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Arboviruses in South Dakota Mosquitoes and Their Pathogenicity to Pheasants

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ARBOVIRUSES IN SOUTH DAKOTA MOSQUITOES AND THEIR PATHOGENICITY TO PHEASANTS

BY

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INTRODUCTION

The extent of arthropod-borne virus activity in humans and domestic and wild vertebrates in South Dakota is not known. The World Health Organization (88) defines an arbovirus as follows: "an arbovirus must produce a viremic in one or more vertebrate species, multiply in some arthropod that feeds on viremic blood and be transmitted through feeding." Mosquitoes or other arthropods fill these requirements because female mosquitoes take blood as a requisite to egg maturation. Blood from any vertebrate host contains the essential nutrients for ovarian maturation (65). Not all mosquitoes prefer blood from the same host so a wide variety of mammals and birds act as sources. Hardy in 1967 (30) reported 21 arthropod-borne viruses were found in North America, of which 20 infected wildlife. Ten of the 21 arboviruses produce clinical disease in man or domestic animals, or both, but usually produce clinically inapparent infections in wildlife. Since mosquitoes are not active during the entire year in South Dakota, resident vertebrate hosts may serve as an overwintering reservoir for the arboviruses. With these facts in mind, a transmission cycle for arboviruses is proposed (Fig. 1). Similar transmission cycles have been proposed by Hess and Holden (35). This proposed transmission cycle places more weight on the pheasant as a host in arbovirus transmission than the other hosts shown in the cycle.

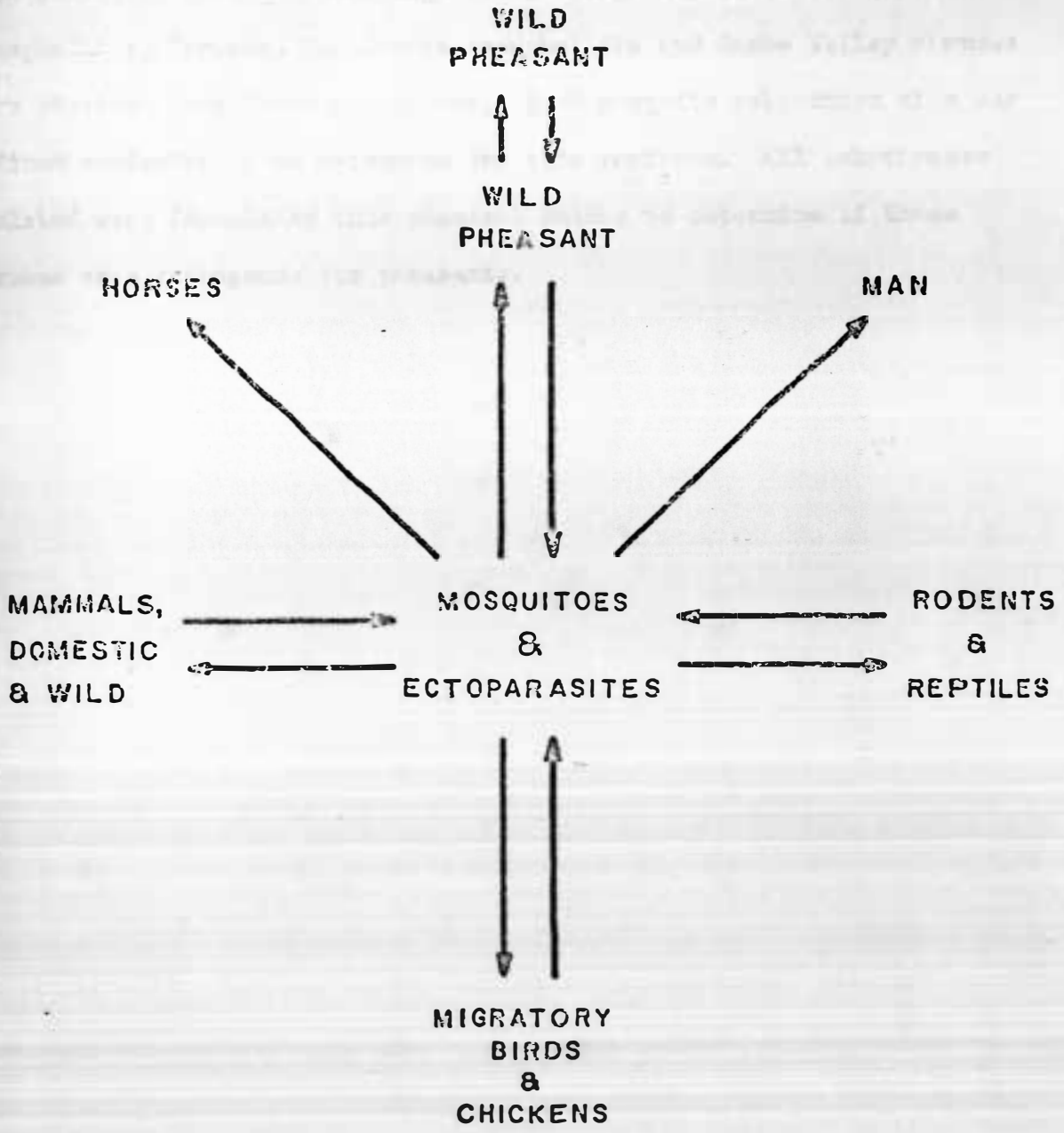

Figure 1. Postulated transmission cycle for arboviruses in South Dakota

Epidemiological surveys of mosquitoes have yielded much of the knowledge concerning arboviruses. In this preliminary study mosquitoes were collected from the Brookings area. Virus isolations of Western encephalitis, Turlock, California encephalitis and Cache Valley viruses were obtained from these mosquitoes. Each mosquito collection site was defined ecologically to determine any site variance. All arboviruses isolated were inoculated into pheasant chicks to determine if these viruses were pathogenic for pheasants.

LITERATURE REVIEW

This review covers the epidemiological features of Western equine encephalitis, Cache Valley, Turlock, California encephalitis, and Eastern equine encephalitis viruses.

Epidemiology of Western Equine Encephalitis

Meyer et al. (53) in 1931 obtained the first isolation of Western equine encephalitis (WEE) virus from the brain of a sick horse in California. Howitt et al. (40) recovered the same virus from the central nervous system and blood of a human case of encephalitis seven years later. Since these early isolations of WEE virus, it has shown a wide geographic distribution. It is predominantly a disease of rural farming areas. It was believed to be limited to the distribution of Culex tarsalis mosquitoes until the virus was isolated from birds in Louisiana, an area where Culex tarsalis is seldom reported (43). To date the virus has been recovered or reported in almost all the United States as well as in Canada and South America (54). The first serious epidemic of WEE occurred in Minnesota, North Dakota, and South Dakota in 1941 (59). According to the available information from North Dakota over 1000 human cases were reported with a mortality rate of 12.5% and 2,500 cases of equine infections were recorded with a mortality rate of 21%. In most of the years following 1941, South Dakota has reported a few confirmed cases of WEE infections in man and horses (16).

The onset of WEE is usually sudden with headache, sweating, disturbance of sleep, confusion and drowsiness. Pain and stiffness in the neck and back occur. Tremors and paralysis may be present (67). The illness lasts for approximately one week. Spastic paralysis has been recorded as sequelae, especially in infants surviving an infection. Sequelae are defined as any lesion or affection following or caused by an attack or disease (17). Infants under 3 months of age infected with WEE virus have had the greatest central-nervous-system damage of which 44% had sequelae. In patients between 1 and 4 years of age, the incidence of sequelae was less (67). Leake (47) and Eklund (18) reported that 70% of the human cases of WEE virus infections in Minnesota and North Dakota in 1941 occurred in males. Hammon (25) concluded that age and sex incidence of WEE infections varied in different geographic areas. Infections of WEE virus occur from May to September, but primarily are in July and August. In the 1941 Minnesota outbreak, 91.3% of the human cases had their onset between July 6 and August 23 (18).

The mosquito species Culex tarsalis has been shown to be the major vector in the transmission of WEE to man and animals (65). Although the virus has been isolated from a number of other mosquito species, a high incidence of C. tarsalis has been found in every epidemic (41). Hammon and Reeves (25) have shown that C. tarsalis mosquitoes feed frequently on birds to obtain blood meals. According to Reeves et al. (64) as high as 84% of blood engorged C. tarsalis from highly endemic areas tested had obtained blood meals from birds.

Avian blood feedings were examined by the precipitin test developed by Tempelis and Lofy (76) and a high degree of preference was shown between different mosquito species. Passerine birds, doves, or domestic fowl may be preferred depending on their relative abundance in the environment (77). It was found that feeding habits of C. tarsalis were not restricted to birds only but have ranged from snakes and lizards to large vertebrates such as man and horses (34,63). These other hosts are secondary to birds in the preference of C. tarsalis. Reeves (65) felt that these hosts other than birds may have disrupted serial transmission of the virus in that they did not have a high titered viremia as was present in avian hosts. Observations made by Beadle (1) indicated that C. tarsalis exhibited a peak biting activity at dusk; whereas, Aedes mosquitoes reached their peak earlier in the evening. Since C. tarsalis had its peak biting activity at dusk, Hess and Holden (35) observed that this was the critical period for transmission of WEE and that the primary reservoir for WEE was among the hosts available to C. tarsalis at this time. Field workers have observed that evening flights of birds to nocturnal roosting sites took place a short while before the peak biting activity of C. tarsalis. Reeves (61) gave a possible explanation of the C. tarsalis-bird feeding association. Birds nested and roosted in trees and foliage which gave off carbon dioxide at night when photosynthesis had ceased. This carbon dioxide would act as an added attractant for C. tarsalis for bird feeding.

Since there are many areas in the United States, such as the Midwest, where mosquitoes are not active during the entire year there is some unknown host that serves as an overwintering reservoir for WEE virus. Cockburn et al. (14) reported isolations of WEE virus from birds and mosquitoes in Colorado from the months June to October but not during the winter months. Blackmore and Winn (6) isolated WEE virus from a pool of 14 hibernating C. tarsalis which were collected in December in an abandoned mine in the foothills of the Rocky Mountains. Rush et al. (69) however, in a study done in Oregon concluded that overwintering C. tarsalis were not a reservoir since no isolations of WEE virus were made from over 2471 hibernating C. tarsalis collected during the winter and early spring. Hess and Holden (35) state that resident (rather than migratory) avian hosts or arthropod vectors are the more likely overwintering reservoirs of WEE virus. Hess and Haynes (37) concluded that mosquitoes serve as both enzootic and epidemic vectors, and birds and possibly other wild vertebrates serve as reservoir hosts. Gebhardt and Hill (21) in experimental studies with snakes, suggest the snake as a possible natural host for maintenance of WEE virus in nature. Reeves et al. (62) working in California were able to isolate WEE virus from C. tarsalis in all months except December. The January to June isolations were all isolated from blood-engorged mosquitoes. Red blood cells in the blood meals of these mosquitoes were all nucleated indicating that they were probably feeding on avian hosts. Bellamy et al. (5) experimentally infected C. tarsalis with WEE virus and

after a 10 to 13 day holding period at 75 to 85 F they were placed in a constant 55 F incubator. Virus persisted for only 41 days; whereas, if these mosquitoes were placed in a cellar during the winter, infective virus would persist for 113 days. Reeves et al. (62) found that infective virus would persist up to 245 days in experimentally infected birds. The role of mammals as an overwintering host is considered to be minor by Kissling (44).

Epidemiology of Cache Valley Virus

In 1956, Cache Valley (CV) virus was isolated from a pool of Culesita inornata mosquitoes from Utah by Holden and Hess (39). Antigenically Casals and Whitman (10) classified this virus in the Bunyamwera group of viruses. This virus was pathogenic in suckling mice when inoculated by intracerebral (IC) or intraperitoneal (IP) routes, but was not pathogenic to weanling mice when inoculated IP. Recently CV virus has been isolated a number of times in the Ohio-Mississippi Basin from Anopheles quadrimaculatus mosquitoes. Precipitin tests on blood meal host preference of this mosquito species have shown that most prefer cattle as hosts (46). Yuill et al. (90) in Maryland have confirmed the work of Kokernot et al. (46) in that they found a large percentage of the dairy cattle were positive for CV virus antibodies. Yuill et al. (90) demonstrated an increase in antibody prevalence with increased age of the cattle. Work by Whitney (86) in New York, also confirms the high prevalence of CV antibodies in dairy cattle. A fairly high percentage of dogs also seem to have antibody titers to CV virus (90). Cache Valley virus

has been isolated from a number of different mosquito species including; Aedes taeniorhynchus and Aedes sollicitans (7) Anopheles quadrimaculatus, Psorophora ferox, and Aedes trivittatus (46) Aedes vexans and Anopheles punctipennis (87). So far CV has been isolated only from mosquitoes. The only other means suggesting presence of the virus has been by immunlogical surveys, therefore; the role of this virus as a disease causitive agent of a disease in man and animals is not yet known.

Epidemiology of Turlock Virus

Repeated isolations were made to an unknown viral agent during arbovirus surveillence studies in California, for WEE and St. Louis encephalitis virus infections in Culex tarsalis mosquitoes. This agent was designated as Turlock virus by Lennette et al. (48, 49). The original isolation by Lennette was made in suckling mice and embryonated chicken eggs. Hartwell et al. (31) reported that chick embryo cell culture was also a suitable means for cultivation of Turlock virus for virus isolation from mosquitoes. Presently the assay system used for Turlock virus is duck-embryo-cell culture plaque-reduction test (33).

This virus is almost exclusively associated with C. tarsalis mosquitoes. Viral infection rates for Turlock virus in sentinel chicken flocks seem to be highest in rural agricultural environments (66). Reeves (66) working with transmission of Turlock virus to mosquitoes has found that mosquitoes can be infected from chickens carrying 2.2 plaque forming units of circulating virus per ml of

blood. These mosquitoes have also been able to transmit the virus back to susceptible chickens. Reeves (66) in experimental studies has been able to induce viremia in chicks when injected with Turlock virus but death has not resulted.

Hall et al. (24) reported isolation of Turlock virus from mosquitoes in Alberta, Canada. In recent studies in Iowa, the virus has been isolated from C. tarsalis (87). Isolations of Turlock virus from field trapped mammals and birds have been accomplished. Hayes et al. (33) isolated Turlock virus from blood samples of one mammal and 4 nestling sparrows. Turlock virus has also been isolated from birds in Brazil by Shope et al. (71). Isolations of this virus have not been made from man or domestic animals so it is not known whether Turlock virus causes an apparent disease.

Epidemiology of California Encephalitis Virus Group

In 1943 and 1944 Hammon et al. (27) isolated a new virus from mosquitoes in California; this virus was later named California encephalitis (CE) virus. This virus was not isolated in California again until 1963, a span of 14 years since the first isolation (28). In other parts of the United States and the world other prototypes of this virus were being isolated from mosquitoes. Eklund, isolated the Trivittatus (TVT) virus in North Dakota in 1948 (28). This virus was found to be antigenically different from the original CE virus isolated by Reeves. Isolations of different prototypes continued and at present the California group of viruses contains 11 types (15). None of these viruses are serologically identical to the original

isolate, so different names have been given to these viruses. Eight of these types have been found in the United States (28). More than 80 isolations of TVT virus were made from mosquitoes in Iowa from 1966 to 1968 (87). Snoeshoe hare virus was isolated in Montana in 1959 (8) and two other types were isolated in Wisconsin; LaCrosse virus in 1964 (79) and Jamestown Canyon virus in 1965 (15).

Since 1963 scores of isolations of the different types of CE have occurred in a large number of states. During the same period serological evidence in man and animals has also been found in most incidences. The first serologically confirmed human case of California virus was reported in California in 1945 (26). Thompson et al. (79) found neutralizing antibodies to the California group of viruses in wild-life workers and in wildlife in Wisconsin. The first, serologically defined, epidemic of CE in man occurred in Indiana during the summer of 1964 (52). So far only one virus isolation has been made from man, this was from a fatal case in Wisconsin (80). While man is frequently infected with the virus it usually is an inapparent infection without residual damage such as sequelae. The more serious cases occur in the younger age group from 1 to 5 years old.

The California group of viruses has also been isolated from hares and rabbits. The viruses of this group seem highly endemic in wild hare and rabbit populations. Hoff et al. (38) reported that California encephalitis group antibodies were present in a high percentage of the hare population in Alberta, Canada. Neutralizing antibodies were present in 58 to 95 percent of the adult hare

population during a six year period when the population dropped from 600 hares per square mile to 3 hares per square mile. Now during years of population recovery the antibody prevalence has dropped to a range of 0 to 43 percent.

The virus cycles in nature are probably from small mammals to mosquitoes and back to mammals, with man appearing as an accidental dead end host (28). Birds do not appear to be involved in the virus cycle. There are areas where the virus has been present serologically in man and animals but there have been no virus isolations from mosquitoes. Gresikova et al. (23) believed that there was some unrecognized vector and transmission cycle effectively maintaining the virus and transmitting infection between small mammals and to man.

Epidemiology of Eastern Equine Encephalitis in Pheasants

Eastern equine encephalitis (EEE) virus was first isolated in New Jersey from a fatal horse infection (78). The first isolation of EEE virus from pheasants was made by Tyzzer et al. (81) in Connecticut. Within a fifteen year period (1939 to 1953) there were 27 major EEE outbreaks in pheasant flocks raised commercially in New Jersey (2,4). Twenty-eight outbreaks of EEE in pheasants in Massachusetts were reported by Faddoul and Fellows (20). Eighty-five percent of the Massachusetts outbreaks occurred during the months of August and September. Luginbuhl et al. (51) reported 15 outbreaks of EEE in pheasants in Connecticut from 1951 to 1956. Natural cases of pheasant encephalitis have also been reported in Rhode Island, Pennsylvania (3) Florida (72) and Maryland (29). The first report

of EEE virus west of the Atlantic seacoast was in Wisconsin (42). Until this report EEE virus was thought to have a definite geographical distribution. In 1967 EEE virus was isolated in South Dakota from pheasants on a commercial pheasant farm at Canton, South Dakota. This isolation was made during an EEE outbreak which killed about 11,000 pheasants between 18 and 24 weeks old (58). Pheasants infected with EEE virus showed symptoms of paralysis in the axial region of the body but showed very little loss of motor control in the head region. The virus has been isolated from a number of mosquito species as well as from mites and lice (85). Wallis (85) also reports that primary contact in pheasants with the virus is probably due to mosquitoes or wild birds and that secondary transmission can result from pheasant to pheasant.

MATERIALS AND METHODS

I. Mosquito Collection

A. Site Selection

The Brookings vicinity was chosen because of the large amount of field work connected with the study. Three sites were selected within a ten mile radius of Brookings. These sites were selected on the basis of varying ecological conditions present. The sites were all farmstead locations, but differed in their flora and fuana. Site 1, which was designated as the "homestead," was located two miles west of Brookings. This farm was located directly on the banks of the Sioux River. Site 2, a horse farm, was located six miles south of Brookings near the river. Site 3, a dairy farm, was located two miles north of Brookings approximately four miles from the Sioux River. These sites are shown on the map in Figure 2.

B. Mosquito Trapping Equipment

New Jersey type light traps (Hauser Mill Works, New Jersey)
Six volt motorcycle batteries
10 ampere battery chargers
Dry ice
Styrofoam chest with dividers and Frig-Paks (Guarantee Fit Inc. Riverdale, New Jersey)
Chloroform
1200 foot 16 mm empty film canisters
16 x 100 mm tubes
Neoprene stoppers size "0"

C. Mosquito Trapping Procedure

It has been shown by past investigators that battery operated mosquito light traps have yielded large numbers of mosquitoes. A

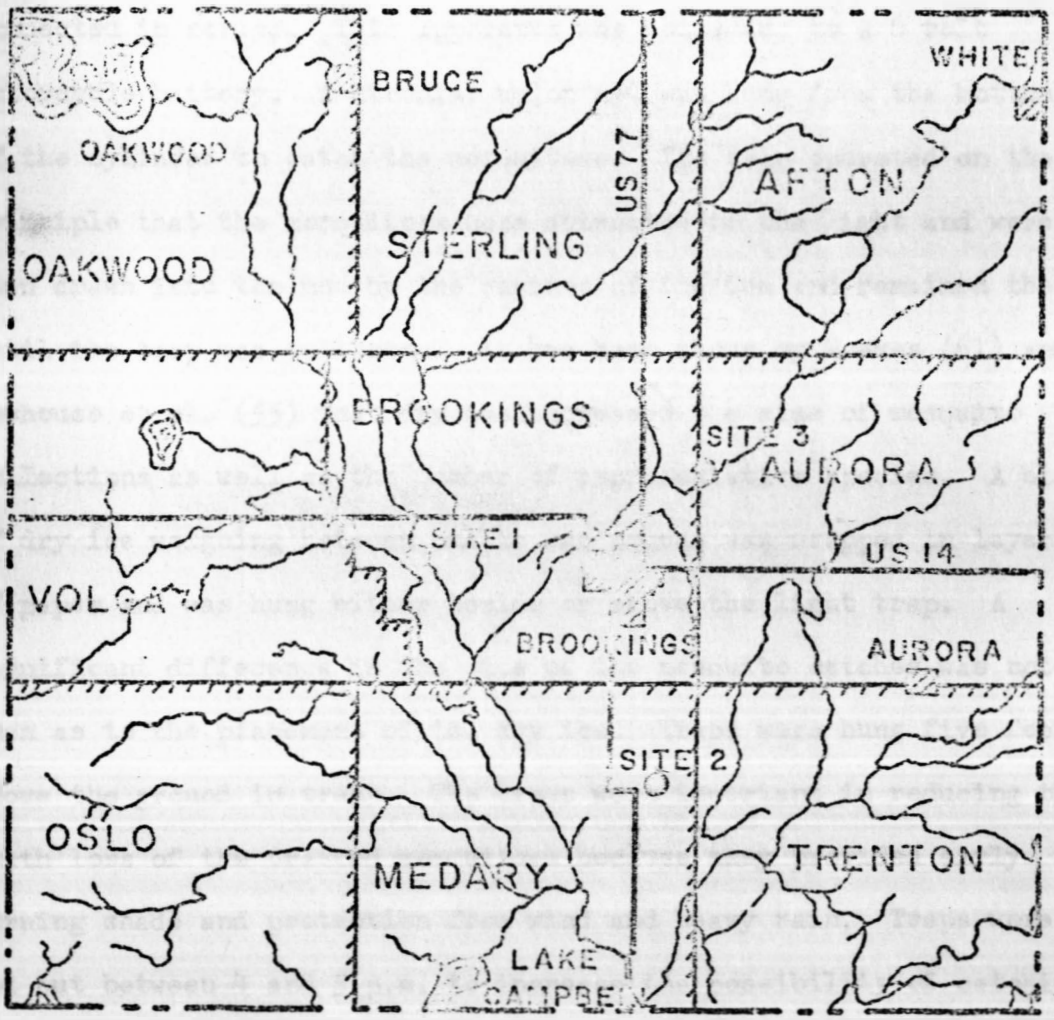

Figure 2. A map of the Brookings area with the mosquito collection sites marked.

good diversified sample of mosquitoes was desired; therefore, New Jersey type light traps were employed (74). These traps (Fig. 3) consisted of a circular metal reflector with an attachment of a plexiglass cylinder which contained a fan motor and a light bulb connected in series. This apparatus was connected to a 6 volt motorcycle battery. A circular nylon net was hung from the bottom of the cylinder to catch the mosquitoes. The trap operated on the principle that the mosquitoes were attracted to the light and were then drawn into the net by the suction of the fan and remained there until the trap was collected. It has been shown by Reeves (61) and Newhouse et al. (55) that dry ice increased the size of mosquito collections as well as the number of representative species. A block of dry ice weighing between one to two pounds was wrapped in layers of paper and was hung either beside or above the light trap. A significant difference in the size of the mosquito catches was not seen as to the placement of the dry ice. Traps were hung five feet above the ground in trees. The trees were important in reducing the death loss of the trapped mosquitoes because they provided early morning shade and protection from wind and heavy rain. Traps were set out between 4 and 5 p.m. to increase the possibility of catching diurnal species of mosquitoes (75). The traps were collected early the following morning and brought to the laboratory. Batteries were also returned to be recharged for the next trap night. The nets were removed from the trap and a knot was tied in the neck portion to prevent mosquito escape. These nets were then collapsed and placed

Figure 3. New Jersey Mosquito Light Trap with a dry ice block hanging beside the trap

in a divided styrofoam chest which contained a frozen Frig-Pak. Using a chest that was dark and cool inside kept the mosquitoes alive and inactive until they were taken to the laboratory. A similar method had been used by Sudia and Chamberlin (75). In the laboratory each net was placed into a circular 1200 foot 16 mm empty film canister which contained a facial tissue dampened with chloroform. The nets were left in the chloroform for about one minute to anesthetize the mosquitoes. After removal from the canister the contents of the traps were transferred onto a white cardboard square. The debri and unwanted insects, as well as the male mosquitoes, were removed with forceps. The female mosquitoes were transferred into 13 x 100 tubes and stoppered with size "0" neoprene stoppers. These tubes were labeled with the date, site, and approximate number in the catch. These were then stored in a -60 C freezer until it was convenient to identify them. Total processing time from the field to freezer was kept at a minimum since the viruses could have been inactivated if they were left at room temperature for too long a time.

II. Mosquito Processing

A. Mosquito Identification Equipment

Chill table
Binocular dissection microscope
Petri dish bottoms
Whatman No. 1 filter paper
2 dram screw cap vials
M-199 tissue culture medium
Antibiotics (penicillin, streptomycin, kanomycin, and mycostatin)
Bovine albumin
Sodium bicarbonate

3 inch O. D. mortars and pestals
Ground glass 200 mesh
Seitz filter
Screw cap conical centrifuge tubes
Refrigerated centrifuge
5 ml serum bottles with crimp caps

B. Mosquito Identification Procedure

All mosquitoes were identified to genus species and divided in pools containing from 1 to 100 mosquitoes depending on their importance as vectors and their abundance. The mosquitoes were identified on a chill table (Fig. 4) that had freezer coils wound under the surface of the table. Refrigerant was circulated through these coils by a compressor. The surface temperature of the table was about 3 to 5 C. The mosquitoes were identified by using an AO dissecting microscope at 30X magnification. The mosquitoes were allowed to thaw in the tube and were then transferred into a petri dish bottom for identification. This was extremely important in the preservation of identifiable parts of the mosquitoes. Other petri dish bottoms were placed on the chill table to hold the various mosquito species as they were identified. To avoid cross contamination of pools a clean moist piece of Whatman No. 1 filter paper was placed in the bottom of each petri dish prior to each sorting. The pool size varied from 1 to 100 mosquitoes depending on the species. Culex tarsilis were pooled in lots of 50 mosquitoes and Aedes vexans and Aedes trivittatus were pooled in lots of 100. The minor mosquito species were pooled in smaller numbers with variation in pool size from 1 to 25 mosquitoes. Each pool of mosquitoes was placed in a 2 dram screw capped vial and

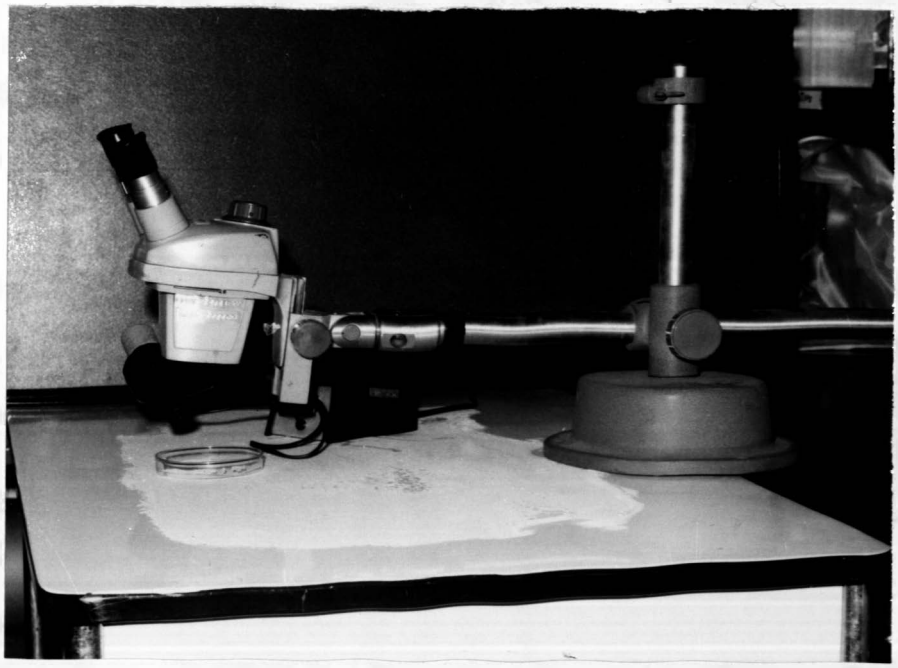

Figure 4. Chill table used for mosquito identification.

stored in a -60 C freezer until grinding. Mosquitoes from different sites and dates were not mixed while being identified and pooled.

C. Mosquito Grinding Stock Solutions

30% Bovine albumin (Pentex Laboratory, Kankakee, Ill.)
Penicillin-streptomycin stock 10,000 units/ml (Difco)
Kanomycin stock 10,000μg/ml (Difco)
Mycostatin stock 10,000μg/ml (Difco)
1X M-199 tissue culture media (Grand Island Biologicals)
3.5% sodium bicarbonate

The sterile 30% bovine albumin was prepared in this concentration from the company. The antibiotic stocks were prepared, using sterile phosphate buffered saline as the diluent, in a concentration of 10,000 units/ml and stored at -20 C. The M-199 medium was prepared in one liter amounts, filtered in a Seitz filter and stored at 4 C.

D. Preparation of Diluent for Mosquito Grinding

	ml
30% Bovine albumin	6
Pen-Strep stock 10,000 units/ml	1
Kanomycin stock 10,000μg/ml	1
Mycostatin stock 10,000μg/ml	1
M-199 1X stock	91
3.5% $NaHCO_3$ as needed to adjust the pH to 7.2 to 7.6	

The above diluent yielded a M-199 diluent containing 1.8% bovine albumin and 100 units/ml of streptomycin, penicillin, kanomycin, and mycostatin.

E. Mosquito Grinding Procedure

Mosquito pools were removed from the freezer and placed in a pan of crushed ice. Each pool was placed into a sterile chilled 3 inch O. D. mortar. A sterile applicator stick was used to remove all mosquitoes from the vial. A small amount of 200 mesh ground glass was added to each mortar to act as an abrasive. The mosquitoes were ground with two to three drops of M-199 diluent until a smooth paste was obtained. Two milliliters of M-199 diluent was added to each suspension and further grinding was continued until there was an even suspension. Suspensions were poured into sterile 12 ml conical screw cap centrifuge tubes which had been previously chilled. These tubes were centrifuged at 4 C for 30 minutes at 1700Xg. After centrifugation the supernatant liquid was decanted aseptically into sterile 5 ml serum bottles which were capped and stored in a -60 C freezer until inoculation into mice.

III. Mouse Inoculation

One to two-day-old suckling mice were used for primary virus isolation (67). Each litter of mice was reduced to a litter size of 6. The mice were inoculated intracerebrally with 0.02 cc of mosquito suspension. A one cc disposible B-D syringe with a 3/8 inch 26 gauge needle was used. Litters were observed daily for viral disease symptoms over a period of 12 days. If no symptoms were observed within this period these mice were killed. If symptoms such as paralysis or morbidity occurred during the 12 day post-inoculation

period these mice were killed and stored in a -60 C freezer until the mouse brains were harvested. If death losses occurred from the first inoculation mosquito pool suspensions were reinoculated into mice as above from the original suspension. The pool was considered to be tentatively positive for virus if the second inoculation also resulted in symptoms in the mice.

IV. Mouse Brain Harvest

Mice previously killed for virus isolation were removed from the freezer and allowed to thaw. The thawed mouse brain was very viscous and was easily removed with a syringe and needle. Mice from suspect mosquito pools were placed on a piece of pasteboard and a piece of tape was placed across the back of the neck of each mouse. The heads were swabbed with 1:1,000 merthiolate before the mouse brain was harvested. Brain material was removed with an 18 gauge needle and a one cc syringe. The needle was inserted at the base of the skull and approximately 0.1 to 0.2 cc of brain material was collected from each mouse. This brain material was dispensed into centrifuge tubes containing one milliliter of M-199 diluent for each mouse brain harvested. This yielded a 10% mouse brain suspension. Suspensions were centrifuged at 1700Xg for 30 minutes at 4 C. The supernatant fluid was decanted and used for virus identification and virus production. These suspensions were stored at -60 C.

V. Virus Identification

Virus identification was attempted after mosquito isolates had been reisolated a second time in suckling mice. The methods employed for virus identification included primary tissue culture cell lines of vero and duck embryo cells (13) and the serology tests of serum neutralization and hemagglutination inhibition (33). All virus identifications were made at the Arboviral Disease Section, USPHS., C.D.C. Ft. Collins, Colorado.

VI. Pheasant Inoculation with South Dakota Arbovirus Isolates

Pheasant chicks were purchased from the South Dakota Pheasant Company, Canton, South Dakota. Pheasants used in the experimental studies varied in age from 1 to 12 days. All birds were kept in series 40 polycarbonate cages (Scientific Products). These cages were placed in plexiglass isolation hoods with an inner working area of approximately 24 square feet (Fig. 5). These hoods were equipped with an air filter and incinerator system. Birds were kept in these hoods for two days before inoculation to adjust to environmental conditions present in the hood. With the use of rubber gloves for protection the birds were held in the palm of the right hand with the head over the thumb and one leg held by the little finger. The birds were inoculated intramuscularly in the leg with 0.2 cc of inoculum using a 20 guage needle and syringe. A daily record was kept as to death or viral disease symptoms such as paralysis in the leg region of the

Figure 5. Plexiglass isolation hood used for pheasant inoculation.

body. Birds were observed for 7 days. All inocula used were 10% suckling mouse brain (SMB) suspensions in M-199 diluent as described above. These inocula had been passed through mice three times. Fifty percent endpoints were determined using the method of Reed and Muench (60). For the "lethal dose 50" determinations of WEE and EEE ten fold dilutions in M-199 diluent were made of the 10% SMB suspensions. These dilutions ranged from 10^{-1} to 10^{-10}. In all pheasant experiments two groups of controls were used, one group was uninoculated and one group was inoculated with M-199 diluent containing no virus.

VII. Egg Inoculation with South Dakota Arbovirus Isolates

Ten-day-old embryonated chicken eggs were obtained from the poultry department at South Dakota State University. These eggs were candled to make sure that the embryo was still alive. During the candling procedure the air sac and the location of the embryo were marked. A small hole was made in the egg shell at the top of the air sac using a vibrating drill (Burgess Vibrocrafts, Grayslake, Illinois). A syringe with a 1 inch 20 gauge needle was used for inoculation. All eggs were inoculated by the yolk sac route. The needle was inserted through the hole in the shell and inserted about three fourths of an inch into the egg, making sure not to hit the embryo. All eggs were inoculated with 0.2 cc of 10% SMB virus properations. After the eggs were inoculated the hole in the eggs were sealed with fingernail polish and placed in an egg incubator at 100 F and 99% humidity. Eggs were checked at 18 hours after inoculation and every hour after

that for 36 hours. Eggs were checked by candling. The embryo was considered dead if blood vessels in the egg shell were not seen or if the embryo did not seem to move. All eggs considered to have dead embryos were harvested. The eggs were broken using a Tri-R egg punch placed on the air sac of the egg. The appearance of the yolk and the embryo gave indication as to cause of death. If the embryo had hemoraghic areas on the body viral death was indicated. If the yolk was milky colored instead of yellow, death was probably caused by bacterial contamination. In all egg embryo experiments the controls were the same as mentioned for pheasants.

RESULTS AND DISCUSSION

Mosquito Collection

Mosquitoes were collected on 40 different trap nights during the period from July 20 to September 9 within a ten mile radius of Brookings at three different trap sites. A trap night is defined as one collection per one trap per one night, therefore, if three traps were set on one night these would constitute three trap nights. Approximately 42,000 mosquitoes were caught on these 40 trap nights. Of these, 22,000 were identified and processed for virus isolation (Table 1). The rest of the mosquitoes were not identified because they constituted extremely high catches the majority of which were on species of mosquitoes from traps on different nights. For example, on the night of August 7, at the horse ranch, site 2 over 10,000 mosquitoes were caught in one trap. About 10% of this catch was identified. Over 75% of those identified were of one mosquito species, Aedes vexans. The rest of this trap catch was not identified since only one virus isolation was obtained from this mosquito species. The same procedure was used for the other large catches of mosquitoes since the majority of these catches consisted of Aedes vexans and Aedes trivittatus mosquitoes. The estimated totals and percentages of the major mosquito species are given in Table 1.

Approximately 93% of the 22,000 mosquitoes identified and processed for virus isolation consisted of three species; Aedes vexans, Aedes trivittatus and Culex tarsalis (Table 1). Fifteen other mosquito

Table 1. Data on Brookings, South Dakota Mosquitoes Caught and Identified from July 17 to September 2, 1969

		Total of Species Identified	% of Total Identified Mosquitoes	Estimated Catch	% Total Estimated & Identified
1.	Aedes trivittatus	7,958	36.32	12,445	30.11
2.	Culex tarsalis	6,765	30.87	8,649	20.93
3.	Aedes vexans	5,567	25.40	17,278	41.81
4.	Aedes species	1,238	5.65	2,448	5.92
5.	Aedes dorsalis	129	0.58	249	0.60
6.	Aedes triseriatus	54	0.24	--	--
7.	Aedes sticticus	47	0.21	--	--
8.	Culex species	31	0.14	--	--
9.	Culiseta inornata	28	0.12	--	--
10.	Culex restuans	27	0.11	--	--
11.	Culex salinarius	16	0.1	--	--
12.	Anopheles punctipennis	14	0.1	--	--
13.	Culex pipens	10	0.1	--	--
14.	Aedes nigromaculus	9	0.1	--	--
15.	Anopheles walkeri	6	0.1	--	--
16.	Aedes flavescens	6	0.1	--	--
17.	Anopheles earlii	2	0.1	--	--
18.	Anopheles quadrimaculatus	1	0.1	--	--
19.	Mansonia pubertans	1	0.1	--	--
20.	Uranotenia sapphirina	1	0.1	--	--
	Total	21,910		41,322	

species were found in the Brookings collections. These included in decreasing abundance: Aedes dorsalis, Aedes triseriatus, Aedes sticticus, Culiseta inornata, Culex restuans, Culex salinarius, Anopheles punctipennis, Culex pipens, Aedes nigromaculus, Anopheles walkeri, Aedes flavescens, Anopheles earlii, Anopheles quadrimaculatus, Mansonia pubertans and Uranctonia sapphirina. Their relative percentages are given in Table 1. All of these species had been described as present in South Dakota by the U.S. Federal Security Agency (83, 84) and Gerhardt (22). These workers also described some species in South Dakota that were not found in the 1969 collections from Brookings County.

Culex tarsalis comprised 31% of the total collection identified. Since this mosquito species is the major vector of Western equine encephalitis (WEE) virus the presence of a high percentage of C. tarsalis is significant. The percentage of C. tarsalis in comparison to collections by other workers seems to be equal or higher than those found in the states of Iowa (87) and Minnesota (19). The average number of C. tarsalis per trap night was over 220 female mosquitoes.

The catches of C. tarsalis varied in the different sites with the dairy farm yielding the most of this mosquito species throughout the collection period, whereas, the horse ranch was the highest in Aedes trivittatus and Aedes vexans with the dairy farm being lowest in these two mosquito species (Table 2). The homestead site had average numbers of all three major mosquito species. C. tarsalis populations reached their peak in the second and third weeks in August (Fig. 6), whereas

Table 2. Number of Major Mosquito Species Processed for Virus Isolation by Week and Site of Collection, Brookings County, South Dakota, 1969

Week of	Culex tarsalis			Aedes trivittatus			Aedes vexans		
	Farmstead	Horse Ranch	Dairy Farm	Farmstead	Horse Ranch	Dairy Farm	Farmstead	Horse Ranch	Dairy Farm
7-27 to 8-2	--*	67	1013	--	347	154	--	430	513
8-3 to 8-9	546	321	251	2480	705	234	571	1030	164
8-10 to 8-16	523	263	657	1076	1835	48	701	474	83
8-17 to 8-23	1379	444	832	56	729	27	205	606	444
8-24 to 8-30		11	--	--	209	--	--	138	--
8-31 to 9-5	--	--	458	--	--	58	--	--	168
TOTAL	2248	1106	3211	3612	3825	521	1477	2678	1412

* Mosquito traps were set at these sites during these weeks, but few, if any, mosquitoes were caught; this was due to bad weather, no mosquitoes, or trap failure.

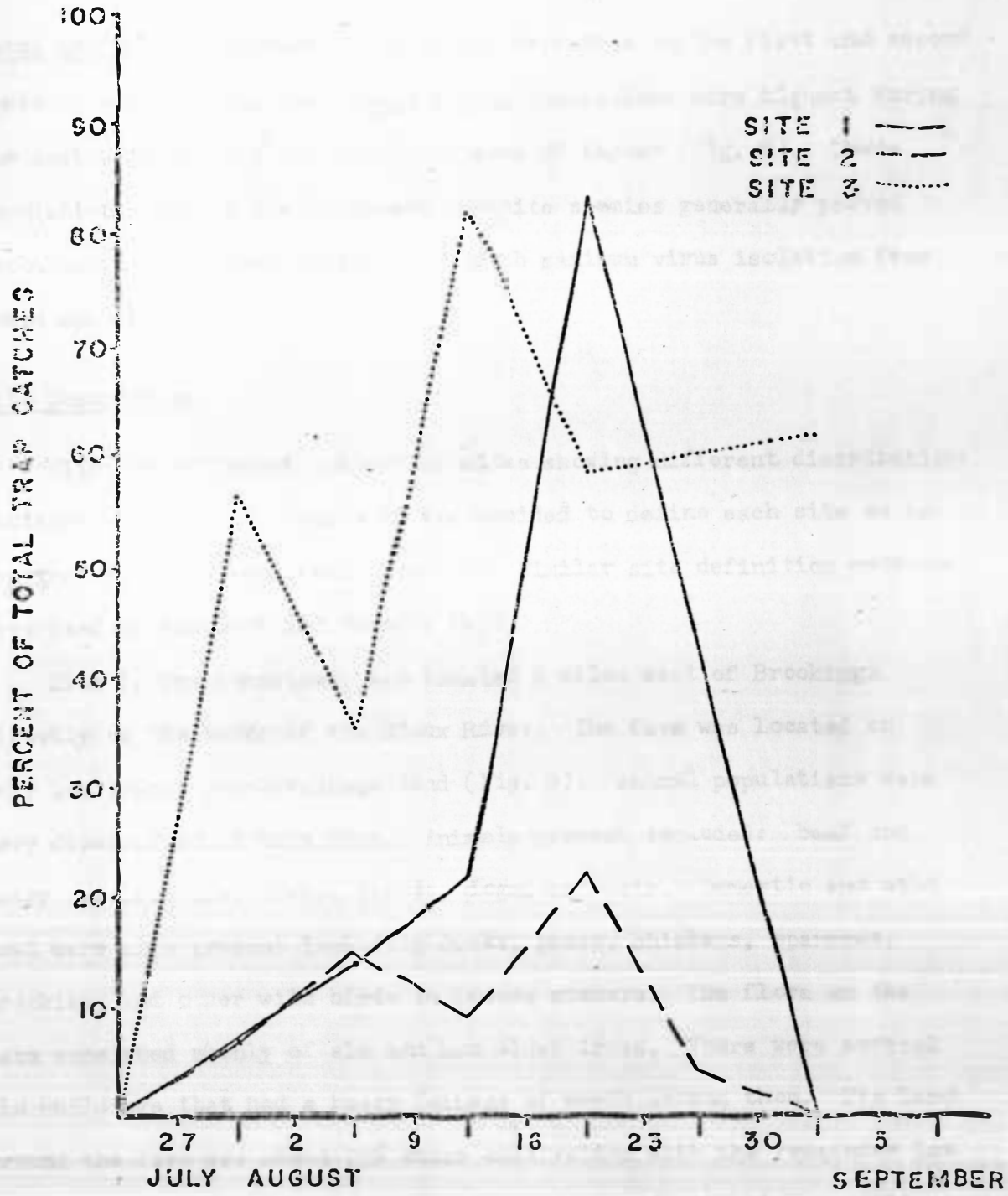

Figure 6. Percentage of Culex tarsalis in the weekly trap catches from the farmstead (site 1), the horse ranch (site 2), and the dairy farm (site 3).

Aedes trivittatus populations reached their peak in the first and second weeks of August (Fig. 7). Aedes vexans populations were highest during the last week of July and the first week of August (Fig. 8). These population peaks of the different mosquito species generally proved important in that they corresponded with maximum virus isolation from these species.

Site Description

With the different collection sites showing different distribution patterns of mosquito species it was decided to define each site as to topography, flora, and fuana present. Similar site definition methods were used by Kokernot and Brandly (45).

Site 1, the homestead, was located 2 miles west of Brookings directly on the banks of the Sioux River. The farm was located on very low lying, poor-drainage land (Fig. 9). Animal populations were very diversified at this site. Animals present included: beef and dairy cattle, pigs, sheep, horses, dogs, and cats. Domestic and wild fowl were also present including ducks, geese, chickens, sparrows, grackles, and other wild birds in lesser numbers. The flora on the farm consisted mainly of elm and box elder trees. There were several old buildings that had a heavy foliage of weeds around them. The land around the farm was about 75% under cultivation with the remainder low lying slough land. The site seems ideal for producing high mosquito populations.

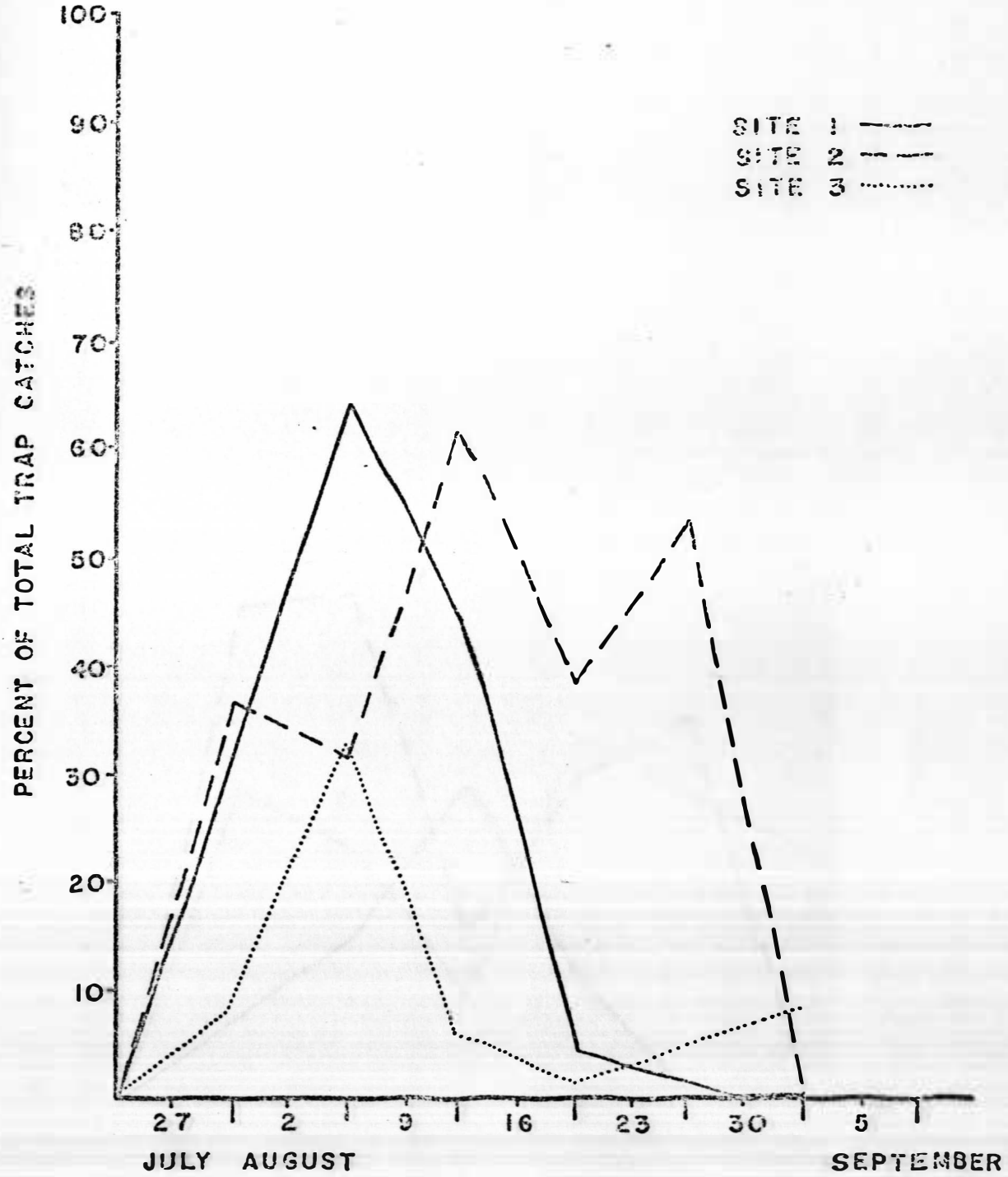

Figure 7. Percentage of Aedes trivittatus in the weekly trap catches from the farmstead (site 1), the horse ranch (site 2), and the dairy farm (site 3).

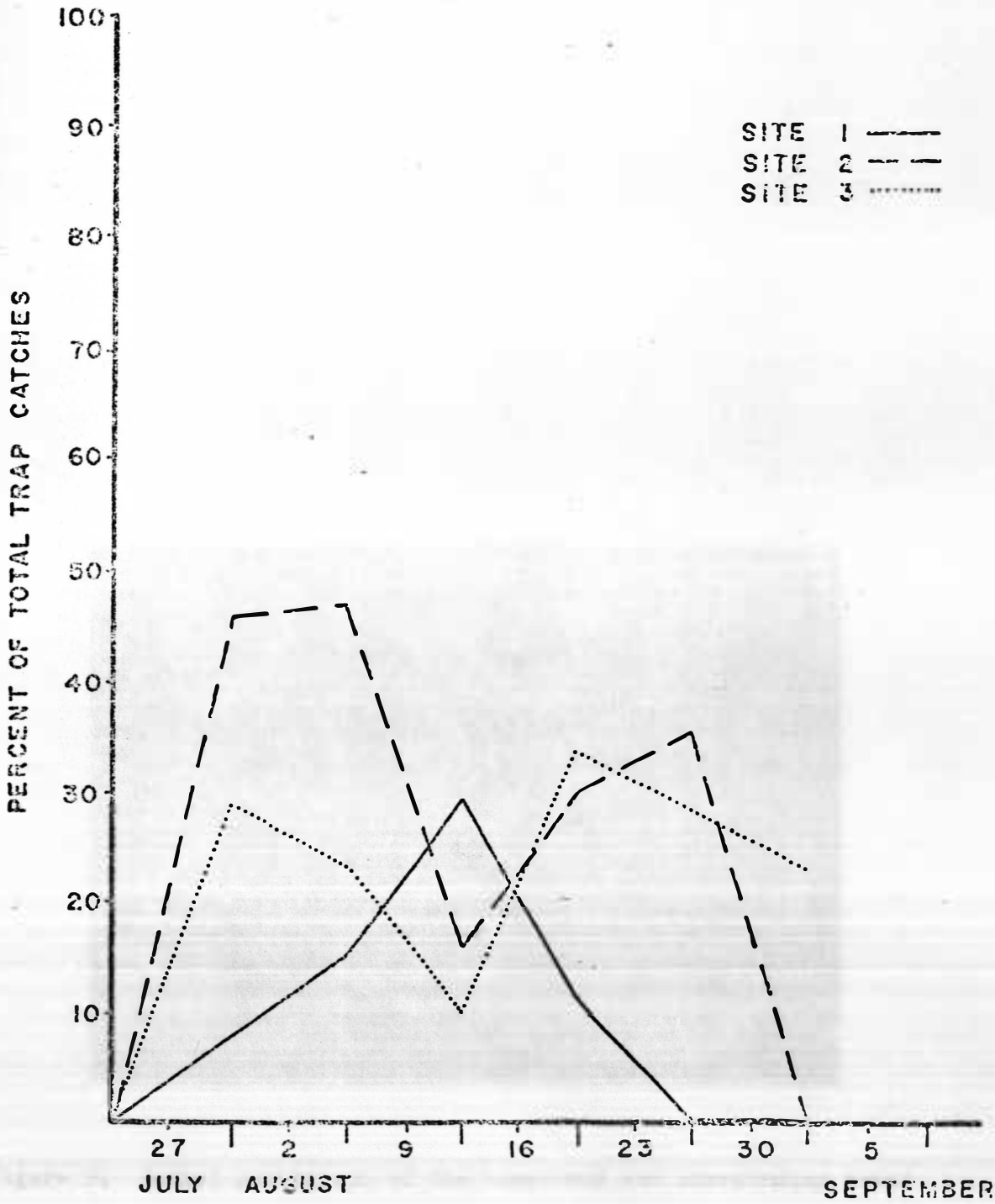

Figure 8. Percentage of Aedes vexans in the weekly trap catches from the farmstead (site 1), horse ranch (site 2), and the dairy farm (site 3).

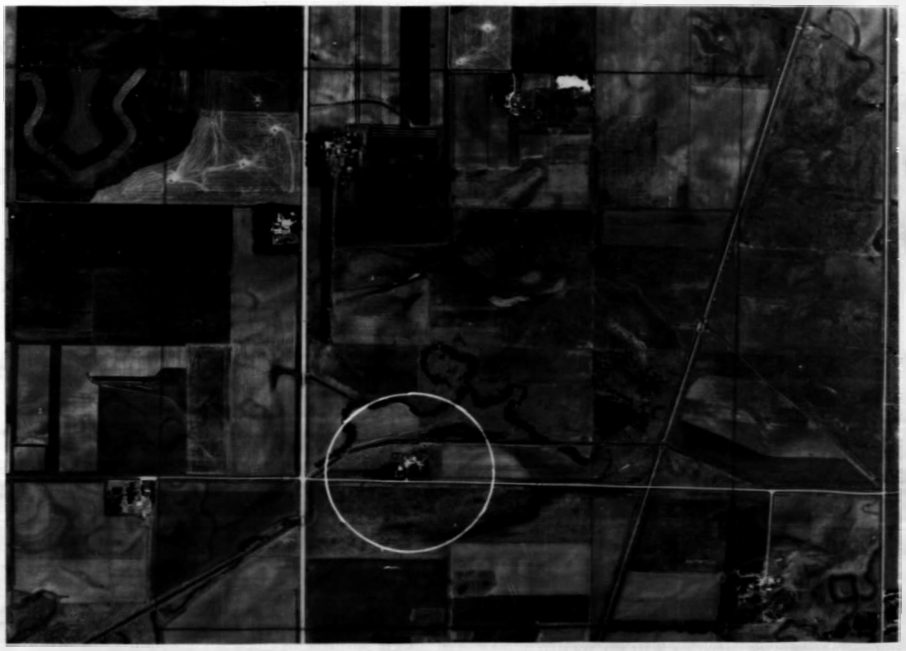

Figure 9. Aerial photograph of the homestead and surrounding area.

Large catches of mosquitoes were made at this site on August 6 and 7. Both of these catches had over 2,000 mosquitoes. Trap catches at this site averaged over 1000 per trap night. This site was the second highest in total numbers of mosquitoes caught during July, August, and September.

Site 2, the horse ranch, was located 5 miles south of Brookings. The ranch was about a mile from the Sioux River. Land surrounding the farm was in the flood plain of the river. This land was very flat and many small sloughs and potholes were present, leaving much of the land uncultivated (Fig. 10). Most of the land adjacent to the ranch was permanent pasture. There was a small creek crossing the pasture near the farm where water stood or flowed all summer (Fig. 11). The farm itself was very clean appearing in that the yard and the grounds on which the buildings were situated were very well kept (Fig. 12). There was no overgrowth of weeds or tall grasses. The buildings were also very clean and therefore there were few bird nests present. The major bird species present were pigeons which were very numerous in an old silo. Trees on the farm were numerous and diversified, a total list as to numbers and kinds is given in Table 3. The only animals present at this site were about 30 horses and 2 dogs. Rodents were also present at this site as well as at the other sites, but species were not determined.

This site yielded the largest overall mosquito collections (Table 2). Aedes vexans and Aedes trivittatus were the major mosquito species at this site. Average daily mosquito collections were over

Figure 10. Aerial photograph of site 2, the horse ranch, and surrounding area.

Figure 11. Standing water near the horse ranch, site 2.

Figure 12. Farm yard at the horse ranch, site 2.

1500, with a high of 10,000 mosquitoes caught on one night (August 7).

Table 3. Trees and Shrubs Surrounding the Horse Ranch

Scientific Name	Common Name	Number
Ulmus pumila	Siberian elm	33
Lonicera tatarica	Tatarian honeysuckle	23
Acer negundo	Boxelder	26
Pyrus malus	Apple	3
Prunus mandshurica	Apricot	1
Prunus americana	Wild plum	3
Syringa vulgaris	Lilac	46
Salixalba "Niobe"	"Niobe" weeping white willow	1
Ribes americana	Wild black currant	3
Juniperus virginiana	Red Cedar	12
Populus deltiodes	Cottonwood	30
Prunus tomentosa	Nanking Cherry	1
Celtis occidentalis	Hackberry	15
Salix amygdaloides	Willow	32

The third site in the study, the dairy farm, was located two miles north of Brookings. This site was located in a very heavily cultivated area. It was removed some distance from any major drainage system such as the Sioux River. The area surrounding the farm was fairly free of sloughs and potholes that could support standing water for mosquito breeding (Fig. 13). Vegetation within the farmstead was heavier than in the other two sites. Grass and weeds in the shelterbelts surrounding the farmstead were quite high (Fig. 14). More trees were present at this site than at the other sites. A complete listing of the trees and shrubs is present in Table 4. The buildings at the

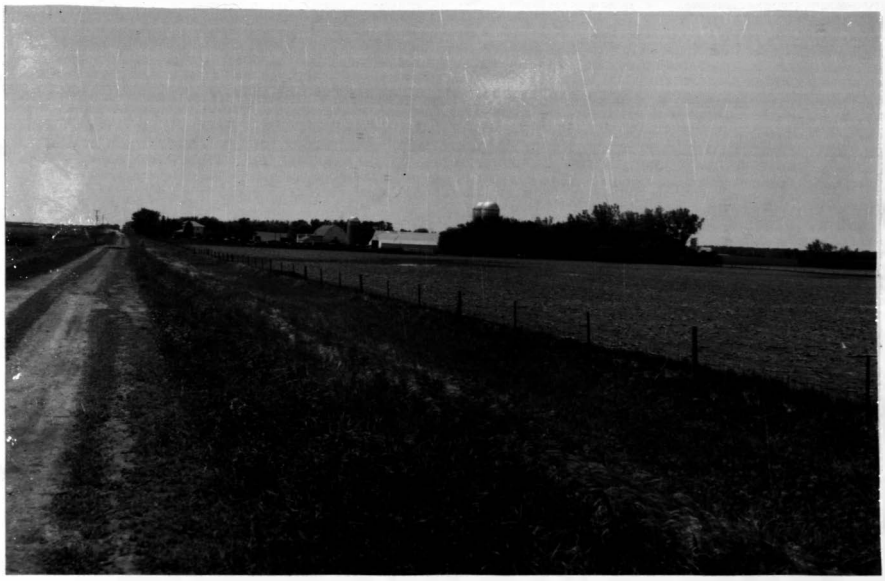

Figure 13. Overall view of the dairy farm, site 3.

Figure 14. Site of mosquito trapping at the dairy farm, site 3, showing overgrowth in the shelterbelt.

dairy farm had numerous bird nests, many of these belonged to sparrows. Other birds were present including the common grackle barn swallow, and mourning dove among others. About 100 dairy cattle were present on the farm. Pigs were also raised on this farm but other livestock were not present. This site also differed from the other sites in that there was a residential development within one-fourth of a mile of the farm (Fig. 15). Around 20 families live in this housing development.

Table 4. Trees and Shrubs Surrounding the Dairy Farm

Scientific Name	Common Name	Approximate Number
Acer negundo	Boxelder	100
Fraxinus pennsylvanica	Green ash	80
Celtis occidentalis	Hackberry	60
Ulmus americana	American elm	60
Acer saccharinum	Silver maple	60
Syringa vulgaris	Lilac	36
Lonicera tatarica	Tatarian honeysuckle	50
Juniperus virginiana	Red cedar	50
Rhamnus cathartica	Buckthorn	40
Prunus virginiana	Chokecherry	20
Cornus stolonifera	Red-osier dogwood	20
Ulmus pumila	Siberian elm	20
Picea pungens	Colorado Spruce	4
Picea glauca	White Spruce	23
Pyrus (hybrid)	Crab apple, ornamental	1

The mosquito collections at the dairy farm were high in C. tarsalis throughout the collection period. The percentage of C. tarsalis in the nightly trap catches averaged around 65%. This was the only site where C. tarsalis existed in such a high percentage.

Figure 15. Aerial photograph of site 3, the dairy farm, and surrounding area.

Site definition is very important since the flight range of most mosquito species is very short (usually less than a mile). With a limitation in flight range the environmental conditions present at the various sites become very important.

Climatic Influence

Climatic conditions have been shown to be very important in the transmission of arboviruses to mosquitoes and finally to a vertebrate host, Hess et al. (36). Hayes and Hess (32) found that high temperatures and abnormally high precipitation correlated with EEE virus infectious in humans. Precipitation plays a role in arbovirus epidemiology. Reeves et al. (65) report a positive correlation between abnormally high precipitation and high incidence of WEE and CE viruses. In the year 1968-1969 South Dakota had very high total precipitation for both rainfall and snowfall (Table 5). The months of September and October were higher in moisture than usual. During the winter months, November through March, unusually high snowfalls were recorded. In Brookings, the snowfall for this period was 73 inches compared with a 70 year average of 23 inches. This snow had a water equivalent of from 6 to 10 inches. In the spring the snow thaw created record floods all over Eastern South Dakota, particularly in the James and Sioux River watersheds. Loomis (50) indicated a relationship between the 1952 outbreak of encephalitis in California and the snowpack in the Sierras. The topography of Eastern South Dakota is very conducive to flooding. A report by the U.S. Federal

Table 5. Precipitation and Snowfall Data for the Period October, 1968, through September, 1969, from Brookings Weather Station (82)

Month	Precipitation in Inches		Snowfall in Inches	
	Monthly Total	Deviation From 30 yr. Normal	Monthly Total	Deviation From 70 yr. average
October, 1968	3.60	+2.38	0	- 0.33
November, 1968	0.54	-0.16	2	- 0.38
December, 1968	2.09	+1.61	22	+18.45
January, 1969	1.11	+0.74	14	+10.12
February, 1969	2.38	+1.91	29	+24.35
March, 1969	0.63	-0.34	6	+ 0.13
April, 1969	1.02	-0.75	0	- 1.99
May, 1969	3.02	+0.23	0	- 0.19
June, 1969	7.20	+3.25	0	0
July, 1969	3.48	+1.33	0	0
August, 1969	1.49	-1.48	0	0
September, 1969	1.32	-0.72	0	0
Total	27.88	+8.00	73	+50.16

Security Agency (84) described the James River Basin as follows:

> "Only a very small percentage of the precipitation which falls over the basin appears as run-off in the streams due to the flat topography of the basin and the collections of surface run-off in numerous sloughs and depressions. Most of the James River Valley has a typical glaciated topography. Glaciers once covered this entire area and the land surface today is much the same as it was left following their recession. The glaciers deposited their loads rather evenly over the area but left many minor irregularities such as low broad mounds and shallow depressions. The basin has a slow and poorly developed drainage system. A considerable amount of the surface water from the area drains into ponds, lakes, depressions, sloughs, marshes, and hardly noticeable but very numerous shallow, enclosed depressions, where it stands until it either evaporates or percolated into the ground. This poor drainage is believed to be due to the level part of the last ice sheet formation known as the Mankato Substage of the Wisconsin. This level land with slow, meandering streams and drainageways is characteristic of this last glacial drift."

It was the presence of similar conditions in the Sioux River Basin that contributed so greatly to the floods. Minor flooding occurred during the first week of April in the Big Sioux River Basin and significant flooding started in the first part of the second week (73). Since the runoff was so high in 1969 the sloughs and potholes throughout Eastern South Dakota were filled and created excellent breeding grounds for mosquitoes throughout the summer.

Reeves and Hammon (63) reported that in Kern County, California, WEE virus was first detected in C. tarsalis during or following half-month periods when the temperature rose above 80 F. Hess et al. (36) indicated that the temperature of 70 F plays a role in the transmission of WEE virus by mosquitoes. When fifty day degrees above 70 F were first accumulated the possibility of WEE transmission from infected mosquitoes to mammals and birds existed. A "day-degree"

is considered any day that the temperature reachs a specified temperature or goes above this temperature. Therefore, when there was a total of 50 days that had temperatures 70 F or above virus transmission could exist. Temperatures in Brookings were an average of 4.2 F below the monthly average temperature for the twelve-month period October 1968 through September 1968 (Table 6). The date when fifty day degrees above 70 F was reached in Brookings was July 18. Cumulative day degrees were also calculated for the temperatures of 64, 68, and 75 F (Table 6). It has been shown by Hess et al. (36) that there is a correlation between the 75 F isotherm and St. Louis encephalitis virus transmission. During the months when the mosquito populations were the highest the temperature in Brookings was about equal to the 30 year normal for those months. The temperatures during April and May were also near normal when the floods occurred and these temperatures combined with the floods had an influence on the early emergence of mosquitoes. By personal observation, mosquito populations seemed quite high in the early part of May but seemed to decrease again by the first part of June. This abnormally high early emergence of mosquitoes could be attributed to the flood waters picking up eggs laid the fall before in dry soil. With the abundance of water there was an immediate flux in the mosquito population when the warmer weather in May allowed these eggs to hatch. This early hatch of mosquitoes had a direct influence on the mosquitoes the rest of the summer. The chances of the mosquitoes having been infected with virus this early were very low.

Table 6. Temperature Data for the Period of October, 1968 Through September, 1969 From Brookings Weather Station (82)

Month	T. Max. Ave.	T. Min. Ave.	Temp. Ave.	Deviation from 30 yr. Normal	Number of Days with Temperature Above 64°F ( )*	68°F ( )*	70°F ( )*	75°F ( )*
October, 1968	57.9	34.5	46.2	- 3.3	12	5	4	0
November, 1968	39.9	23.3	3.16	0.0	1	1	0	0
December, 1968	22.5	7.0	14.8	- 4.8	0	0	0	0
January, 1969	15.9	- 5.2	5.2	- 8.4	0	0	0	0
February, 1969	23.5	5.9	14.7	- 2.9	0	0	0	0
March, 1969	26.8	3.9	15.4	-13.8	0	0	0	0
April, 1969	54.3	33.0	43.7	- 1.5	7(7)	3(3)	1(1)	0
May, 1969	68.9	44.9	56.9	- 0.7	22(29)	17(20)	14(15)	9(9)
June, 1969	69.8	47.2	58.5	- 8.6	22(51)	19(39)	18(33)	12(21)
July, 1969	80.6	58.5	69.6	- 3.6	31(82)	30(69)	28(61)	26(47)
August, 1969	82.3	57.1	69.7	- 1.5	31(113)	31(100)	30(91)	28(75)
September, 1969	72.7	46.8	58.8	- 1.5	27(140)	24(124)	21(112)	12(87)
Total				-50.4	140	124	112	87

( )* Cumulative total of day degrees above the given temperatures from April to September.

Virus Isolations

The 22,000 mosquitoes that were identified were tested in suckling mice for virus. Rooyen and Rhodes (67) reported that suckling mice are one of the best hosts for arbovirus isolation. If only one mosquito in a pool is infected, the mice are sensitive enough to detect the virus. A varying pool size was used since some mosquitoes such as C. tarsalis are proven vectors of arboviruses. A pool size of 50 was selected for C. tarsalis. Work by Hayes et al. (33) has shown that for Hale County, Texas, the pool size for C. tarsalis had to be as low as 10 to get an accurate picture of WEE infection rates. Aedes vexans and Aedes trivittatus mosquitoes were pooled in lots of 100 since few virus isolations have been made from these mosquito species. The pool size of the minor mosquito species varied from 1-25 mosquitoes.

A total of 320 mosquito pools collected during the summer of 1969 was tested for arboviruses (Table 7). One pool each of Culex pipens, Aedes nigromaculus, Anopheles walkeri, Aedes flavescens, and Anopheles earlii were tested also but are not included in Table 7. These five pools were all negative for virus in suckling mice. From these 320 pools 14 virus isolations were made. Of these isolates 8 were WEE virus, 2 were Trivittatus virus, 3 were CV virus and 1 was Turlock virus. The WEE virus isolates were all obtained from C. tarsalis (Table 8). All of these isolates were reisolated in duck embryo cell culture (DECC) where typical small plaques appeared 24 hours after inoculation. All isolates were serologically confirmed as WEE virus with the serum neutralization test (SN) or the hemagglutination

Table 7. Arbovirus Isolation from Mosquitoes Caught, Pooled, and Tested in 1969

Mosquito Species	Number of Pools Tested	Virus Isolates of				Total Number of Isolates
		WEE	California	CV	Turlock	
Culex tarsalis	132	8	-	1	1	10
Aedes trivittatus	74	-	2	1	-	3
Aedes vexans	58	-	-	1	-	1
Aedes species	20	-	-	-	-	-
Aedes dorsalis	10	-	-	-	-	-
Aedes triseriatus	5	-	-	-	-	-
Culex species	3	-	-	-	-	-
Culiseta inornata	3	-	-	-	-	-
Culex restuans	3	-	-	-	-	-
Culex salinarius	2	-	-	-	-	-
Anopheles punctipennis	2	-	-	-	-	-
TOTAL	315	8	2	3	1	14

Table 8. 1969 Laboratory Data on WEE Virus Isolates from *Culex Tarsalis* Mosquitoes

Trap Night Date	Number of Mosquitoes in Pool	Test Used to Identify the Virus	Titer of Virus in DECC[a]
7-31-69	50	SN[b]	$10^{5.41}$[e]
8-14-69	50	HAI[c]	NT[d]
8-15-69	50	HAI	NT
8-15-69	50	HAI	NT
8-19-69	50	SN	$10^{5.69}$
8-19-69	50	SN	$10^{5.69}$
8-21-69	50	SN & HAI	$10^{7.60}$
8-21-69	50	HAI	NT

[a] DECC = Duck embryo cell culture.

[b] SN = serum neutralization test.

[c] HAI = hemaglutination inhibition test.

[d] NT = not tested.

[e] Titer = expressed as number of plaque forming units 0.2 ml of inoculum.

inhibition test (HAI). Titers of these isolates in DECC ranged from $10^{-5.41}$ to $10^{-7.60}$ plaque forming units (Pfu) of virus/0.2 cc of infected fluid.

Seven of the eight WEE isolations were obtained from the dairy farm. This was also the site with the highest percentage of C. tarsalis throughout the summer. Chiang and Reeves (12) developed a method of statistically determining the infection rate in mosquitoes. This method was based on the assumption that the infection rate was small and it was impossible to make a determination on each individual sample. They derived the following formula:

$$P = 1 - \frac{(n-x)^{1/m}}{n}$$

P = the infection rate in mosquitoes expressed by number per 1,000 mosquitoes.

n = the number of pools of mosquitoes tested for a given period of a given species of mosquitoes.

x = the number of "n" pools tested that are positive for virus.

m = the number of mosquitoes in each pool which should be constant for all "n" pools.

This formula was used to determine the weekly infection rates in the various mosquitoes from which virus isolation was accomplished. An increase in the infection rate per 1,000 mosquitoes was shown for WEE virus in C. tarsalis over the collection period at the dairy farm. From July 27 when the first isolation was made till August 21 when the last isolation was made the infection rate increased from 1.03 to 7.39/1,000 mosquitoes (Table 9). The infection rate in C. tarsalis

Table 9. Virus Isolations by Date, Site and Mosquito Species with Weekly Infection Rates

Date by Week	Site	Virus--No. of Isolates	Mosquito Species	Infection rate (per 1000 mosq.)
7-27 to 8-2	Dairy farm	WEE-1	C. tarsalis	1.03*
	Dairy farm	CV-1	A. vexans	2.87
	Horse ranch	CE-2	A. trivittatus	10.73
8-3 to 8-9	Dairy farm	None	C. tarsalis	--
	Horse ranch	None	A. trivittatus	--
8-10 to 8-16	Dairy farm	WEE-2	C. tarsalis	3.39*
	Dairy farm	CV-1	C. tarsalis	1.03
	Horse ranch	WEE-1	C. tarsalis	4.45
	Horse ranch	CV-1	A. trivittatus	0.73
8-17 to 8-23	Dairy farm	WEE-4	C. tarsalis	7.39*
	Dairy farm	Turlock-1	C. tarsalis	1.03
	Horse ranch	None	--	--

*This shows an increased infection rate of C. tarsalis with WEE at the dairy farm.

of 7.39 is higher than the infection rates of 3 to 4/1,000 mosquitoes reported in Kern County, California, studies by Hammon and Reeves (25) when a number of human cases of WEE were reported. This infection rate is lower than the infection rates in C. tarsalis reported by Hayes et al. (33) in Hale County, Texas, during years when few cases of WEE were reported. Since this site, the dairy farm, was located near a residential development the high number of WEE isolates were of public health importance. The infection rate at this site was high enough in C. tarsalis to have caused infections in man. No known human cases of central nervous system involvement were reported from this area by local physicians during the summer of 1969.

The other WEE isolate came from the horse ranch during the third week of August. The infection rate on this date was 4.45/1,000 C. tarsalis present. This was the only WEE virus isolation from this site during the summer collection period and it was impossible to come to any conclusion about the public health importance of this isolate. Rueger et al. (68) and Olson et al. (56) have shown in studies in Minnesota that the pigeon is a very good indicator of WEE and St. Louis encephalitis activity. Studies were not done at the horse ranch to determine the percentage of the pigeons that were positive for WEE antibodies.

Culex tarsalis has been shown to be the major vector of WEE virus to birds and humans as well as to domestic and wild animals. Eklund (19) reported that birds are the most probable reservoir of WEE virus. The English sparrow and the grackle are the species of birds most

often incriminated. This could have influenced the number of isolates of WEE virus from the dairy farm as these two bird species were observed in greater numbers at this site than at the other two study sites. Attempts to isolate WEE virus from birds were not made at these sites.

The arbovirus isolations other than WEE were all confirmed in Vero cell cultures using the SN test. The isolates of Trivitattus virus in Aedes trivittatus had titers in Vero cells of $10^{-4.63}$ and $10^{-5.51}$ Pfu/ 0.2 ml of infected fluid (Table 10). The CV isolates were from three different mosquito species: Aedes trivittatus, Aedes vexans, and Culex tarsalis. Titers of these viruses are given in Table 10. Only one isolation of Turlock virus was obtained in C. tarsalis.

The two isolations of Trivittatus virus from Aedes trivittatus mosquitoes were the first reported isolations of this virus in South Dakota. Trivittatus virus is only one of the 8 known types of California virus that exist in the United States (89). This strain has not been isolated from human origin as has the LaCrosse strain (80). Both of the Trivittatus viruses were isolated on July 31 at the horse ranch. The infection rate on this day in Aedes trivittatus was 10.73/1,000 mosquitoes. Unfortunately no more isolations of this virus were made during the rest of the study. It is hard to determine if this virus existed in a high enough percentage of the mosquitoes to have infected any other host. These isolations were important because Trivittatus virus is known to cause infections in man and

Table 10. Laboratory Data on CV, Turlock, and Trivittatus Viruses Isolated from Mosquitoes in 1969

Trap Night Date	Mosquito Species	No. of Mosquitoes in pool	Test used to Identify the Virus	Virus Identity	Titer of Virus in Vero[a]
7-27-69	Aedes trivittatus	100	SN[b]	Trivittatus	$10^{4.63}$[c]
7-27-69	Aedes trivittatus	100	SN	Trivittatus	$10^{5.51}$
8-14-69	Aedes trivittatus	96	SN	Cache Valley	$10^{7.65}$
8-1-69	Aedes vexans	100	SN	Cache Valley	$10^{7.36}$
8-11-69	Culex tarsalis	50	SN	Cache Valley	--
8-21-69	Culex tarsalis	50	SN	Turlock	$10.^{5.96}$

[a] Vero = indicates Vero cell culture passage under agar.

[b] Titer = expressed as number of plaque forming units 10.2 ml of inoculum.

[c] SN = serum neutralization test.

and the knowledge of its presence in South Dakota adds to the geographical distribution of the virus.

The isolations of Cache Valley (CV) virus were made from three different mosquito species and from two different trap sites. Two of the isolations were from mosquitoes trapped at the dairy farm. The first of these isolations on July 1 was from Aedes vexans. Wong (87) gives the only other report of this virus from Aedes vexans. The other isolation of CV virus from the dairy farm was on August 11 from C. tarsalis. This is also a rare isolation, in that C. tarsalis are rarely found infected with this virus. The isolations of CV virus from the dairy farm are interesting in that it has been reported by Kokernot et al. (46) and Yuill (90) that a large majority of the dairy cattle tested had CV antibodies. Kokernot, has also found that a large percentage of the mosquitoes that have CV virus have obtained blood meals from dairy cattle. Dairy cattle at the dairy farm were not tested for virus nor were blood meal studies done on mosquitoes so our findings could not be compared with the findings of Kokernot (46) and Yuill (90).

The other isolation of CV virus was made at the horse ranch on August 14. The infection rate at this site was very low in Aedes trivittatus on this date (Table 9). Since CV virus has never been isolated from sources other than mosquitoes its importance as an infectious agent is not known.

A single isolation of Turlock virus was made from a pool of C. tarsalis mosquitoes. This isolation was made on the 19th of

August at the dairy farm. Turlock virus does not resemble any of the other viruses antigenically and therefore it is placed in a group by itself. Like CV virus Turlock virus has not been isolated from man. There have been reported isolations from birds and small mammals (33, 71). The virus is usually isolated from C. tarsalis mosquitoes. This mosquito species has proven to be a very effective vector of WEE and possibly with further research Turlock virus will be found to cause an infection in some mammalian host.

Virus isolations were made from mosquitoes from the dairy farm and the horse ranch during the summer but no isolations were made from those from the third study site, the homestead. The reason or reasons for this are not known. Mosquito catches at this site were as high as those at the other two sites. High populations of C. tarsalis were found in a number of catches. The other two major mosquito species Aedes vexans and Aedes trivittatus were also found in high numbers at this site. One possible explanation for the lack of virus could be that the mosquito trap was too close to the mosquito breeding grounds and they were caught before they had a chance to take a blood meal from an infected host. Mosquitoes from the different sites were not checked to determine the percentage of the mosquitoes that had taken recent blood meals so this possibility could not be checked. Another possible explanation is that there was such a diversified animal population present at the site that the mosquitoes did not feed on the animal or bird sources of the arboviruses. Use of the precipitin test developed by Tempelis and Lofy (77) to

determine the source of mosquito blood meals could have given information about this. There might have been completely different feeding patterns at the three sites for the three major mosquito species trapped.

Mortality in Pheasants Inoculated with Arbovirus Isolates from South Dakota

Byrne and Robbins (9) have demonstrated that pheasant chicks are very susceptible to EEE virus. The objective of this experiment was to see if the arboviruses isolated in South Dakota would produce fatal infections in young pheasant chicks. The arthropod-borne isolates of CV, Trivittatus, Turlock, and WEE were experimentally inoculated into 3-day-old pheasant chicks. Also included in this experiment was the EEE virus strain isolated from pheasants in South Dakota in 1967 (58). Three-day-old pheasant chicks adapted best to environmental conditions and handling in the hood. If birds less than 3 days old were used non-specific deaths occurred either from environmental changes present in the hood or from inoculation procedures and handling.

Of the 1969 arbovirus isolates WEE virus produced the highest mortality in the inoculated pheasant chicks. The experiment was repeated 4 times and in all trials 100% mortality resulted (Table 11). Mortality from WEE virus infection in chicken chicks had been demonstrated by Chamberlin et al. (11). Mortality patterns with EEE virus were the same as those observed with WEE. Similar results were observed with EEE virus experimentally inoculated into pheasant chicks by Byrne and Robbins (9) and Hanson et al. (29). With WEE

Table 11. Experimental Study to Determine the Mortality in 3-Day-Old Pheasant Chicks Inoculated with Five Arbovirus Isolates from South Dakota

Virus	Inoculum Preparation	Number of Birds per Experiment		Times test repeated	% mortality observed 72 hrs. postinoculation
		Controls	Inoculated		
WEE	SMB_3 10%*	12	12	4	100
EEE	SMB_3 10%	12	12	4	100
Cache Valley	SMB_3 10%	10	10	2	40
Trivittatus	SMB_3 10%	10	10	2	10
Turlock	SMB_3 10%	10	10	2	10

* SMB_3 10% = 10% suspension of infected suckling mouse brain with virus at third passage level in mice.

and EEE viruses symptoms of paralysis in the leg region of the body (Fig. 16) appeared about 24-36 hours after inoculation. Symptoms of paralysis in the axial region of the body in birds up to 18-24 weeks old have been observed during EEE epizootics in penned pheasants (Fauddoul et al., 20 and Parikh et al., 58). Mortality in pheasants inoculated with either WEE or EEE viruses will occur as early as 36 hours after inoculation with the majority occurring by 72 hours (Fig. 17). A similar time pattern of death was observed in all age groups of pheasants inoculated. Death losses with the CV, Trivittatus, and Turlock viruses from South Dakota were not as high as those with WEE and EEE viruses. Cache Valley virus killed approximately 40% of the 3-day-old pheasant chicks inoculated (Table 11). This mortality rate is high enough to suggest the possibility that infections may occur in pheasants in the wild if the virus is present and if there is an appropriate factor to transmit the virus to pheasants. Since there has been no reported CV virus isolations from pheasants or serological evidence of CV infection in pheasants further work has to be done to determine if mortality from CV infection does occur in pheasants in the wild. Holden and Hess (39) in preliminary work with the original isolation of CV virus were not able to demonstrate viremia in 0.5 day old chicken chicks.

Mortalities observed in pheasants inoculated with Turlock and Trivittatus viruses were less than 10%. Deaths that did occur with these viruses occurred at least 5-6 days after inoculation. The low percentage mortality in pheasants with Turlock and Trivittatus

Figure 16. Pheasant chicks inoculated with WEE virus showing symptoms of paralysis in the leg region of the body.

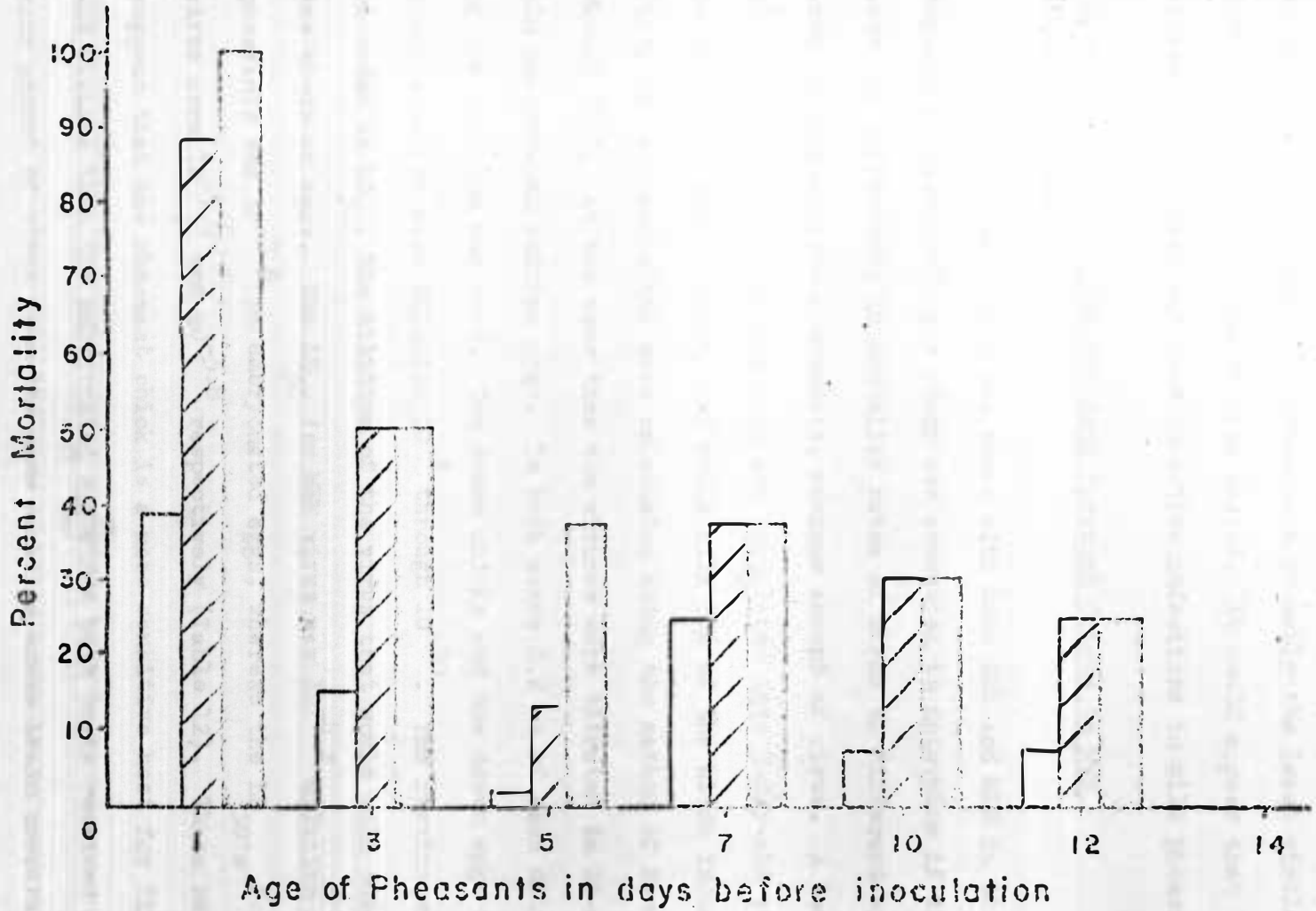

Figure 17. Variation in daily cumulative mortality in different aged pheasant chicks. All chicks were inoculated with one chick LD_{50} of EEE virus estimated in 3-day-old pheasant chicks. ☐ 48 hrs., ▨ 72 hrs., and ☐ 96 hrs postinoculation.

viruses indicates that the viruses are probably the least virulent for pheasants of all the viruses tested. It would appear that these viruses do not play any role in active infections in wild pheasants.

Susceptibility of Different Aged Pheasant Chicks to WEE and EEE Viruses

Since 100% mortality was shown with both EEE and WEE in three-day-old pheasant chicks a study was conducted to determine if there were any differences in mortality rates of birds of different ages when inoculated with a constant, minimum amount of virus. A ten fold dilution of each virus was made and inoculated into 3-day-old pheasants to determine the dilution that would kill 50% of the birds inoculated. Fifty percent endpoints were calculated using the method of Reed and Muench (60). At the same time the viruses were titrated in 10-day-old embryonated chicken eggs. In both cases 0.2 cc of each dilution of the inoculum was used. One dozen chicks and one dozen eggs were inoculated for each dilution 10^{-1} through 10^{-10}. The results were recorded as LD_{50}, the dilution of the virus that would kill 50% of the birds or eggs. The LD_{50} for WEE virus was $10^{-4.2}$ dilution in pheasants and $10^{-3.4}$ in embryonated eggs, whereas the LD_{50}'s for EEE virus were $10^{-7.2}$ and $10^{-5.8}$ respectively (Table 12). These results suggest that the pheasant chick is a more sensitive host for EEE and WEE viruses than the embryonated egg since both hosts received the same amount of virus from the same suckling mouse brain preparation. It was not possible from these results to conclude that the EEE virus

was more virulent than WEE virus to the pheasants and in the embryonated eggs since the virus concentrations in the original mousebrain preparations used were not determined.

Table 12. Relative Titers of EEE and WEE Viruses in 10-Day-Old Chick Embryonated Eggs and Three-Day-Old Pheasant Chicks

Virus and Strain Number	LD_{50} of Virus in 10-day-old chick embryonated eggs	LD_{50} of Virus in 3-day-old pheasant chicks
WEE-S.D. CT-57, '69	$10^{-3.4}$	$10^{-4.2}$
EEE-S.D. PH-1, '67	$10^{-5.8}$	$10^{-7.2}$

Byrne and Robbins (9) had shown that as the age of the pheasant increased so did its resistance to EEE virus. To determine if this was true, with EEE and WEE viruses isolated in South Dakota pheasants of different ages were inoculated with one LD_{50} for a 3-day-old pheasant chick. Birds ranging from 1 to 12 days old were used. The same procedures for inoculation and observation were used as described for the previous experiment. All deaths occurring after 24 hours and up to 72 hours after inoculation were recorded. Pheasants inoculated with EEE virus showed a decrease in mortality with an increase in age (Fig. 18). The mortality had dropped from 100% in the one-day-old birds to 30% in the 12-day-old pheasants. Pheasants older than 12 days were not used in the study so the maximum age of chicks that would die when inoculated with one LD_{50} of the virus was not determined.

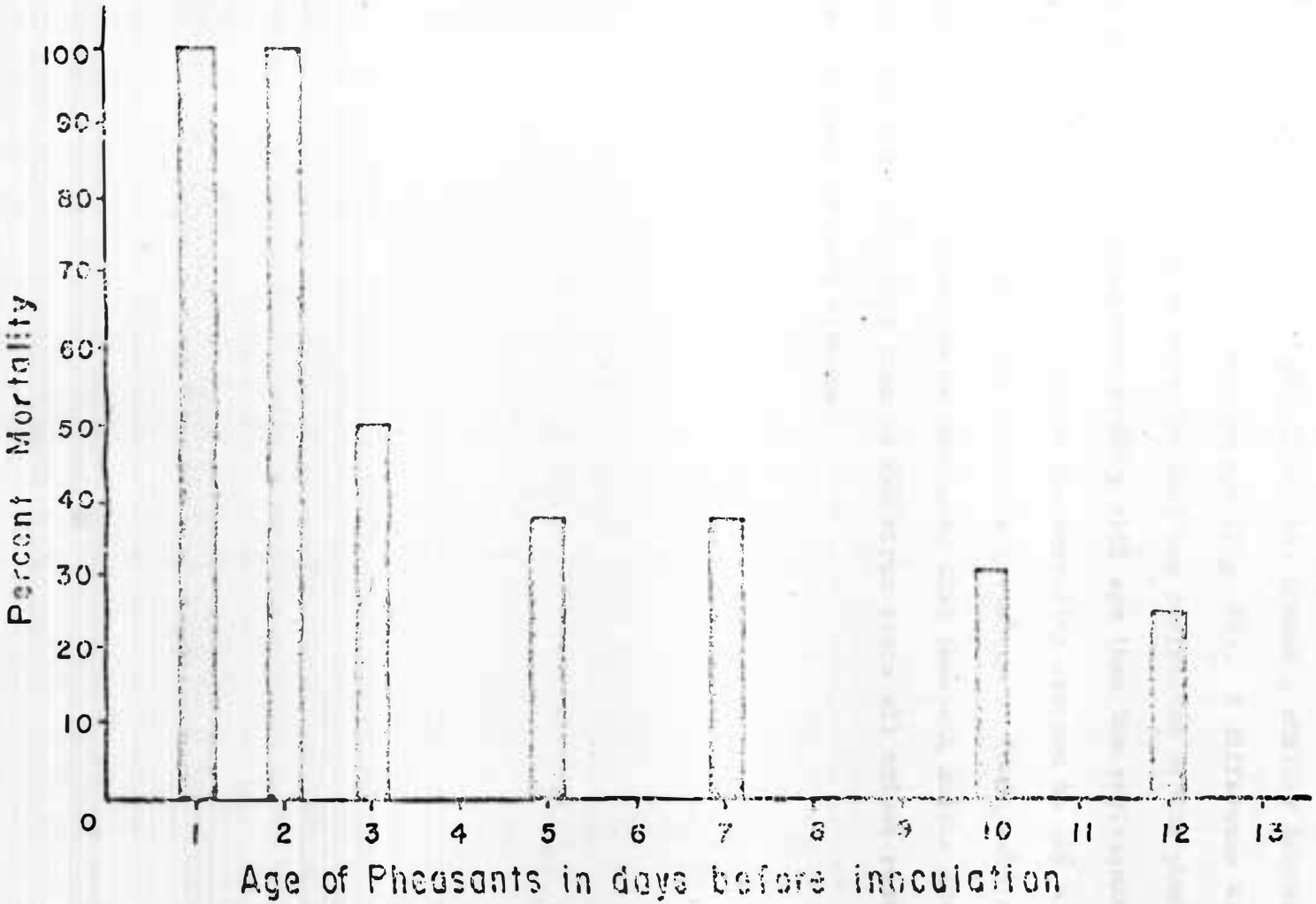

Figure 18. Mortality patterns of different aged pheasant chicks inoculated with one LD_{50} of EEE virus estimated in 3-day-old pheasant chicks. All results were recorded 72 hours post-inoculation.

Pheasants inoculated with WEE virus also showed a similar decrease in mortality with an increase in age (Fig. 19). A difference was shown between the two viruses in that the resistance of the pheasants to WEE virus increased more rapidly with age than the resistance to EEE virus. In 10-day-old chicks the mortality dropped to 10% with WEE virus, whereas, with EEE virus the mortality in 10-day-old chicks was 30%. It can therefore be concluded that pheasant chicks are more susceptible to EEE virus than to WEE virus since all chicks received the same dose of both viruses.

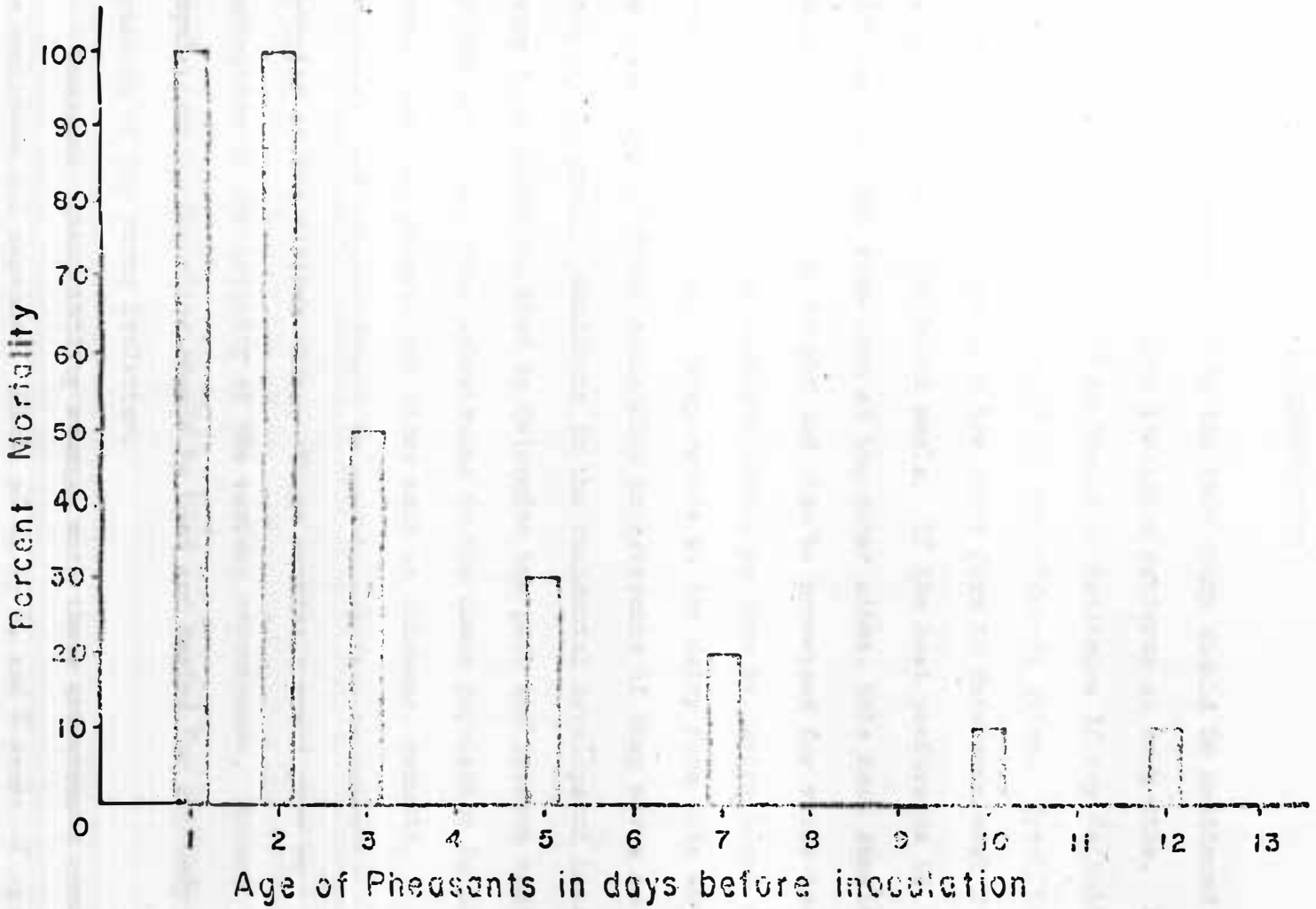

Figure 19. Mortality patterns of different aged pheasant chicks inoculated with one LD_{50} of WEE virus estimated in 3-day-old pheasant chicks. All results were recorded 72 hours post-inoculation.

RECOMMENDATIONS

The study areas used in the 1969 study should be continued to further understand the virus isolation patterns at each site. Blood engorged mosquitoes should be tested to determine if any definite host preference patterns exist at the different sites. Specific attention should be given to the dairy farm to determine what host C. tarsalis prefer for blood meals. If the host preference is different at this site than at the other sites, this host should be tested for antibodies to WEE and also be processed for virus isolation. Virus isolation attempts should be done by taking blood samples from the suspect hosts. Dairy cattle at the dairy farm site should be tested for CV virus antibodies to determine if they serve as a host for CV virus. Residents in the residental development near the dairy farm should be bled to determine the past and present status of WEE virus and other arboviruses in the human population in this area. Sentinal animals and birds such as chickens, rabbits, pheasants, and pigeons should be used in addition to mosquito trapping at the various sites. These sentinals would give an indication of the activity of the various arboviruses. Rodent populations at the sites should be bled and tested for antibody presence or for virus isolation.

Pheasant susceptibility studies with these arboviruses should be continued and expanded. Birds of 2, 4, 6, and 8 weeks of age should be inoculated with 100 to 10,000 LD_{50}'s to determine how

important the quantity of virus is in causing death in older birds. Experiments with CV, Turlock, and Trivittatus viruses in pheasant chicks should be repeated to confirm the results obtained. Age susceptibility studies should also be done with these viruses. Antibody levels of WEE and EEE viruses should be determined experimentally in pheasants over an 18 month period. Pheasants in egg production should be inoculated with EEE virus to determine if the virus causes any effect on pheasant reproduction.

CONCLUSIONS

Ninety-three percent of the 22,000 mosquitoes identified were of three species: Culex tarsalis, Aedes vexans, and Aedes trivittatus. Fifteen other mosquito species were found in South Dakota but in smaller numbers. The dairy farm site had the highest C. tarsalis populations throughout the summer. The horse ranch site was highest in Aedes vexans and Aedes trivittatus mosquitoes. The third study site, the homestead, had approximately equal percentages of all three major mosquito species.

From the mosquitoes processed for viruses 14 arbovirus isolations were made. Eight Western equine encephalitis (WEE) virus isolates were obtained from C. tarsalis. Seven of these WEE virus isolates were obtained from the dairy farm. The infection ratio for WEE in C. tarsalis increased from 1.03/1,000 to 7.38/1,000 during the summer at the dairy farm. The other WEE isolate was obtained from the horse ranch. Three isolations of Cache Valley virus were obtained, one each from C. tarsalis, A. vexans and A. trivittatus. Two of these isolates were obtained from the dairy farm. Two pools of A. trivittatus mosquitoes caught at the horse ranch were positive for Trivittatus virus, a serotype of California virus. Turlock virus was isolated from one pool of C. tarsalis caught at the dairy farm. None of these viruses had been previously isolated from mosquitoes in South Dakota.

Western and Eastern equine encephalitis (EEE) viruses caused 100% mortality in 3-day-old pheasant chicks inoculated with 0.2 cc of 10% SMB preparation of these viruses. Cache Valley virus caused 40% mortality while Turlock and Trivittatus viruses caused less than 10% mortality in 3-day-old pheasant chicks. When pheasant chicks ranging from 1-12 days old were inoculated with 1 LD_{50} of either WEE or EEE virus a decrease in susceptibility with increasing age of pheasants was shown. The EEE virus strain was more pathogenic to pheasant chicks than the WEE virus strain.

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