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FIELD AND LABORATORY STUDIES ON THE HOSTS AND VECTORS OF THE SNOWSHOE HARE STRAIN OF CALIFORNIA VIRUS

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INTRODUCTION. Snowshoe hare (SH) virus, which is closely related to California encephalitis virus (CEV), is endemic in certain mountainous areas of the northwestern United States and British Columbia (Burgdorfer *et al.*, 1961; Newhouse *et al.*, 1963). Antibody was found in snowshoe hares and golden-mantled ground squirrels; virus isolations were made from the blood of a snowshoe hare and from ticks removed from a chipmunk, a golden-mantled ground squirrel and a snowshoe hare. Newhouse *et al.* (1963) attempted to infect ticks in the laboratory; however, virus was not recovered from the ticks for more than 48 hours after an infected blood meal.

This paper presents additional serological information on probable SH virus infection in the Northwest. Also, experimental viremia and antibody studies were done in several species of common small mammals to determine which ones might be potentially important in the natural cycle of SH virus. Field studies on mosquitoes in Montana yielded isolations of SH virus from *Aedes fitchii* (Felt and Young) and from *Culiseta impatiens* (Walker).

DESCRIPTION OF STUDY AREAS. Mammal collections were made in several canyons in the Bitter Root Mountains, but primarily in Lost Horse Canyon, approximately 12 miles southwest of Hamilton, Montana. The mouth of this canyon lies about 4,000

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feet in altitude and its floor rises for several miles. The sides of the canyon at the lower end are steep and pine-covered, with high rock cliffs and talus slides. Lost Horse Creek, a shallow mountain stream, flows along the canyon floor and into the Bitter Root River. Mammals were trapped along a road extending from the canyon floor to approximately 2,000 feet up the north side.

In this area the soil-temperature inversion occurs about the end of March, and by mid-April most of the snow is gone from the valley floor. As the creek rises in May, seepage pools form in the lower spots throughout the woods. Summer is short and hot, and by the end of August or the first of September the first frost occurs.

MATERIALS AND METHODS. Collection of small wild mammals in Lost Horse Canyon was carried out from 1960 to 1963 by shooting or live trapping. Blood samples were taken by cardiac puncture and either placed directly on dry ice or refrigerated with wet ice and were taken to the Rocky Mountain Laboratory (RML) and centrifuged.

Vertebrate sera were tested for the presence of antibody reactive against SH virus by a suckling mouse (SM) intraperitoneal (IP) neutralization test according to the methods described previously (Newhouse *et al.*, 1963).

Mammals used for viremia studies were first determined to be SH seronegative and then were inoculated subcutaneously (SC) in their hind quarters with varying dilutions of a 13th mouse-brain passage of SH virus. Blood samples were taken from the heart by syringe moistened with heparin sodium, U.S.P. at 12 and 24 hours post inoculation and at 24-hour intervals thereafter for at least 8 days. Suckling mice were inoculated IP with 0.05 ml of ten-fold dilutions of each blood sample, and LD_{50} endpoints were calculated by the method of Reed and Muench (1938). Titers are expressed as $SMIPLD_{50}/0.05$ ml.

Mosquitoes were collected in Lost Horse Canyon from June through September of 1962 and March through September of 1963 in rabbit-baited traps of a modified lard-can design and a goat-baited trap of the Magoon-type (Magoon, 1935). Traps were placed several hundred feet up the sides of Lost Horse Canyon early in the spring and were moved down into the bottom in early June as overwintering mosquitoes disappeared and spring broods of *Aedes* emerged. Mosquitoes were killed by freezing and stored in rubber-stoppered vials at —60° C until tested. They were placed in dishes chilled on cracked ice and identified. They were pooled by species in groups of 50 or less, and ground with mortar and pestle in 1.5 ml phosphate buffered saline containing 50 percent normal rabbit serum, 300 units sodium penicillin G and 300 micrograms streptomycin sulfate, U.S.P. per ml. Suspensions were centrifuged for 10 minutes at 3,000 RPM and the supernatant fluids were inoculated into SM, 3 to 4 days old, both intracerebrally (IC) and IP (0.02 and 0.05 ml, respectively).

Viral isolates were identified as members of the California complex by SM neutralization tests at the RML. They were subsequently identified as the SH type at the Center for Disease Control, by Dr. Fred Murphy, by gel diffusion (Murphy, 1966, personal communication).

RESULTS. Of 10 species of small wild vertebrates tested (Table 1), 2 species were found to have antibodies to CEV: the snowshoe hare (5/104) and the golden-mantled ground squirrel (2/60). Numbers of animals collected of other species were too small for the negative results to be of significance.

Six of 10 species tested developed demonstrable viremias (Table 2). Three of these, the chipmunk, the golden-mantled ground squirrel, and the meadow vole, developed titers of virus in their blood that ranged from 3.5 to 4.4 logs/0.05 ml. Their period of viremia also persisted the longest, 2 to 4 days. The snowshoe hares, on the other hand, had blood virus titers which were somewhat lower (no greater

TABLE 1.—Neutralizing antibodies to the snowshoe hare strain of California encephalitis virus in blood or serum of wild mammals from Montana, 1960–1963

Species	No. Positive / No. Tested
Porcupine *Erethizon dorsatum*	0/1
Meadow Vole *Microtus pennsylvanicus*	0/1
Ground Hog *Marmota flaviventris*	0/3
Red Squirrel *Tamiasciurus hudsonicus*	0/13
Chipmunk *Eutamias amoenus*	0/20
White-footed Mouse *Peromyscus maniculatus*	0/18
Bushy-tailed Wood Rat *Neotoma cinerea*	0/19
Golden-mantled Ground Squirrel *Spermophilus lateralis*	2/60
Columbian Ground Squirrel *Spermophilus columbianus*	0/80
Snowshoe Hare *Lepus americanus*	5/104
Small Vertebrate Totals	7/319

than 2.6 logs) and the viremic period persisted for no more than 2 days. A bushy-tailed woodrat had a low-titered viremia, and 1 of 4 Columbian ground squirrels circulated only a trace of virus in the blood.

All species tested produced antibody which neutralized SH virus (Table 2). Four vertebrates (elk, dog, ground-hog and white-footed mouse) produced antibody, although no viremia could be detected during the post inoculation sampling period.

During the summers of 1962 and 1963, we collected 19,321 mosquitoes of 14 species in the Bitter Root Mountains. They were tested for virus in 869 pools (Table 3). Six isolations of SH virus were made: 5 from *Aedes fitchii* (F&Y), in 1962, and 1 from *Culiseta impatiens* (Walk.), in 1963. The overall infectivity rates calculated for these years are 1:1518 for *A. fitchii* and 1:4763 for *C. impatiens*, for all months during which the mosquitoes were collected (June–September 1962; March–September 1963). The rates were somewhat higher when only collections made during the months which included the first and last isolations were calculated: 1:1326 for *A. fitchii* and 1:93 for *C. impatiens*. These last figures may provide a somewhat more realistic impression of the involvement of these species during the period of virus amplification.

All 5 isolations from *A. fitchii* were from mosquitoes collected between August 1 and September 2, 1962. The single pool of *C. impatiens* that contained SH virus was collected on June 10, 1963.

DISCUSSION. Of the 10 field-collected species of small mammals tested, only 2, the snowshoe hare and golden-mantled ground squirrel, had antibodies reactive against SH virus. These antibodies were assumed to have been stimulated by SH virus since that was the only California-group virus isolated in the area during this study.

There are few published studies on California group arboviruses in western United States with which these antibody rates can be compared. Hammon and Reeves (1952) found CEV neutralizing antibody in cottontail rabbits (14 percent), jack rabbits (18 percent) and California ground squirrels (19 percent) in California. Gresikova *et al.* (1964), also working in California, found CEV HI antibody in cotton tail rabbits (6 percent), jack rabbits (12 percent) and California ground squirrels (7 percent). Other species, such as the grasshopper mouse, were reported to be as high as 33 percent seropositive for the small number sampled.

Other vertebrates which we did not collect in large numbers have been found by others to have neutralizing antibody; finding antibody in porcupines and ground-hogs in Ontario and New York (Newhouse *et al.*, 1964; Whitney *et al.*, 1969) has been particularly interesting.

The results of serological tests on field-

TABLE 2.—Experimental viremias in small vertebrate species and subsequent neutralizing antibody.

Species	No. Tested	SH Virus Dose Inoculated *	Peak Viremia/Hours * (Duration of Viremia in Days)	Representative Log_{10} Neut. Indices **
Golden-mantled Ground Squirrel *Spermophilus lateralis*	7	4.8–6.1	$\frac{3.5}{24}$ (2), $\frac{2.9}{48}$ (4), $\frac{2.5}{24}$ (3), $\frac{2.4}{72}$ (4), $\frac{1.5}{24}$ (2), $\frac{1.2}{24}$ (2), 0	5.0, 3.6
Chipmunk *Eutamias amoenus*	7	2.7–4.4	$\frac{4.1}{48}$ (3), $\frac{3.8}{48}$ (2), $\frac{3.5}{24}$ (2), $\frac{2.8}{24}$ (3), $\frac{2.5}{24}$ (2), $\frac{2.3}{48}$ (3), $\frac{2.0}{24}$ (2)	
Snowshoe Hare *Lepus americanus*	5	3.0–5.4	$\frac{2.6}{24}$ (2), $\frac{2.0}{24}$ (1), $\frac{1.6}{21}$ (1.5), $\frac{1.4}{29}$ (2), $\frac{1.0}{47}$ (2)	>6.0
Columbian Ground Squirrel *Spermophilus columbianus*	4	4.8–5.5	$\frac{0.7}{24}$ (1), 0, 0, 0	>2.7, >2.0
Meadow Vole *Microtus pennsylvanicus*	2	4.8	$\frac{4.4}{48}$ (3), $\frac{3.8}{48}$ (3)	>3.6
Bushy-tailed Wood Rat *Neotoma cinereus*	2	5.0	$\frac{1.6}{72}$ (3), 0	>3.0
Ground Hog, *Marmota flaviventris*	2	5.3	0, 0	>3.0
White-footed Mouse *Peromyscus maniculatus*	2	4.8	0, 0	>2.6, >2.0
Domestic Dog (Beagle)	2	5.0	0, 0	>3.7, >3.7
Elk Calf, *Cervus canadensis*	1	4.5	0	>4.1

* $SMIPLD_{50}$/.05ml, log_{10}

** Approximately 30 days post inoculation.

collected mammals are consistent with those of the experimental viremia studies. The snowshoe hare, chipmunk and golden-mantled ground squirrel were found to circulate virus from 1 to 4 days after inoculation with SH virus. Since all of the animals were adults, differences in viremias probably did not reflect age resistance of these species. The lowest peak viremia titers and the shortest viremic periods were more frequently found in snowshoe hares; golden-mantled ground squirrels and chipmunks generally circulated relatively larger amounts of virus for longer periods of time. The number of meadow voles tested was low, but this species also had viremias equivalent to that seen in the chipmunk. If viremia could be detected in the other species tested, including the Columbian ground squirrel, the titer was very low. Nevertheless, all animals tested developed antibody as a result of the inoculation, even those in which no virus could be detected.

The isolation of five strains of SH virus from *A. fitchii* suggests that this species should be considered a potentially important vector; certainly the isolations demonstrate that the species is susceptible to infection and suggests close association with natural vertebrate hosts. For accepted vector criteria to be met (Reeves, 1960), it must be demonstrated that the species becomes infected when it feeds upon viremic hosts in the laboratory and is capable of transmitting the virus by bite under controlled conditions. The data of Carpenter and Nielson (1965) support the vector potential of *A. fitchii;* they reported that *A. fitchii* is capable of surviving at least 4 ovipositions and has a biting period in California of up to 53 days, adequate to serve an active role in virus buildup. Our collections in the Bitter Root Mountain area indicated a biting period of at least 63 days. In 1963, the first female *A. fitchii* was caught on June 3, the first blooded female on June 12, and the last blooded female on August 14. Had collections been made beyond mid-August, an even

TABLE 3.—Mosquitoes collected in the Bitter Root Mountains, Montana, 1962 and 1963, and tested for virus

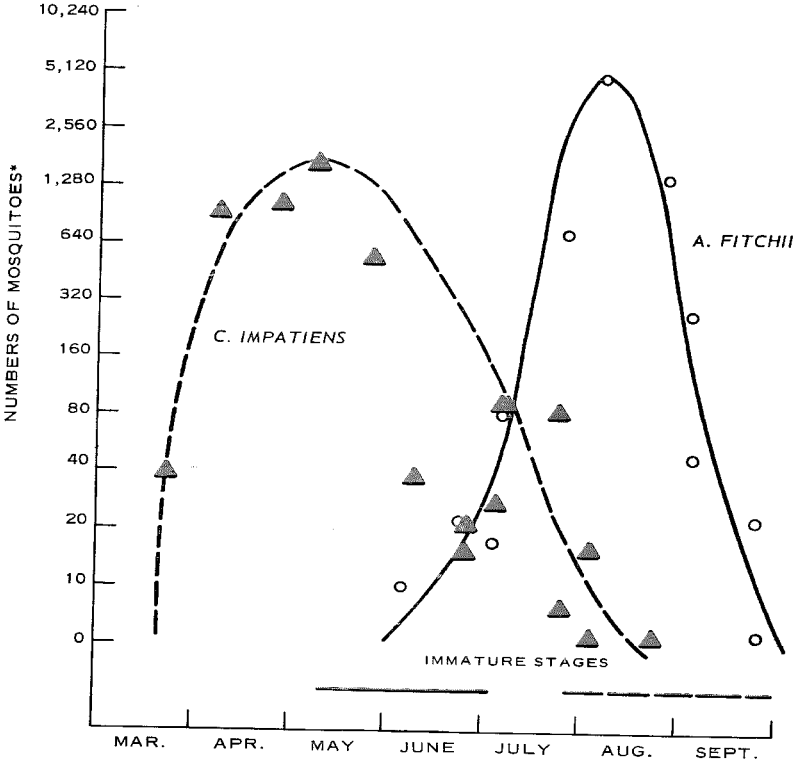
Species	Mosq. Tested	Pools Tested	Snowshoe Hare Strains Isolated
Aedes canadensis (Theo.)	3,875	173	
Aedes excrucians (Walk.)	89	41	
Aedes fitchii (F&Y)	7,592	276	5*
Aedes increpitus Dyar	1	1	
Aedes sierrensis (Lud.)	5	5	
Aedes vexans (Meig.)	8	4	
Aedes sp. nr. *punctor*	2,728	123	
Anopheles freeborni Aitk.	2	2	
Culex tarsalis Coq.	13	7	
Culiseta alaskaensis (Lud.)	201	26	
Culiseta impatiens (Walk.)	4,763	188	1*
Culiseta incidens (Thomp.)	29	12	
Culiseta inornata (Will.)	12	8	
Mansonia perturbans (Walk.)	3	3	
TOTALS	19,321	869	6*

* 25 *A. fitchii* collected Aug. 1, 1962.
50 *A. fitchii* collected Aug. 20, 1962.
50 *A. fitchii* collected Aug. 20, 1962.
50 *A. fitchii* collected Aug. 24, 1962.
50 *A. fitchii* collected Sept. 2, 1962.
3 *C. impatiens* collected June 10, 1963.

longer biting period might have been demonstrated.

Perhaps the most interesting isolation of SH virus was that from a pool of 3 blooded *C. impatiens* collected from a goat-baited trap on June 10, 1963. Frohne (1953) found this mosquito species to be single-brooded in Alaska; it enters a period of aestivation-hibernation immediately after reaching imago and does not feed on blood until the following spring.

In the Bitter Root Mountain area, evidence also indicates a single brood. Overwintered adults were most numerous during the last half of April and first half of May (Figure 1). Their population decreased to about one-third during the last half of May and to about one-tenth during the first week of June. The new adults began to emerge (as evidenced by empty pupal skins) during the last week of July. There was no indication of the new brood



*MOSQUITOES COLLECTED BI-MONTHLY IN RABBIT AND GOAT BAITED TRAPS.

FIG. 1.—Probable adult population curves of *Culiseta impatiens* and *Aedes fitchii* in Lost Horse Canyon based on combined 1962-1963 collection records.

entering traps, since the numbers collected continually decreased until August when they completely disappeared.

If, in the Bitter Root area these mosquitoes are indeed single-brooded and aestivate upon emergence, the blooded mosquitoes from which the isolation was made must have fed at least once before the June 10 goat blood meal to acquire the infection. If, as it appears, they do not feed before spring, either another vector species carried the virus over the winter to initiate the virus infection in vertebrates earlier than June 10, or the virus actually overwintered in some vertebrate. Only 30 mosquitoes, other than *C. impatiens, Culiseta incidens* (Thomp.) or *Culiseta alaskensis* (Lud.), had been seen by June 15, and these were all *A. fitchii* or *Aedes vexans* (Meig.) mosquitoes which do not overwinter as adults.

Danielova *et al.* (1968) and Danielova and Minar (1969) were able to demonstrate experimentally that Tahyna virus could be isolated from *Culiseta annulata* (Schrk.) after the mosquitoes had been in hibernation for as long as 6 months. However, in the Bitter Root Mountains, *C. incidens* appears to be the only early emerging *Culiseta* species which, like *annulata,* feeds prior to hibernation; and it is an unlikely overwintering vehicle in view of its relative rarity. Only 129 specimens were collected during a 2-year period, although the methods used capture this species in great abundance where it is more common. *C. alaskaensis,* like *C. impatiens,* apparently does not feed on blood until after hibernation. Thus, the possibility of SH virus overwintering in a *Culiseta* mosquito in the study area seems remote. The same is true of *A. fitchii* and *A. vexans.* Since these mosquitoes survive the winter in the egg, transovarial transmission of the virus would be required for the species to serve as overwintering reservoirs; effective transovarial passage of an arbovirus in mosquitoes via infected embryos has not yet been demonstrated.

In view of these considerations, the hypothesis that certain vertebrates serve as overwintering reservoirs of SH virus seems most plausible. Thus far, in warm-blooded vertebrates, viremias of long enough duration for overwintering of a virus have been demonstrated only under conditions of induced hibernation. The findings of Simkova (1966) are of particular interest; she discovered that Tahyna virus persisted in hibernating hedge hogs for as long as 140 days and could be recovered for several days after their being aroused from hibernation. Similarly, Emmons (1966) reported that Colorado tick fever virus persisted in blood of hibernating golden-mantled ground squirrels up to 124 days and continued to persist up to 47 days after the animals' emergence from hibernation.

Columbian and golden-mantled ground squirrels come out of hibernation early in May, nearly a month after *Culiseta* mosquitoes emerge from hibernation and at the time when the avid *Culiseta* population is at its peak.

In terms of abundance, emergence dates, viremia peaks and duration of viremia following experimental infection, the golden-mantled ground squirrel appears to be a more likely candidate as an overwintering reservoir host than the Columbian ground squirrel. However, the need for more study in this area is obvious.

SUMMARY. A total of 319 small wild vertebrates collected in the Bitter Root Mountains of Montana from 1961 through 1963 were tested for neutralizing antibody to California encephalitis virus. Two of the 10 species represented, the snowshoe hare and the golden-mantled ground squirrel, were found to have CEV antibody, with a rate of 4.8 percent in the former and 3.3 percent in the latter.

In the laboratory, snowshoe hares, golden-mantled ground squirrels, chipmunks, and meadow voles were shown to develop viremias of moderate titers which persisted up to 3 days after inoculation with snowshoe hare (SH) virus. Columbian ground squirrels and bushy-tailed woodrats reacted inconsistently. Ground-hogs, an elk calf, domestic dogs, and white-footed mice did develop a detectable viremia. All species tested developed neutralizing antibody reactive

against SH virus as a result of the inoculation.

Mosquitoes were collected for virus isolation in the Bitter Root Mountains of Montana in 1962 and 1963. From 19,321 mosquitoes collected, 6 isolations of the SH virus were made: 5 from *Aedes fitchii* collected in the late summer of 1962, and 1 from *Culiseta impatiens* collected in early June 1963.

Other aspects of the ecology of CEV in the Bitter Root Mountains are discussed.

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