Western Equine Encephalitis in Saskatchewan Reptiles and Amphibians, 1961-1963

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ABSTRACT

Western equine encephalitis (WEE) antibodies were found in blood samples from garter snakes and leopard frogs collected in Saskatchewan but WEE virus was not recovered from any of the specimens. Evidence of natural WEE infection in snakes was found in 8 different localities while in frogs in two only. Experimentally, garter snakes were readily infected and developed a high, relatively sustained viremia without signs of disease. After experimental exposure, viremia persisted regularly for 10 to 12 days, while the longest observed duration of viremia was 30 days. Anamnestic responses were elicited in snakes as a result of second inoculations of virus after the antibody levels from first exposures had fallen. Newborn snakes were observed to be more sensitive to infection than adults. The possibility of virus and antibody transmission from infected pregnant garter snakes to their offspring was investigated. Snakes and frogs were both susceptible to infection by the oral route. Two bull snakes collected at Steveville, Alberta, were found to have antibody for St. Louis Encephalitis virus.

Introduction

Studies of the natural history of western equine encephalitis in Saskatchewan were commenced in 1959, and results of wild duck studies (1) and mosquito studies in 1962 (2) have been reported. The present paper deals with reptiles and amphibians.

There are a few publications on the subject of reptiles as possible reservoir hosts of arboviruses. Experimental studies carried out by Thomas, Eklund and Rush (3, 4, 5) showed that garter snakes (*Thamnophis spp.*), experimentally infected with WEE virus, developed viremia of high titre and long duration. Gebhardt and Hill (6) reported that experimentally infected garter snakes maintained WEE virus for 139 days during winter hibernation. Rehacek *et al.* (7) experimentally studied the susceptibility of the lizard, *Lacerta viridis* to tick-borne encephalitis virus: relatively large doses of virus, while productive of transient viremia, failed to elicit the production of neutralizing antibody. Karstad (8) presented evidence of natural and experimental infection of poikilotherms with eastern equine encephalitis (EEE) virus. Graighead *et al* (9) found EEE neutralizing antibody in the blood of lizards collected in Panama.

Our search of the literature failed to yield records of the natural occurrence of WEE antibody in garter snakes or reports of the susceptibility of frogs. In the study being reported here a series of experiments were carried out in an attempt to establish the susceptibility of reptiles and amphibians to infection with WEE virus under laboratory conditions. These were aimed at the study of production and duration of viremia and the study of antibody responses. Subcutaneous and oral routes of exposure were used with a view to simulating possible modes of infection in nature. Anamnestic responses were studied in a few snakes by giving second or "booster" doses of virus and noting the responses. Experiments were carried out with pregnant garter snakes in efforts to determine whether virus might be transmissible to their offspring. Lastly, cold blooded ani-

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mals (five species) were exposed in late fall to determine if they would maintain virus over winter, or over the hibernating season, under laboratory conditions.

Materials and Methods

A search of the literature yielded little information on methods of collecting, maintaining and utilizing snakes in experiments involving infectious agents. The methods adopted by the authors are separately described (10).

A total of 820 snakes, 533 frogs and toads, three salamanders and one turtle were collected from 25 different localities in Saskatchewan. In 1961, 1962 and 1963 the numbers of snakes collected were 22, 368 and 430 respectively. A total of 756 snakes were examined for the presence of WEE virus and antibody, while 64 escaped, lost their identification or died. Of the 756, 123 were kept and used in experiments. Of the frogs and toads, 256 were examined for virus, 173 for WEE neutralizing antibody and 111 used in experiments. The salamanders and the turtle were used in an experiment.

As soon as the live snakes reached the laboratory they were labelled and blood samples were collected for tests by bleeding from ophthalmic vessels. The small samples of blood (0.1 - 0.2 ml.) collected in this way were diluted 1:10 or 1:5 with beef heart infusion broth containing 1000 I.U. of potassium penicillin and 2.5 mg. of streptomycin sulfate per ml. The diluted blood was centrifuged for 10 minutes at 2000 r.p.m. and the supernatant was tested for the presence of antibody or virus as desired. Larger samples of blood, collected by more extensive bleeding or exsanguination, were allowed to clot and the serum was separated from the clot and saved.

Frogs were pithed, the thorax opened and blood drawn directly from the heart with a hypodermic syringe fitted with a fine gauge needle. The blood samples were transferred to diluent as with small samples of snake blood.

Blood samples from newly born and very small snakes were collected using paper discs (11) after decapitation.

The turtle, laid on its back with head secured in an extended position, was bled from the heart using a syringe with a 3-½ in., 20 gauge needle. The needle was inserted dorsal to the midline of the anterior end of the plastron until the heart was punctured as evidenced by flow of blood into the syringe.

All blood samples or sera were heat inactivated (30 min. at 56°C) before testing for neutralizing antibody.

Embryonated hens eggs were used for virus isolation attempts and in carrying out tests for neutralizing antibody in serum samples. Five or 10 eggs taken on the 10th day of incubation were each inoculated by the allantoic route with 0.1 ml. of the prepared supernatant, serum or tissue suspension. Inoculated eggs were candled at least once a day and those showing dead embryos were removed for examination. The absence of bacterial infection of dead embryos was taken as evidence of possible virus multiplication and serial passages were made from such eggs.

Organs (brain, lung, liver, spleen and kidney) examined for virus were prepared by grinding in a TenBroeck grinder with nine volumes of beef heart infusion broth containing penicillin and streptomycin. The 10% suspensions were centrifuged and the supernatant fluids saved to examine for virus.

Tests for neutralizing antibody were conducted using serial 10-fold dilutions of virus mixed with equal amounts of undiluted serum, or with blood diluted 1:10 as described above. Results were recorded up to 72 hrs. after egg inoculation.

In routine and experimental work the viremia and neutralizing antibody titers are recorded as chicken embryo (C.E.) log 10 LD50 units of WEE virus per /ml. of blood or sera. End points were computed by the method of Reed and Muench (12). Neutralization indices from 1.00 to 1.50 were interpreted as suspicious, and indices higher than 1.75 as positive. Throughout the studies a human strain of WEE virus (R_1) was used. This strain was isolated during an epidemic in Saskatchewan in 1941, since which time it has been maintained in the laboratory by serial passage. As a source of virus for experimental and routine work, amniotic and allantoic fluids from dead embryos were harvested and stored in a freezer at -20°C. The stock virus contained $10^{8.5}$C.E LD50, per/ml. of egg fluid.

Snakes and frogs were experimentally exposed to R_1 virus by different routes and in different dosages. Virus recovered from blood and tissues of experimentally infected animals was identified through

TABLE I — Results of WEE neutralization tests on blood and sera of reptiles and amphibians collected in Saskatchewan in 1961, 1962 and 1963.

TOTAL	COMMON NAME	SCIENTIFIC NAME	POSITIVE (indices > 1.75)	SUSPICIOUS (indices 1.00—1.50)	NEGATIVE (indices < 1.00	TOTAL TESTED
REPTILLIA	Western Plains Garter snake	**Thamnophis radix haydeni**	12	78	485	575
	Red-sided Garter snake	**Thamnophis sirtalis parietalis**	32	43	98	173
	Wandering Garter snake	**Thamnophis vagrans**	1	1	1	3
	Bull snake	**Pituophis catenifer**		1	2	3
	Prairie Rattle snake	**Crotalus viridis viridis**			2	2
	Painted turtle	**Chrysemys picta**			1	1
AMPHIBIA	Leopard frog	**Rana pipiens**	30	52	184	266
	Wood frog	**Rana sylvatica**			20	20
	Plains Spade-foot toad	**Scaphiopus bombifrons**			50	50
	Devil's Lake salamander	**Amblystoma tigrinum diaboli**			3	3

use of a known WEE antiserum. The antiserum was produced in rabbits with mosquito strain virus (999 - 57) supplied by Dr. L. A. Thomas, Rocky Mountain Laboratory, Hamilton, Montana. This strain had undergone one chick embryo passage and three mouse passages before it was received. Antibody responses in experimentally exposed reptiles and amphibians were measured using the neutralization test with R_1 virus in chick embryos. Some of the sera positive with R_1 virus were retested using 999-57 virus.

In all experiments, the number and frequency of bleedings were scheduled with a view to obtaining information on the existence of virus in the bodies of the animals and on the development of antibody. Positive antisera were prepared in snakes and the hyperimmunization procedures are recorded. Four serum samples from snakes collected in nature and 6 sera from experimentally infected snakes were sent to Drs. Casals and Clarke, Rockefeller Foundation Laboratory, New York, to prove and compare our results with the HI tests, for WEE and other arboviruses.

Results

Natural infection in reptiles and amphibians. All attempts to recover WEE virus or other viruses from the blood and organs of reptiles and amphibians (total of 1161), collected in nature, failed. Blood samples from a number of specimens showed the presence of WEE neutralizing antibody. The results are recorded in Table I. Forty five (6%) of 756 snakes had antibody titres in the positive range (indices 1.75 - 3.00). In 1962, of 368 snakes examined, 17 (5%) were positive. In 1963, of 366 snakes examined, 28 (8%) were positive. Positive snakes were from eight locations in the province: Beadle (3), Coleville (1), Estevan (32), Kindersley (1), Maidstone (4), Onion Lake (2), St. Walburg (1) and Swift Current (1).

Of the 45 positive snakes, 32 were red-sided garter snakes, 26 of them from Estevan. Of the snakes examined for neutrallizing antibody from Estevan, 131 (22 pos-

itive) were red-sided garter snakes and 24 (4 positive) were plains garter snakes. The respective proportion of positives in both species at Estevan was 17%.

Three hundred and thirty-six frogs and toads were collected throughout the province and examined for WEE neutralizing antibody. Thirty (9%) had significant titres (indices 1.75 - 2.50). All 30 positives were leopard frogs and the percentage of positive leopard frogs was 12%. Positive leopard frogs were found at two locations in the province: Elmore (14) and Alsask (16). Frogs with borderline titres (indices 1.50 - 1.75) were found at two other localities: North Battleford and Beaver Creek. Points where positive snakes and frogs were found are shown in Figure I.

Experimental infections in snakes. In four different experiments, 99 garter snakes of Western plains species (*Thamnophis radix haydeni*) were used. The size of adult snakes varied between 45/265 gm. in weight and 40/100 cm. in length. The pregnant snakes varied between 67/153 gm. and 98/400 cm. The average length of day-old snakes was 17 cm.

Experiment 1. — Twenty-two snakes, collected in the field and found negative for WEE virus and neutralizing antibody were used. Twenty were experimentally infected by the subcutaneous route with $10^{5.8}$C.E. LD_{50} of WEE virus while the remaining two were kept as controls. Fifteen of the 20 snakes developed viremia. Virus was first detected at 24 hrs. after exposure and rose to a peak titer of $10^{4.6}$C.E. LD_{50} per/ml. whole blood on the fourth day. In some of the snakes the viremia, after the peak on the fourth day, declined to undetectable levels, but then rose again to a second peak, lower than the first, on the 10th or 12th day after exposure. The longest observed duration of viremia in the experiment was 30 days. In five snakes, we failed to detect viremia. Specific neutralizing antibody first was detected 12 days after exposure and reached peak titers about the 60th day. There were variations between individuals but all 20 snakes inoculated with virus developed antibodies. In some snakes, antibody titers had declined to insignificant levels by the 90th day.

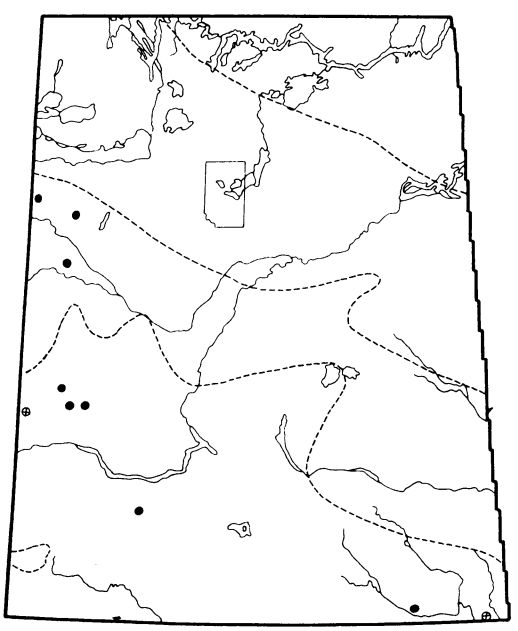

Figure 1. Map of Saskatchewan showing points (solid circles where garter snakes were found serologically positive for WEE, and points (crossed circles) where positive frogs were found.

On the 90th day after first exposure, three snakes still with positive (indices 1.75 - 2.50) titers of antibody and three with insignificant titers were each given a second dose ($10^{3.5}$C.E. LD_{50}) of WEE virus. These six snakes were examined at regular intervals over the next 270 days. Detectable viremia failed to develop after the second exposure. In three of the snakes —those with insignificant antibody titers —anamnestic responses were produced with 100-fold to 10,000-fold increases in antibody titers on the second, third and fifth days respectively. The other three snakes failed to produce significant responses to the second inoculations of virus. On the 270th day after the second exposure, the six snakes were bled and killed. Organs were examined for virus with negative results. Sera from all six snakes showed significant antibody titers.

After a year's observation, the remaining 16 snakes (14 principals, two controls) were killed and examined for virus and antibody. Attempts to recover virus failed; seven of the 14 principles still had positive titers of neutralizing antibody. Neither virus nor antibody were detected in the two control snakes. During the period of observation, no signs of ill health were noted in the infected snakes.

Experiment 2. — A pregnant female garter snake, collected in nature, gave birth in the laboratory to 50 young, and these were used to determine ease of infectivity

TABLE II - Detection of viremia and virus neutralizing antibody in day-old garter snakes infected with WEE virus. (August 13, 1962 — September 10, 1962)

Virus dose	Day after exposure	Number snakes killed to make 1:10 blood pools principals/controls	Group of 25 infected snakes		Group of 25 Control snakes	
			Viremia	Antibody	Viremia	Antibody
$10^{3.2}$ C.E. LD_{50} (0.05 ml. of diluted C. E. fluid, S/C)	0	3/3	—	—	—	—
	3	3/3	4.50	—	—	—
	6	3/3	8.50	—	—	—
	9	3/3	2.50	—	—	—
	12	3/3	—	1.50	—	—
	15	2/2	—	3.00	—	—
	18	2/2	—	3.50	—	—
	21	2/2	—	3.00	—	—
	24	2/2	—	3.50	—	—
	27	2/2	—	3.00	—	—

of the newborn in comparison to adults. Twenty-five day-old snakes were kept as controls and 25 were infected with WEE virus. The controls and principals were kept together in the same container. Blood samples were collected for examination every three days up to and including the 27th day after infection. Data obtained are summarized in Table II.

Viremia was first found on the 3rd, 6th and 9th days. The highest virus titer of $10^{8.5}$C.E. LD_{50} per/ml. blood was found on the 6th day and this was about 10,000-fold higher than peak titer found in experimentally infected adult snakes. Detectable antibody appeared in the blood on the 12th day and reached significant levels that were still present at termination of the experiment on the 27th day. The 25 controls failed to develop either viremia or antibody. No signs of disease was observed in any of the 50 snakes.

Experiment 3. — Attempts were made to infect snakes by the oral route. Five snakes were exposed by instillation of virus into the mouth cavity. In a second group of 10

TABLE III - Detection of viremia and virus neutralizing antibody in garter snakes orally exposed to the virus of WEE. (November 5, 1962 — November 23, 1962).

Virus dose	Virus recovery from blood (1:10) — after day						Antibody indices after day						Virus recovery from organs *1
	0	3	7	10	15	23	0	3	7	10	15	23	
$10^{7.8}$ C.E. LD_{50} (0.2 ml. of undiluted C.E. fluid, per /Os)	—	—	—	Pos. (1.00)	—	—	—	—	—	—	—	2.50	Negative
	—	—	—	—	—	—	—	—	—	—	—	1.50	"
	—	—	—	—	—	—	—	—	—	—	—	1.24	"
	—	—	—	—	—	—	—	—	—	—	—	0.74	"
	—	—	—	—	—	—	—	—	—	—	—	0.25	"

*[1]All snakes were killed 23rd day after exposure and heart, lung, liver, spleen, kindey and brain examined for WEE virus.

TABLE IV - Detection of virus and virus neutralizing antibody in garter snakes orally exposed to the virus of WEE. (January 21, 1963 — April 24, 1963).

Virus dose	Antibody response days after exposure					Virus recovery from organs
	0	23	39	45	95	
Approximately $10^{6.9}$ C.E. LD_{50} (average 2.5 ml. of infected water)	—	—	—	—	—	—
	—	—	—	—	—	—
	—	—	—	—	—	—
	—	—	—	—	—	—
	—	—	—	—	—	—
	—	1.26	—	—	—	—
	—		—	—	—	—
	—	1.36	1.00	—	—	—
	—	2.76	1.00	—	—	—
	—	2.76	1.50	1.00	1.24	—

*[1] All snakes were killed 95th day after exposure and heart, lungs, liver, spleen, kidney, and brain examined for WEE virus.

snakes, virus was administered in the drinking water. To accomplish the latter the snakes were deprived of water for eight days. On the day of exposure, water containing virus was placed before the snakes for a few minutes and then removed. The average consumption of infected water was 2.5 ml. Results obtained in these two lots of snakes are shown in Tables III and IV. Table III shows that one of the snakes exposed by placement of virus into the mouth cavity, manifested a low viremia 10 days later and a positive (index 2.50) titer of antibody 23 days later. Four of the snakes failed to exhibit viremia or to develop positive titers of antibody. None of the snakes exposed through drinking water developed detectable viremia; two developed positive antibody titers; two suspicious titers; six remained negative. Snakes of the lot exposed through placement of virus into the mouth were killed 23 days after exposure and tissues— heart, lung, liver, spleen, kidney and brain were examined for virus with negative results. Snakes given virus in drinking water were killed 95 days after exposure; tissues were examined with negative results.

Experiment 4. — Eight pregnant garter snakes, tested and found negative for WEE virus and antibody, were infected subcutaneously using a dose of $10^{3.5}$ C.E. LD_{50} of WEE virus (0.1 ml. diluted C.E. fluid). Four additional snakes were kept as controls. Blood samples from the mothers were collected for examination on the day of parturition. Blood examined from newborn snakes consisted of pools of blood from 10 snakelings in each litter. Results are summarized in Table V.

Attempts to recover virus from mother snakes at time of parturition failed. The blood pool from one litter (No. 109) yielded WEE virus while the other seven pools

TABLE V - Detection of viremia and virus neutralizing antibody in pregnant garter snakes and their offsprings, exposedt o the virus of WEE. (May 25, 1962 — August 22, 1962).

Snake Number	Date of infection	Date parturition	Number days between infection and parturition	Number of baby snakes born		Results in mother snakes		Results in new born (blood pool of 10 snakelings)	
				Alive	Dead	Viremia	Antibody	Viremia	Antibody
109	May 25	Jul 31	66	30	3	Negative	2.00	Positive 1.00	1.75
141	Jul 14	Aug 19	35	31	11	"	2.00	Negative	1.75
143	Jul 14	Aug 15	31	25	4	"	2.00	"	1.00
146	Jul 14	Aug 13	33	42	0	"	2.74	"	1.00
151	Jul 24	Aug 9	15	52	3	"	1.00	"	0.00
158	Jul 24	Aug 8	14	48	12	"	1.75	"	0 00
159	Jul 24	Aug 7	13	48	1	"	1.50	"	0 00
157	Jul 24	Aug 5	11	47	4	"	2.00	"	0 00
152	control	Aug 5	—	28	0	"	0.00	"	0.00
153	control	Aug 22	—	10	28	"	0.00	"	0.00
162	control	Aug 10	—	42	3	"	0.00	"	0.00
165	control	Aug 14	—	41	2	"	0.00	"	0.00

TABLE VI - Detection of viremia and virus neutralizing antibody in leopard frogs experimentally infected with WEE virus. (July 19, 1961 — August 2, 1961)

Virus dose	Day after infection	Viremia and antibody response in pools of blood from pairs of frogs diluted 1:10						Examination of organs for virus at termination of experiments (after 14 days)
		20 frogs infected subcutaneously		20 frogs infected orally		20 control frogs		
		Viremia	Antibody	Viremia	Antibody	Viremia	Antibody	
$10^{5.8}$ C.E. LD_{50} (0.2 ml. of C.E. fluid, s/c or per/os)	0	—	—	—	—	—	—	—
	2	Positive 1.00		—		—		—
	3	—		—		—		—
	5	—		—		—		—
	7	—		—		—		—
	9	—		—		—		—
	12	—		—		—		—
	14	—	1.74	—	1.24	—	0.00	—
	14	—	2.48	—	1.74	—	0.76	—
	14	—	1.00	—	0.00	—	0.25	—

were negative. Positive titers of antibody (indices 1.75 - 2.74) were noted in six of the eight mother snakes and in two of the eight pools of snakeling blood, including No. 109. The four controls proved negative for virus and antibody. There were snakelings dead at birth in seven of the eight principals and in three of the four controls. Attempts to recover virus from dead snakelings and from the organs of killed mothers and offspring at termination of the experiment failed.

Experiment infections in frogs. In order to establish the susceptibility of frogs to WEE virus, two experiments were performed using 105 adult frogs of the leopard species (*Rana pipiens*). The average weight of frogs was 25 gm.

Experiment 1. — Sixty frogs were distributed in three groups of 20 each. Frogs of the first group were each inoculated subcutaneously and those of the second were given virus in the same dosage by instillation into the mouth cavity. The third group served as controls. During the experiment, the frogs were kept separately in 3 aquaria at room temperature. Two frogs from each group were killed at varying intervals between days 0 and 14 inclusively. Blood from each pair of frogs was pooled and the pools examined separately. Results are summarized in Table VI.

TABLE VII - Detection of viremia and virus neutralizing antibody in leopard frogs experimentally infected with WEE virus. (August 13, 1962 — September 10, 1962)

Virus dose	Day after infection	Number frogs for each respective group used to make up blood pools (Blood dilution 1:10)	Group of 15 frogs infected subcutaneously		Group of 15 frogs infected intramuscularly		Group of 15 control frogs	
			Viremia	Antibody	Viremia	Antibody	Viremia	Antibody
$10^{3.5}$ C.E. LD_{50} (0.1 ml. of diluted C.E. fluid, s.c. or i.m)	0	1/1/1	—	0.00	—	0.0	—	0.26
	3	1/1/1	—	0.76	—	0.76	—	0.25
	6	1/1/1	—	0.75	—	0.00	—	0.76
	9	1/1/1	—	1.00	—	0.00	—	0.00
	12	1/1/1	—	0.00	—	0.76	—	0.00
	15	2/2/2	—	1.00	—	1.50	—	0.26
	18	2/2/2	—	0.76	—	1.26	—	0.00
	21	2/2/2	—	0.76	—	0.00	—	0.76
	24	2/2/2	—	0.76	—	2.00	—	0.00
	27	2/2/2	—	2.00	—	2.00	—	0.76

TABLE VIII - Antibody response in reptiles and amphibians experimentally infected with WEE virus. (November 26, 1962 — May 8, 1963)

Common name	Scientific name	Number of positive/over total tested (index range)
Western plains garter snake	**Thamnophis radix haydeni**	18/20 (2.00 — 3.00)
Bull snake	**Pituophis catenifer**	2/2 (1.75 — 2.00)
Painted turtle	**Chrysemys picta**	1/1 (3.76)
Devil's lake salamander	**Amblystoma tigrinum diaboli**	2/3 (1.75 — 2.00)
Leopard frog	**Rana pipiens**	4/6 (2.00 — 2.24)
		TOTAL: 27/32 (84% positive)

Virus was recovered from only one blood pool. This was the pool taken two days after subcutaneous exposure. Antibody responses were noted in two out of three pools taken from the subcutaneously infected group on the 14th day. Control frogs failed to develop significant antibody titers.

Experiment 2. — Forty-five frogs were divided into three groups of 15 each. The first group was injected subcutaneously; the second group intramuscularly; and the third group, kept as controls. Blood samples were taken at three day intervals between day 0 and day 27 inclusively. Up to and including day 12, frogs were examined individually; after day 12, in pools of two. Results are shown in Table VII.

Virus was not found at any time in any of the blood samples. A significant antibody response was found in one pool of blood taken on the 27th day after subcutaneous exposure. Two significant titers were found in pools 24 and 27 days after oral exposure. Controls yielded no significant antibody responses.

Experimental infections in various cold blooded animal species. In this experiment 32 cold-blooded animals, negative for WEE virus and antibody were infected subcutaneously with $10^{7.5}$C.E. LD_{50} of WEE virus (0.1 undiluted C.E. fluid, s/c) on November 26, 1962, and observed throughout the following winter. The animals comprised 10 adult garter snakes (*Thamnophis radix haydeni*), 10 young garter snakes (same species) born in the laboratory in August 1962, two bull snakes (*Pituophys catenifer*), three salamanders (*Amblystoma tigrinum diaboli*), six frogs (*Rana pipiens*), and one pointed turtle (*Chrysemys picta*). The species were kept separate from each other but the individuals of the same species were kept together. With the exception of the 10 adult garter snakes which were torpid and refused to eat during December and January, the animals were fed once a week.

TABLE IX - Detection of viremia and virus neutralizing antibody in garter snakes hyperimmunized with WEE virus.

Virus dose	Viremia titer indices on day								Antibody titer indices after last exposures on day					Pooled serum antibody titer index	Virus recoveries from tissues
	0	7	14	21	28	35	42	49	0	28	35	42	49		
$\Sigma 10^{8.8}$ C.E. LD_{50} (2.2 ml. undiluted C.E. fluid, s/c and i/per	—	6.5	—	—	Killed				—	3.26					(28) Spleen
	—	4.74	—	—	—	Killed			—		3.00				(35) Spleen
	—	4.75	—	—	—	—	Killed		—			3.54		3.24	—
	—	6.24	—	—	—	—	—	Killed	—				2.00		—

No signs of illness were observed during the experiment. On May 8, 1963, 153 days after exposure, all animals were killed. Blood and organs were collected from each individual. Tests for WEE virus were negative throughout. Antibody findings are summarized in Table VIII. Significant titers of antibody were found in all or some individuals of each species.

Antiserum against WEE virus produced in Garter Snakes. This experiment was carried out to study the hyperimmune response of snakes. Four negative adult snakes (*Thamnophis spp.*) were each inoculated with large doses of WEE virus, administered as follows: day 0, 0.2 ml. subcutaneously; day 7, 0.5 ml., subcutaneously; day 14, 0.5 ml., subcutaneously; and day 21, 1.0 ml., intraperitoneally. One snake was exsanguinated and killed for examination on each of day 28, day 35, day 42 and day 49. Results are tabulated in Table IX.

All snakes on 7th day showed viremia titers (indices 4.75 - 6.50), higher than those obtained in other experimentally infected adult snakes. Antibody levels obtained in the serum of each snake had indices between 2.00 and 3.54. The pooled sera of all four snakes had a titer index of 3.24. Antibody levels, in part at least, were slightly higher than any found in snakes collected in nature or experimentally exposed but once. The spleens of the snakes killed on day 28 and 35 respectively, both yielded WEE virus.

Evidence of infection in snakes with WEE virus and other arboviruses. As mentioned above, 10 samples of snake serum were sent to Drs. Casals and Clarke of the Rockefeller Foundation Laboratory, New York, to test for WEE and other arboviruses using the haemagglutination inhibi-

TABLE X - Results of SN and H1 tests with snake sera for antibodies to the WEE virus and other arboviruses — 1*

Source of Serum				SN Test		H1 Test		H1 Test
SPECIES	**COLLECTED**	**LOCATION**	**INFECTION**	**WEE**	**EE**	**WEE**	**EEE**	**SLE**
Garter snake (**T. r. haydeni**)	Sept. 27/62	Onion Lake Sask.	Exptl. with WEV (R_1)	2.75	Neg	Pos (1:80)	Neg (1:10)	
"	July 20/62	Milestone Sask.	"	1.76		Neg (1:10)		Neg (1:10)
"	"	"	"	1.76		Neg (1:10)		Neg (1:10)
Bull snake (**Pituophis Catenifer**)	May 21/62	Steveville Alta.	"	2.50	Neg	Neg (1:10)		Pos (1:20)
"	"	"	"	Neg	Neg	Neg (1:10)		Pos (1:20)
"	May 23/62	Estuary Sask.	"	2.50	Neg	Neg (1:10)		Neg (1:10)
Garter snake (**T. r. haydeni**)	May 14/62	Coleville Sask.	Natural	1.76		Pos (1:20)		Neg (1:10)
"	July 20/62	Milestone Sask.	"	Neg		Neg (1:10)		Neg (1:10)
"	Sept. 27/62	Onion Lake Sask.	"	1.26		Neg (1:10)		Neg (1:10)
Garter nsake (T. r. parietalis)	May 7/62	Estevan	"	Neg		Neg (1:10)		Neg (1:10)

1* Nine out of ten snakes SERA examined for RSSE, Powassan, C. E. Tick-borne and Tahyna viruses, in H1 test only, resulted negative.

tion, in addition to the serum neutralization test. Results are shown in Table X. Two bull snakes, collected in Dinosaur Provincial Park, Steveville, Alberta, were found to have positive titers of St. Louis Encephalitis (SLE) antibody. Tests (HI) for antibody against Russian spring-summer encephalitis (R.S.S.E.), Powassan, C.E. Tick-borne and Tahyna viruses were negative.

Discussion and Conclusions

In view of the data obtained, it is possible that reptiles and amphibians could develop specific antibodies as a result of WEE virus exposure, under natural and experimental conditions. The natural antibodies in reptiles and amphibians were detected only in restricted geographical areas in which WEE also occurred in horses, or where WEE was known to be endemic in Saskatchewan previously. In 15 out of 25 different localities the reptiles and amphibians collected and examined, were found free of neutralizing substances. This is inconsistent with the idea of the existence of non-specific neutralizing substances, in few randomly distributed individuals. Noteworthy is the fact that the same pattern of virus-antibody response which are known for warm blooded animals, were observed also in experiments done with reptiles and amphibians. It should be emphasized that neutralizing and HI antibodies were found only for WEE and SLE viruses, and none were detected for 5 other arboviruses.

There is no reason to believe that the presence, under natural conditions, of positive titers of WEE antibody in the blood of Saskatchewan snakes and frogs does not constitute evidence of WEE transmission to these animals. However, the mode of transmission of the infection in nature has not been clarified, and the part played by snakes and frogs in the ecology of WEE virus is not known. In general, it is to be anticipated that the natural ecological relationship will probably conform to patterns established in laboratory experiments.

Thomas and Eklund (4) have shown experimentally that the mosquito *C. tarsalis* can transmit the virus to garter snakes. If this mosquito is a vector in nature, transmitting the infection to snakes, then this could be one explanation for the positive findings in the Saskatchewan snakes in 1962-63. In 1962, *C. tarsalis* was a dominant species in the mosquito population of the province (13), and in addition yielded recoveries of WEE virus (2).

Most of the garter snakes were collected either at the time of leaving hibernacula in the spring or entering hibernacula in the fall. Very few snakes were collected during mid-summer when they remain hidden during the day in various natural retreats, or are able to elude capture in rank summer vegetation. Too few snakes were collected during the peak of *C. tarsalis* abundance, the time when they perhaps were most likely to have been recently bitten by infected mosquitoes. Consequently, failure to recover WEE virus from snakes was most likely the result of failure to time snake collecting with abundance of vectors.

Studies of experimental infections in garter snakes (3, 4, 5, 6) suggest these animals are likely reservoir hosts, capable of harbouring the virus over the winter. Our findings failed to confirm the possibility of overwintering; but in our experiments the snakes were not permitted to go into normal hibernation. Perhaps entering hibernation shortly after exposure is a condition necessary for the overwintering of the virus. In nature this would presume a fortuitous sequence and concurrence of events involving weather, vectors and snakes. In Saskatchewan in 1962, garter snakes were observed to be entering the hibernaculum at Onion Lake during the third week of September. While a few *C. tarsalis* continued to be taken in a light trap at Saskatoon until October 6, very few mosquitoes of any species were on the wing anywhere in the province after August 26.

In our experiments, garter snakes regularly developed a viremia that persisted for 10 to 12 days after inoculative exposure. This, in comparison to the viremia that develops in horses, or was found to develop in wild ducks (1), is of relatively long duration. In some snakes the duration was even longer. In Experiment 1, one snake still showed virus in its blood 30 days after exposure, while in Experiment 4, virus was recovered from a blood pool of new-born snakelings 66 days after experimental infection of the mother during pregnancy. Virus was not recovered from day-old snakes where exposure of the pregnant mother was only 11 to 35 days prior to parturition; but too few infections of

pregnant snakes were studied to establish conclusively whether transfer of infection requires maternal exposure very early in pregnancy.

In the experiment on infection of day-old snakes, contact controls remained uninfected. Thus, in this as well as other experiments no evidence was obtained to suggest that actively infected snakes shed virus into the environment where it was picked up and produced infection in contact snakes.

It was found possible to infect snakes by the oral route; but this was accomplished only in a few individuals, after oral instillation of a relatively large dose of virus or by inducing them promptly to drink water containing virus. It is difficult to visualize the occurrence of natural water with such high concentrations of virus. Frogs are the preferred food of garter snakes and it is fairly easy to visualize a garter snake eating a frog with viremia. In the laboratory garter snakes were observed to be devoid of a cannibalistic tendency. Sometimes a bigger snake did ingest a smaller one, when both simultaneously seized and started at opposite ends to swallow the same piece of food and could not break it in two or release their toothholds. We would suspect that this was strictly a laboratory conditioned phenomenon, the result of feeding rather tough strips of fish tissue and that it was not something likely to happen frequently under natural conditions. While infection following ingestion of virus is possible, its occurrence in nature is considered to be of relatively infrequent occurrence.

In the experiment in which adult snakes were infected repeatedly with relatively large doses of virus, high antibody levels and viremia developed. In two of the four snakes WEE virus had localized in the spleen and was recovered respectively on the 7th and 14th days after the last doses of virus. If more snakes had been used in the experiment it is anticipated that perhaps some individuals might have exhibited splenic localizations of longer duration. This seems to us to be not an extraordinary assumption—one snake was observed to manifest viremia for as long as 30 days and another to harbour virus for 66 days after exposure.

In our studies of experimental WEE infections, at no time did any of the garter snakes exhibit signs of disease. Along with their failure to show disease, experimentally they exhibited, as noted above, ability to support virus multiplication, to develop viremias of relatively long duration and high intensity, to localize the virus in the spleen, and to transmit the virus to the offspring. These observations encourage us to assume that garter snakes may be a natural vertebrate host of the virus.

The two principle species of garter snakes in Saskatchewan are the plains garter snake (*T. radix haydeni*) and the red-sided garter snake (*T. sirtalis parietalis*). Table I shows 18% of all red-sided garter snakes collected in nature with positive titers of antibody as compared to 2% of plains garter snakes. This difference perhaps gives a false impression. Most of the red-sided garter snakes were collected at Estevan, which is known to be in an area of high WEE virus activity. Plains garter snakes collected at Estevan, yielded an almost identical percentage of positives as red-sided garter snakes. Most plains garter snakes were collected in areas far to the north and west of Estevan where virus activity, except locally, was lower. Large numbers of negative plains garter snakes were taken at hibernacula in the extreme north west of the study area (e.g. Onion Lake) and inclusion of these lowered the percentage of positives found in the endemic area. However, we have not as yet studied more than a very few red-sided garter snakes collected in the normal geographic range of the species. The distribution of garter snakes in Saskatchewan is dealt with elsewhere (10).

Attempts to infect frogs experimentally with WEE virus during the summer months yielded indifferent results (Experiments 1 and 2); viremia was detected once, two days after subcutaneous infection; low positive titers of antibody were noted in a few frogs infected parenterally. However, 4 out of 6 frogs infected in late November and carried over winter to the following May showed positive titers of antibody.

In mid-July, 1962, frogs with suspicious titers of WEE antibody were collected at North Battleford and Beaver Creek. On August 31 of the same year, 20 frogs were collected at Elmore in extreme southeastern Saskatchewan; 14 had positive and 6 suspicious antibody titers. A few days later (September 4 to 7), 113 frogs were collected at Alsask, 16 of which were pos-

itive and 33 suspicious.

The findings indicate that frogs can be infected in the laboratory, and in addition become infected under natural conditions. How frogs acquire their infections in nature is not known. In the laboratory we were unable to induce *Culex tarsalis* to bite frogs. In early September, 1963, *Culex territans* was collected at two points in the province—Asquith and Lloydminister. This species is reported to feed on amphibians and snakes (14, 15) but we were unable to find any references in the literature to its potential as a vector of WEE. In view of the data obtained it seems possible to draw the following conclusions:

1. The presence of WEE antibodies in the blood of garter snakes may be taken as evidence of natural exposure to WEE virus in Saskatchewan.

2. Experimentally, garter snakes can be infected with moderate doses of WEE virus, as a result of which they develop an intense viremia of relatively long duration, without overt illness.

3. Experimentally, garter snakes and leopard frogs can be infected by the oral route, but less easily than when WEE virus is injected parenterally. This points to the possibility of a mode of exposure in nature by which frogs and snakes may develop WEE antibodies without vectors.

4. Experimentally, new born garter snakes were found to be more sensitive to WEE infection than adult snakes.

5. The possibility of virus and antibody transmission from pregnant snakes to their offspring was demonstrated.

6. Based on the presence of WEE antibody in frogs collected in nature and on the results of experimental exposure, amphibians cannot be excluded as vertebrate hosts of WEE virus.

7. Under laboratory conditions (at room temperature) we were not able to preserve WEE virus in reptiles and amphibians over the winter months and obtain the viremia stage in the early spring, except that a significant antibody response was noted 135 days after experimental exposure.

8. The successful WEE virus isolation from reptiles and amphibians in nature could give us more definite answers concerning cold-blooded animals as potential reservoir hosts for WEE virus and other arboviruses.

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