Dulbecco (*4*) and modified by Hsiung and Melnick (*5*).

Sources of EE virus were titrated in cell cultures. Clarified extracts of *P. regina* were inoculated in tenfold dilutions in both cell cultures and agar overlaid monolayers. The latter were used only to detect the presence of EE virus in insect extracts.

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*Dr. R. C. Wallis, chief, section of medical epidemiology, Yale University School of Medicine, supplied the blow flies and advised on the investigations.*

Adult and larval stages of *P. regina* were sampled from colonies maintained at the Connecticut Agricultural Experiment Station. We obtained the brain material and its extracts in the laboratory from Swiss albino mice. The white rock chick that served as a source of EE virus in one experiment was sampled from a flock hatched from embryonated eggs in the laboratory.

### Experiments

Three experiments were devoted to the larval stages of *P. regina*.

In the first experiment, 43 fourth-instar larvae were placed in 16 grams of coarsely chopped horsemeat mixed with sawdust, to which had been added 1 ml. of a 1:10,000 dilution of clarified mouse brain extract containing $1\times10^5$ $TCD_{50}$ of EE virus. After given periods of incubation at 30° C. in a relative humidity of 80 percent, insects were sampled and tested for virus (see table). At the same time insects were sampled from a control colony and similarly processed to exclude the presence of naturally occurring viruses.

Thirty-one infected suckling mouse brains containing $2\times10^7$ $TCD_{50}$ of EE virus per milligram served as a source of infection in the second experiment. Approximately 100 first-instar larvae were placed in direct contact with a mixture of brain material and sawdust, and incubated for 24 hours at 30° C. in a relative humidity of 80 percent. They were then trans-

**Pools of *Phormia regina* sampled at various incubation periods for EE virus extraction**

| Number of days of incubation at 30° C. in relative humidity of 80 percent | Number of insects extracted per pool in given volume of extracting fluid × number of pools sampled | | | Virus yields $Log_{10}$ $TCD_{50}$ |
|---|---|---|---|---|
| | Larval pools | Pupal pools | Imaginal pools | |
| *4th-instar larvae* | | | | |
| 1 | 2/2 ml. × 2 | | | <1.0 |
| 2 | 2/2 ml. × 3 [1] | 2/2 ml. × 1 | | <1.0 |
| 3 | 2/2 ml. × 2 [2] | | | <1.0 |
| 6 | | | 1/2 ml. × 2 | <1.0 |
| *1st-instar larvae* | | | | |
| 1 | 10/2 ml. × 1 | | | <1.0 |
| 2 | 5/2 ml. × 1 | | | <1.0 |
| 3 | 5/2 ml. × 1 | | | <1.0 |
| 4 | 2/1 ml. × 1<br>5/2 ml. × 1 [3] | | | <1.0 |
| *3d- to 4th-instar larvae* | | | | |
| 3 | 10/20 ml. × 1 | | | <1.0 |
| 7 | | 10/20 ml. × 1 | | <1.0 |
| 13 | | | 4/4 ml. × 1 | <1.0 |
| *4- to 5-day-old adults* | | | | |
| 0 | | | 2/2 ml. × 1 | 4.3 |
| 1 | | | 2/2 ml. × 1 | <1.0 |
| 2 | | | 2/2 ml. × 2 [4] | <1.0 |
| 3 | | | 2/2 ml. × 1 [5] | <1.0 |
| *2-day-old adults* | | | | |
| 0 | | | 4/4 ml. × 1 | 4.7 |
| 1 | | | 4/4 ml. × 1 | <1.0 |
| 3 | | | 4/4 ml. × 1 | <1.0 |
| 5 | | | 1/1 ml. × 1 | <1.0 |

[1] Included one pool of recently dead larvae.
[2] Consisted of recently dead larvae only.
[3] Consisted of recently dead larvae.
[4] Included one pool of recently dead adults.
[5] Consisted of recently dead adults only.

NOTE: Recently dead insects represent those that died within 24 hours.

ferred to an EE-free medium of coarsely chopped horsemeat mixed with sawdust and reincubated as before. An approximately equal number of larvae were similarly placed in a mixture of brains of normal suckling mice and sawdust, and then transferred to a mixture of coarsely chopped horsemeat and sawdust. Insects from both the potentially infected and the control colonies were sampled at given intervals and tested for virus (see table).

In the third experiment, the carcass of an infected chick was used as the source of virus. A freshly hatched chick was given an intramuscular dose of approximately $1\times10^7$ $TCD_{50}$ of EE virus. When it became moribund 20 hours later, its brain was harvested and the blood titrated for virus. Its thorax and abdomen were then opened to receive approximately 100 third- to fourth-instar larvae. After given periods of incubation at 30° C. in a relative humidity of 80 percent, insects were sampled and tested for virus (see table). The brain sample yielded $5\times10^6$ $TCD_{50}$ of EE virus per mg., and the blood sample, $>3\times10^5$ $TCD_{50}$ of EE virus per 0.2 ml.

Two experiments were designed to infect *P. regina* in the adult stage with oral doses of EE virus in 20 percent sucrose solution. Under $CO_2$ anesthesia each fly was suspended from a paraffin wax mount by embedding the tips of its spread-eagled wings with heated forceps. Thus restrained, the fly was fed a measured amount of the infected solution from a 0.1 ml. pipette with a suitably curved tip. After the feeding, the flies were removed from the mounts, placed in glass containers with food and water, and incubated at 30° C. in a relative humidity of 80 percent.

In the first experiment, twelve 4- to 5-day-old flies were each given 0.005 ml. of sucrose solution containing approximately $5\times10^4$ $TCD_{50}$ of EE virus. At the same time eight controls were also fed similar volumes of virus-free sucrose solution. Flies were sampled and tested for virus immediately following the feeding, and after incubation periods ranging from 1 to 3 days (see table).

The second experiment was similar to the first, except that thirty 2-day-old flies were each fed 0.01 ml. of sucrose solution containing approximately $1\times10^5$ $TCD_{50}$ of EE virus. They were sampled and tested for virus immediately following the feeding and after incubation periods ranging from 1 to 5 days (see table).

Apart from those adult flies sampled immediately after the infective feeding, EE virus was not recovered from any of the sampled insects in the five experiments.

## Discussion

The results suggest that the virus of EE does not multiply in *P. regina*. During an epizootic in Connecticut, Dr. Wallis failed to transmit EE to susceptible pheasants by feeding them with *P. regina* larvae taken from the carcasses of infected pheasants. He was also unable to recover the virus from adult flies caught in the area surrounding the pheasant pens. Thus the available evidence suggests that *P. regina* plays no role in the transmission of EE virus.

## Summary

Five experiments were performed on *Phormia regina*. The first three were attempts to infect larvae by placing them in infected food media, and the last two were attempts to infect adults by individually feeding them virus in 20 percent sucrose solution. Apart from freshly fed adults, the sampled insects failed to yield virus in fluid and agar-overlaid cultures of chick embryo fibroblasts. It is concluded that the species plays no role in the transmission of eastern encephalitis.

## REFERENCES

(1) American Public Health Association: Diagnostic procedures for virus and rickettsial diseases, New York publication office, 1956.

(2) Webster, L. T., and Wright, F. H.: Recovery of eastern equine encephalomyelitis virus from the brain tissue of human cases of encephalitis in Massachusetts. Science 88: 305–306 (1938).

(3) Reed, L. J., and Muench, H.: A simple method of estimating fifty percent endpoints. Amer J Hyg 27: 493–497 (1938).

(4) Dubecco, R.: Production of plaques in monolayer tissue cultures by single particles of animal virus. Proc Nat Acad Sci 38: 747–752 (1952).

(5) Hsiung, G. D., and Melnick, J. L.: Comparative susceptibility of kidney cells from different monkey species to enteric viruses (poliomyelitis, coxsackie and ECHO groups). J Immun 78: 137–146 (1957).