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THE DEVELOPMENT OF THE VIRUS OF YELLOW FEVER IN HAEMAGOGUS MOSQUITOES[1]

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I. INTRODUCTION

In a previous article (Bates and Roca-García, 1945a) we described the establishment of laboratory cycles of yellow fever virus using haemagogus mosquitoes and local monkeys. In these first experiments we observed that the infection of the mosquitoes seemed to depend on several factors, especially the amount of virus ingested by the mosquito and the environmental temperature at which the mosquitoes were maintained. More detailed studies of the factors governing virus establishment and development in these mosquitoes seemed to be warranted, and with this object in view continuous monkey-haemagogus transmission cycles, using a recently isolated virus strain, were maintained under laboratory control for a period of a year—fourteen complete mosquito-monkey cycles. A chart of these passages has been published in connection with a study of the behavior of the virus in the mammalian hosts (Bates and Roca-García 1946), and the serial position of any particular lot of infected mosquitoes can be determined by reference to this chart.

When we published our first paper on haemagogus transmission of yellow fever virus, we were still impressed by the technical difficulties of maintaining cyclic transmissions under laboratory control, and we concluded that special studies of virus behavior in mosquitoes would be most appropriately made by using as source animals monkeys inoculated with known amounts of virus. With more experience, we have reversed our opinion, and we now believe that a virus strain continuously maintained by "natural" cyclic passage provides the most satisfactory source of material for behavior experiments in either mosquitoes or mammals. The maintenance of a virus strain by this means cannot be undertaken as a casual or subordinate part of a laboratory program, but it is far from presenting insuperable difficulties if adequate facilities and personnel are dedicated to the project. It would probably be inadvisable to maintain more than one strain of a given virus by this means in a small laboratory because of the danger of possible confusion of material—always a very real hazard in virus experimentation. For this reason, our experiments have been largely made with a single strain of yellow fever virus, and it is thus impossible in some cases to be sure to what extent results are properties of yellow fever virus in general, and to what extent properties of the particular strain we used. The extent of variation among virus strains has been indicated in recent publications by work-

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ers in Brazil (e.g. Laemmert, 1944). In general, however, the various South American strains seem more similar to each other than any are to the African Asibi strain, which has been so extensively used in experimental studies of yellow fever; and we have observed nothing that would lead us to suspect that our Rodas strain was atypical.

As we have pointed out previously, the principal factors governing mosquito infection with virus seem to be (1) the characteristics of the virus strain, (2) the characteristics of the mosquito, (3) the virus dosage ingested, and (4) the environment of the mosquito, particularly the environmental temperature. We have tried to keep the virus strain factor constant, except for a few special experiments with possible modification through mouse brain passage. The mosquitoes used have in all cases been wild caught, and thus presumably represent a genetically diverse population. It seems very possible that a given wild population of haemagogus mosquitoes would include strains of varying virus susceptibility, but since we have not succeeded in establishing laboratory colonies of the Villavicencio species, it has been impossible to study this factor. The virus dosage factor is unquestionably of overwhelming importance in determining mosquito infection, but the lack of a precise method of quantitative estimation of the virus greatly complicates the study of this factor; this, and the effect of environmental temperature, have been the chief objects of our study.

The taxonomy of the mosquitoes of the genus *Haemagogus* is currently the subject of intensive study by several entomologists. The Villavicencio population, referred to in previous papers as *Haemagogus capricornii*, has been classified as a form of *Haemagogus spegazzinii* in a recent paper by Kumm, Osorno and Boshell (1946). The term "haemagogus" in publications from the Villavicencio laboratory may always be taken to refer to this specific population, since no other species of the genus seems to be represented in the region.

II. MATERIALS AND METHODS

The materials and methods used in the present series of transmission experiments have been described in some detail in a previous article (Bates and Roca-García, 1945a). Mosquitoes for mouse inoculation were invariably killed with potassium cyanide, since Waddell (1945) has found that this method has no injurious effect on the virus content of the insects. We used a large "cyanide jar" made up according to the method long used by butterfly collectors: a few lumps of potassium cyanide are placed in the bottom of a jar and covered with a layer of two or three centimeters of sawdust; a layer of plaster of Paris is poured over this, and the plaster is allowed to dry and harden by leaving the jar open for 24 hours (somewhere outside of the laboratory!). A jar made up in this fashion will continue to give off cyanide fumes for a year or so, and the mosquitoes can be quickly and conveniently killed by placing the tubes that contain them inside of the jar; if the tubes are plugged with cotton, this need not be removed. Our infected haemagogus were always maintained in small tubes plugged with wire gauze, and these were very easily handled in the large cyanide jar. We also use this method for killing mosquitoes brought in from the field

for identification, making a jar large enough to hold our small field cages. The method is more convenient and cheaper than the use of chloroform.

We have not calculated precise titers and dosages in terms of mouse m.l.d. in the present article. The results of adult mouse inoculations with Rodas virus are very similar to those obtained with the Perez strain (Bates and Roca-García, 1945a), and the calculation of definite titers on such results would seem to us to be an example of the fallacy of misplaced concreteness. We believe that a better idea of the amount of virus of these strains in circulation can be obtained by giving the final serum dilution causing adult fatalities; this is meant to serve as an indication of the order of magnitude of the virus titer. We made a series of parallel inoculations in baby and adult mice, and then tested the surviving adult mice with "challenge" inoculations of fixed neurotropic virus according to the method of Fox (1943). We could thus compare mortality and immunity in adult mice with mortality in baby mice that had received

TABLE 1

Parallel titration of Rodas virus in white mice of various age groups and in saimiri monkeys (serum of Saimiri 295, 4th day after infection by haemagogus of lot 284)

DILUTION OF SERUM	MOUSE MORTALITY (INTRACEREBRAL INOCULATION OF VIRUS),* AGE OF MICE			CIRCULATION OF VIRUS IN MONKEY (INTRAMUSCULAR INOCULATION, 0.03 CC.)	
	7 days	21 days	44 days	Monkey no.	Result.
1:10			3/6		
$1:10^2$			4/6		
$1:10^3$		5/6	1/6		
$1:10^4$		5/6	3/6	310	Positive
$1:10^5$	3/4	3/6	0/6	331	Positive
$1:10^6$	2/4	2/6	0/6	343	Negative
$1:10^7$	1/4	0/6	0/6	345	Negative
$1:10^8$	0/4	1/6	0/5	347	Negative

* Number dying over number inoculated.

identical inocula. The results were not consistent, in that adult mice frequently succumbed to challenge inoculations when the "baby" controls showed that the original inoculum had contained appreciable amounts of virus.

We have continued to find that "baby mice" (5 to 7 days old) give very regular results in titrations by intracerebral inoculation; and sound quantitative studies of virus behavior could be made using such animals. The results of a parallel titration in mice of various age groups and in saimiri monkeys were given in a previous article (Bates and Roca-García, 1945b, table 2), and the results of a similar but more complete titration are given in table 1 of the present article. We judge from this that 7-day-old mice on intracerebral inoculation are just as susceptible to virus of this strain as are saimiri monkeys on intramuscular inoculation; that the minimum lethal dose for 7-day-old mice corresponds very closely to the minimum infectious dose for saimiri monkeys. In the course of the experiments we have made many parallel titrations in baby

and adult mice. The end point with baby mice is very generally in the next tenfold dilution beyond the final dilution causing adult deaths; the titration reproduced in table 1 is unusual in this respect: we would have expected at least one adult death in the $1:10^5$ dilution.

We also made a series of parallel tests for virus by inoculating 5- to 7-day-old mice intracerebrally and 3-day-old mice subcutaneously. The results of the subcutaneous inoculations were almost identical with those of the intracerebral inoculations. We judge from this that baby mice are sensitive indicators of virus transmission by mosquitoes, as reported by Bugher (1941). It would perhaps be more satisfactory to use the subcutaneous rather than the intracerebral inoculation of baby mice for tests of the presence of virus in mosquitoes, since intercurrent mortality from contamination or toxic materials in the inoculum would be greatly reduced.

III. EFFECT OF TEMPERATURE ON VIRUS DEVELOPMENT

Constant temperature: 30°C.

The usual method of following the course of virus development in mosquitoes is by the inoculation of suspensions of pooled mosquitoes (Whitman, 1937). Since we found considerable variation in the virus content of individual mosquitoes, we decided to attempt to follow the history of the virus by the inoculation of suspensions of individual mosquitoes at regular intervals. The first such experiment was made with haemagogus of lot 181 which fed on Saimiri 176 on the 4th day after infection with rehydrated serum of Saimiri 99, the original animal of the Rodas series. The virus was thus 2 saimiri passages from man. The monkey circulated a considerable amount of virus, the serum on the 4th day killing 5/6 adult mice in $1:10^4$ dilution (the highest dilution tested). The mosquitoes were kept at a constant temperature of 30°C. Five were inoculated in parallel groups of baby and adult mice immediately after feeding; 5 others were treated in the same way at 24-hour intervals through the first 10 days, and at 48-hour intervals through the next 10 days. Seventy-five mosquitoes were thus inoculated separately in mouse groups in the course of the experiment.

The mouse mortalities from these inoculations are given in detail in table 2, and the percentage of mice killed by mosquito inoculation on each day is plotted in graph form in figure 1. It will be noted that virus was recovered from all mosquitoes after the 3rd day except for 2 inoculated on the 14th day, i.e., from 53 of 55 mosquitoes (96 per cent). It seems hardly fair to include these 2 negatives in the mean for the 14th day, so this is based on the 3 positives only. Only 3 mosquitoes were killed on the 18th day, and 2 on the 20th day; these are averaged and the result plotted for the 19th day. Five mosquitoes bit baby mice on the 12th day, but none transmitted; 2 of 3 that bit on the 14th day transmitted. The "extrinsic incubation period" in this experiment can thus be taken as 13 days.

The curve for these mouse mortalities seems very similar to the curve that one

TABLE 2

Mouse mortalities caused by the inoculation of individual mosquitoes in parallel groups of adult (45 days) and baby (5–7 days) mice at regular intervals after the infectious meal (Haemagogus of lot 181 infected on Saimiri 176 and maintained at 30°C.)

DAYS AFTER INFECTIOUS MEAL	MOUSE MORTALITIES (A = ADULT MICE; B = BABIES)										TOTAL MORTALITIES (IN PER CENT OF MICE)	
	A	B	A	B	A	B	A	B	A	B	Adults	Babies
0	2/5	5/5	3/6	4/4	4/6	5/5	3/5	5/5	3/6	4/4	50	100
1	0/6	1/5	2/5	5/5	3/6	3/5	1/6	5/5	0/3	0/5	23	56
2	1/5	2/5	0/6	0/0	0/4	0/0	0/6	1/5	0/6	2/5	4	33
3	1/6	5/5	1/6	5/5	0/6	0/5	4/5	5/5	0/6	3/5	20	64
4	0/5	3/5	2/5	3/5	1/5	3/5	1/6	5/5	2/6	4/4	22	75
5	3/5	5/5	2/5	5/5	2/5	5/5	2/5	5/5	0/6	4/5	36	96
6	0/6	1/4	1/6	5/5	5/6	4/4	0/6	2/5	2/6	5/5	24	74
7	0/6	4/5	0/6	1/5	1/6	3/5	0/6	5/5	1/6	5/5	7	72
8	2/6	5/5	2/6	5/5	2/6	5/5	2/6	5/5	2/5	5/5	38	100
9	3/6	5/5	3/6	5/5	4/6	5/5	1/6	5/5	4/6	5/5	50	100
10	3/6	3/3	2/6	4/4	6/6	5/5	1/6	4/5	2/6	5/5	47	96
12	4/6	5/5	4/6	5/5	2/6	5/5	1/5	5/5	3/6	5/5	48	100
14	3/6	4/4	0/4	0/4	2/6	4/4	4/5	5/5	0/6	0/5	(53)	(100)
16	4/6	5/5	3/6	4/4	3/6	5/5	3/6	4/5	6/6	5/5	63	96
18	5/6	5/5	4/6	5/5	6/6	4/4						
20	4/6	5/5	3/6	4/5							72	96

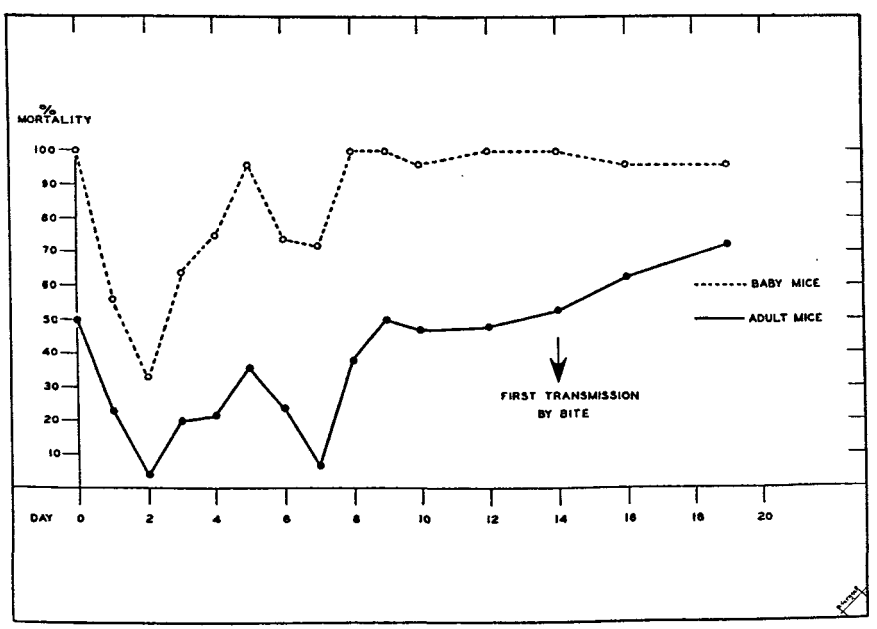

FIG. 1. MOUSE MORTALITIES FROM INOCULATION OF INDIVIDUAL MOSQUITOES. HAEMAGOGUS OF LOT 181, CONSTANT TEMPERATURE 30°C.

would expect for virus development in the mosquitoes, and it seems to be an index of the average amount of virus contained in the mosquitoes. The amount of virus measurable by this means has a definite limit: reached for baby mice on the 8th day, and by extrapolation for adult mice on about the 26th day. We have not been able to devise a satisfactory method of interpreting these mouse mortalities in terms of titer, even when the "average survival time" of the mice is taken into account: there are too many variable factors. Apparently there

TABLE 3

Titration of pools of 5 mosquitoes each (Lot 234, infected on Aotus 9, kept at 30°C.)

DAY	MOUSE MORTALITIES (5-TO-7-DAY-OLD MICE)				
	Pure	1:10	$1:10^2$	$1:10^3$	$1:10^4$
0	4/4	4/4	3/4	0/4	0/4
2	1/2	2/4	0/4		
4	4/4	1/4	0/4	0/4	
6	4/4	0/4	0/4	0/4	0/4
8	4/4	4/4	1/4	1/4	0/4
10	4/4	0/4	0/4	0/4	0/4
12	2/3	1/4	0/4	0/4	0/4
14	3/4	0/4	0/4	0/4	0/4
16	4/4	4/4	1/4	0/4	0/4
18	4/4	3/4	0/4	0/4	0/4
20	4/4	2/4	1/4	0/4	0/4
36	4/4	4/4	4/4	3/4	2/4

TABLE 4

Titration of pools of 5 mosquitoes each (Lot 289, infected on Saimiri 342, kept at 30°C.)

DAY	MOUSE MORTALITIES (5-TO-7-DAY-OLD MICE)					
	Pure	1:4	1:16	1:64	1:256	1:1024
0	4/4	4/4	4/4	3/4	1/4	1/4
2	0/4	0/4	0/4	0/4	0/4	
4	4/4	1/4	1/4	0/4	0/4	
10	4/4	1/4	0/4	0/4	0/4	
20	4/4	4/4	4/4	3/4	3/4	0/4

is a steady loss of virus during the first 48 hours after the infectious meal, but after 48 hours the virus has become established and starts to multiply. This multiplication is very slow, as compared with virus multiplication in the mammalian host. Transmission may simply represent a certain threshold of virus concentration in the mosquito, reached at 13 days in the case of this particular experiment.

We made several attempts to measure the rate of growth of virus in mosquito tissue by means of titrations of pools at regular intervals. To make the results comparable with those obtained from the inoculation of individual mosquitoes,

the pools were prepared by grinding 5 mosquitoes in 2.5 cc. of diluent (in our other experiments individual mosquitoes were ground in 0.5 cc. of diluent each) and this suspension was taken as the starting point in making serial dilutions. The results of 2 such titrations are given in accompanying tables: in table 3 those of lot 234, tested in tenfold dilutions; and in table 4 those of lot 289, tested in fourfold dilutions. The source animals for both of these experiments were circulating moderate amounts of virus: in the case of Aotus 9, the final serum dilution killing adult mice was $1:10^5$ (3/6 mortality); in the case of Saimiri 342, $1:10^4$ (4/6 mortality). The irregularity of the titration results is probably due to the considerable variation in virus content of the mosquitoes, the sample of 5 specimens for each pool not being large enough to eliminate this factor.

The titration experiments agree with the experiments involving individual mosquitoes in that there is a rapid loss of virus in the first 48 hours, followed by a slow gain. The gain is so slow during the first 10 days that it is hardly measurable in terms of titer. Yet the ultimate concentration of virus in the mosquitoes may be considerable, as shown by the results obtained with pools of 20- and 36-day-old mosquitoes in these experiments.

Constant temperature: 20°C.

In experiments reported in a previous paper (Bates and Roca-García, 1945a) we failed to recover virus from 10 mosquitoes kept for 22 days at 20°, though virus was recovered from 60 per cent of a parallel lot kept at 30°. Once virus was established in the mosquito, however, exposure to 20° seemed to have no adverse effect. In our first experiment using Rodas virus at 20°, we kept a group of mosquitoes for 10 days at this temperature and then transferred them for 10 more days to a constant temperature of 30°; 10 mosquitoes inoculated separately into mice after this treatment all showed virus. A parallel group kept at 30° from the beginning were also 100 per cent infected, and it is probable that these mosquitoes had ingested a considerable amount of virus: serum of the source saimiri killed 3/6 adult mice in the $1:10^5$ dilution, the highest tested.

We then arranged an experiment with haemagogus lot 197 from which we hoped to determine the rate of loss of virus at 20°, what happened to virus in mosquitoes kept constantly at 20° (did it finally die out, or multiply slowly) and the rate of development of virus in mosquitoes transferred at 10 days from a temperature of 20° to one of 30°. The mosquitoes of this lot fed on Aotus 2 on the 4th day after infection; the animal must have been circulating a tremendous amount of virus, since serum diluted $1:10^7$ (the highest dilution tested) killed 4/5 adult mice. The "dosage factor" in this experiment would thus be optimum. A parallel group kept constantly at 30° transmitted virus to Aotus 3 ten days after the infectious meal—the shortest incubation period we have encountered.

Five mosquitoes were inoculated separately into mice at 24 hour intervals for the first 5 days; at 10 days 5 more mosquitoes were tested, and the survivors were then divided into 2 lots: 1 lot was transferred to a constant temperature of

TABLE 5

History of virus recovery from mosquitoes of Lot 197 (Haemagogus capricornii *infected on Aotus 2, 4th day*)

DAY	MORTALITY			
	Adult mice*	Per cent	Baby mice	Per cent
Mosquitoes kept at a constant temperature of 20°C.				
0	25/27	93	23/23	100
1	13/27	48	20/24	83
2	13/25	52	19/24	79
3	4/23	17	12/23	52
4	8/28	29	16/23	70
5	1/29	3	7/23	30
10	2/22	9	16/25	64
22	1/30	3	11/20	55
Mosquitoes transferred from constant temperature of 20°C. to constant temperature of 30°C. on 10th day				
12	8/26	31	18/25	72
14	23/28	82	20/23	87
16	16/29	55	24/24	100
18	17/28	61	23/23	100
20	20/29	69	19/19	100
22	21/25	84	18/18	100

* These represent the sum of the mortalities from five mouse groups inoculated on a given day with suspensions of individual mosquitoes.

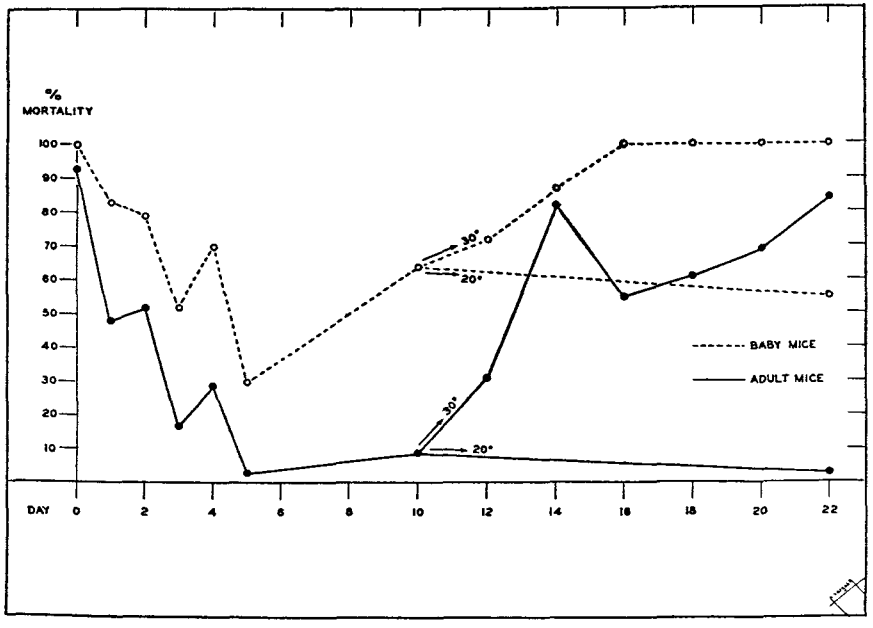

FIG. 2. MOUSE MORTALITIES FROM INOCULATION OF INDIVIDUAL MOSQUITOES OF LOT 197, AT CONSTANT TEMPERATURE OF 20°C. UNTIL 10TH DAY, WHEN PART OF THE GROUP WAS TRANSFERRED TO A CONSTANT TEMPERATURE OF 30°C.

30°, and 5 individuals were tested for virus at 48 hour intervals for the next 12 days; the others were left at 20°, and 5 were tested after 22 days to see whether virus was still demonstrable. The results of this experiment are summarized in table 5; since the mosquitoes were 100 per cent infected, there seems no point in giving the individual results. The mouse mortalities are plotted in graph form in figure 2.

From this experiment, it seems that virus dies out slowly in mosquitoes kept at 20°, reaching a minimum concentration on about the 5th day. It apparently persists at this minimum level indefinitely, since the mosquitoes inoculated on the 5th, 10th and 22nd days showed very similar mouse mortalities. One can imagine that if the original virus dosage ingested by the mosquitoes had been smaller, the virus would have dropped below the threshold detectable with mice and persisted at that level; or that with an even smaller dosage, the virus would have died out during the first 5 days without becoming established in the mosquito at all. When the mosquitoes were transferred to a temperature of 30°

TABLE 6

Recovery of virus from haemagogus of Lot 298, kept at a constant temperature of 25°C. (per cent mortality from 5 groups of mice inoculated with suspensions of individual mosquitoes on a given day)

	DAY					
	0	2	4	6	8	10
Per cent mortality in 7-day-old mice.....	100	83	76	95	88	86
Per cent mortality in 45-day-old mice.....	97	4	0	19	30	33

the virus started multiplying, at a rate closely comparable to that shown by lot 181 (fig. 1). These mosquitoes were allowed to feed on baby mice before being killed. There were no transmissions until the 16th day (6 days at 30°) when 2 of the 5 mosquitoes transmitted; 3 of 5 transmitted on the 18th day.

Constant temperature: 25°C.

Two experiments were made in which mosquitoes kept at a constant temperature of 25° were tested individually at regular intervals for the presence of virus. The results of the first of these (lot 208, infected on Aotus 3) are summarized in table 6. These mosquitoes ingested a very large virus dose (the aotus serum killed 3/6 adult mice in the dilution $1:10^8$, the highest tested), and the mosquitoes were 100 per cent infected. Virus apparently reached its minimum level in the mosquitoes on the 4th day.

A more detailed experiment was carried out with lot 246, infected on Aotus 11 at a time when the animal was circulating a more moderate amount of virus (2/5 adult mouse mortality with serum diluted $1:10^5$). Five mosquitoes were tested daily for the first 10 days, and at 48-hour intervals for the next 10 days. The results are summarized in table 7. In this case virus was recovered from all of the mosquitoes during the first 2 days, but recovery was more irregular

TABLE 7

History of virus recovery from daily inoculation of 5 mosquitoes of Lot 246, kept at constant temperature of 25°C.

DAY	NUMBER OF MOSQUITOES SHOWING VIRUS	MOUSE MORTALITIES*			
		Adults	Percentage	Babies	Percentage
0	5	17/27	63	20/20	100
1	5	26/30	87	20/20	100
2	5	0/24	0	8/16	50
3	2	3/21	14	4/17	23
4	1	0/28	0	1/18	6
5	1	2/28	7	2/18	11
6	5	2/28	7	10/20	50
7	2	0/30	0	4/20	20
8	5	17/29	59	19/20	95
9	5	12/30	40	19/20	95
10	4	5/23	22	12/15	80
12	4	16/24	67	16/16	100
14	5	11/28	39	19/20	95
16	5	20/30	67	18/19	95
18	2	10/12	83	8/8	100
20	3	11/17	65	11/12	92

* For the 10th day and after, these are based only on mouse groups that included infected animals: i.e., that had been inoculated with mosquitoes known to be infected.

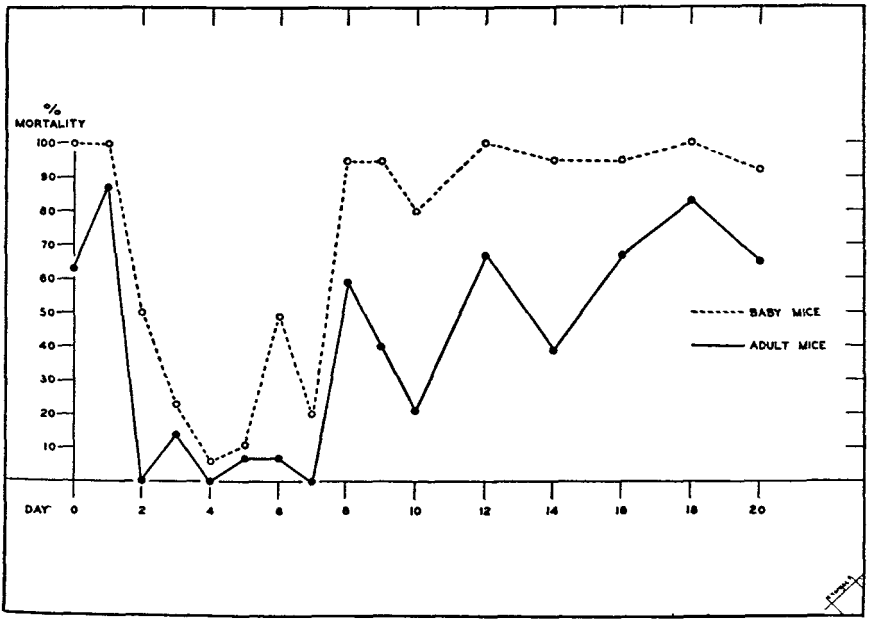

FIG. 3. MOUSE MORTALITIES FROM INOCULATION OF INDIVIDUAL MOSQUITOES OF LOT 246, KEPT AT CONSTANT TEMPERATURE OF 25°C.

subsequently. From the 10th day on, when one would expect the virus in all mosquitoes to have passed above the threshold detectable on mouse inoculation, only 23 of the 30 mosquitoes showed virus (77 per cent). It seems likely that the rest of these mosquitoes had failed to become infected, and in consequence mouse mortalities for this period have been based only on the positive mosquitoes, with the hope of thus giving a better idea of the rate of virus development after establishment in the mosquito. These results are presented in graph form in figure 3. Again the minimum virus level is reached on the 4th day. Unfortunately, no attempt was made to determine the onset of transmission with these mosquitoes.

TABLE 8

Mouse mortalities from inoculation on alternate days of 5 mosquitoes from Lot 278, kept at 25°C. for 20 hours and 35°C. for 4 hours daily

	DAY						
	0	2	4	6	8	10	12
Per cent mortality in baby mice.....	100	78	84	100	70	70	100
Per cent mortality in adult mice.....	76	35	55	50	50	57	65

Alternating temperature: 25°–35°C.

Results are summarized in table 8 of an experiment with mosquitoes of lot 278, which were kept for 20 hours daily at 25° and for 4 hours daily at 35°. These were infected on Oedipomidas 3 at a time when the animal showed a high titer of circulating virus (2/6 adult mice killed by serum dilution $1:10^7$). Results are very similar to those obtained with a constant temperature of 30°, in that the minimum concentration of virus appears to be on the second day after the infectious meal.

IV. THE DOSAGE FACTOR

It is impossible to give a precise evaluation of the dosage factor in these experiments because of the uncertainty of virus titrations and because, in most cases, the number of mosquitoes tested for virus was not large enough to be given statistical significance. A survey of the whole series of experiments, however, gives certain clear impressions that seem to be valid. The experiments are not uniform enough for concise tabular summary from this point of view, and perhaps the best method of review is by the citation of examples. The source animals can conveniently be classified into four groups according to the apparent titer of virus in circulation; haemagogus maintained at a constant temperature of 30° show the following behavior for these four categories of virus dosage:

Trace of virus (serum of source animal not infecting adult mice in dilutions greater than 1:10). We have no evidence that haemagogus ever become infected under these circumstances. The best example is lot 204, which fed on Cebus 10 on the 5th day after infection, when the 1:10 serum dilution caused

fatal infection in 2/5 adult mice. No virus was recovered at 20 days from the total suspension of a pool of 11 surviving mosquitoes inoculated subcutaneously in a saimiri monkey.

Small amount of virus (serum of source animal not infecting adult mice in dilutions above $1:10^3$). In this category, virus is recovered from an occasional specimen; we have no instance of transmission by bite, but attempts were not made after more than 18 days and the incubation period of the occasional infected haemagogus would presumably be prolonged. Examples: Lot 193, infected on Saimiri 182, serum dilution 1:100 causing 1/5 mouse mortality; 1 of 6 mosquitoes showed virus at 17 days. Lot 241, infected on Metachirus 238, serum dilution 1:100 causing 2/6 mouse mortality; 1 of 10 mosquitoes showed virus at 17 to 29 days. Lot 189, infected on Saimiri 180, serum dilution 1:1000 causing 1/6 mouse mortality; 1 of 11 mosquitoes showed virus at 15 days. Lot 174, infected on Saimiri 108, serum dilution 1:1000 causing 2/6 mouse mortality; 3 of 7 mosquitoes showed virus at 14 to 18 days.

Moderate amount of virus (serum of source animal causing mouse infections in $1:10^4$ and $1:10^5$ dilutions). In this category, virus is recovered from a majority of the mosquitoes and the minimum incubation period is 13 days. Most of the infections of haemagogus on saimiris fall in this class.

Large amount of virus (serum of source animal causing mouse infections in the dilution $1:10^6$ or more). These titers of circulating virus occur occasionally with saimiris, more frequently with aotus. We have always recovered virus from over 90 per cent of the haemagogus infected on such animals, and one is tempted to think that the occasional specimen not showing virus represents a mistake made somewhere in the course of the experiment. The incubation period may be as short as 10 days.

In short, the percentage of mosquitoes showing virus is a function of the amount of virus in circulation in the source animal at the time of feeding, other factors (virus strain, environmental temperature) being constant. In the case of experiments in which only a proportion of the mosquitoes show virus, it would be interesting to know whether the negative mosquitoes are really completely uninfected, or whether their virus content is below the threshold detectable on mouse inoculation. We made two attempts to check this by inoculating saimiri monkeys with the total suspension obtained by grinding individual mosquitoes, on the theory that if any virus was present in such a mosquito the monkey would become infected. In both cases we tested mosquitoes that had been maintained at 25° after feeding on saimiris circulating moderate amounts of virus, since as a general rule only about 50 per cent of the mosquitoes show virus on mouse inoculation under such circumstances. In the first instance 10 mosquitoes were tested in mice at 20 days, and virus was recovered from 7; 4 mosquitoes were then tested at 28 days in saimiris, and all 4 saimiris were infected. In the second instance, 10 mosquitoes were tested in mice at 24 days, and virus was recovered from 6; 5 were then tested at 39 days in saimiris, and 4 of the 5 saimiris became infected. Thus in both cases a higher proportion of the mosquitoes showed virus on saimiri inoculation than did on

mouse inoculation, but the numbers are so small that this result could be accounted for on a chance basis. The failure of 1 saimiri to become infected shows that, in some cases at least, the mosquitoes are probably completely free of virus. In some cases, however, failure to recover virus from mosquitoes on mouse inoculation surely means simply that the virus is below the threshold detectable by this means.

It is interesting to speculate as to why certain individual mosquitoes in a given experiment become infected, or show demonstrable virus, while others do not. The most likely explanation would be the varying amounts of virus ingested by the mosquitoes at the time of feeding: those engorging fully would acquire a larger absolute number of virus particles than those taking less blood. This does not, however, seem to be the entire explanation. In one experiment, we divided the mosquitoes into 2 lots after they had fed. One lot included only specimens whose abdomens had become distended with blood; the other, specimens in which the abdomen had not obviously become distended. After 22 days at 30°, 10 specimens of each group were tested in mice and virus was recovered from 9 specimens in each case: the mouse results were closely similar in both cases, the positive mosquitoes showing large amounts of virus.

It seems entirely possible that the factor of mosquito strain enters in such cases: that some individual haemagogus are more susceptible to infection than others. Where an overwhelming dosage is ingested and the mosquitoes are kept under optimum temperature conditions, they all become infected; but where the virus dosage is smaller, and the temperature conditions less favorable, individual differences in susceptibility can enter in. This hypothesis could be tested with mosquito species that can be maintained as laboratory colonies so that genetic lines could be separated out. This would be analogous with the situation described by Huff (1934) for *Culex pipiens* and plasmodium infection.

V. THE VIRUS STRAIN FACTOR

Our success in maintaining laboratory transmissions of yellow fever virus with the Perez and Rodas strains of virus contrasted strongly with the partial or complete failure of our experiments during previous years. Undoubtedly, two important factors in the later success were the maintenance of the mosquitoes at relatively high temperatures, and the improved entomological techniques that resulted in a greater mosquito longevity. We thought, however, that the use of new virus strains might have been an additional factor. Our early work was carried out with strains that had been subject to considerable laboratory manipulation; they had, in particular, been passaged several times through mouse groups, and had been maintained for prolonged periods in a desiccated state. We thought it worth while to check the possible effect of mouse brain passage on virus infectivity for mosquitoes.

We made 3 separate series of experiments in which "pantropic" virus of the regular haemagogus cycles was passaged through 6 mouse groups and then inoculated into a saimiri, which was used as a source animal for haemagogus infections. The virus acquired a "fixed" character in the first 3 or 4 brain

passages, the mouse mortality becoming regular, with death on the 6th and 7th days, in contrast with the irregular mortality and prolonged incubation period of the unmodified virus.

Two such experiments were made with Perez virus and 1 with Rodas virus. The mosquitoes were kept at 30° in all cases. In the first experiment 62 haemagogus fed on the saimiri, and 25 of these were tested individually for virus from 12 to 29 days later, without virus being recovered in any case. The titer of virus in circulation in the monkey must have been low, as the mosquito "controls" (inoculated directly after engorging) infected only about half of the test mice: the titration of the monkey's serum was incomplete. Still it seemed remarkable that virus was recovered from none of the 25 mosquitoes.

Data on the second experiment are more complete. Perhaps the best method of summary is by comparing the results of the experiment with mouse adapted virus (Lot 176) with those of an experiment with unmodified virus (Lot 172) that was carried out at the same time under the same conditions.

Serum titrations of the source monkeys were:

Serum dilutions	1:10	10^2	10^3	10^4	10^5
Saimiri 108 (unmodified virus)	4/6	1/6	3/5	3/5	0/6
Saimiri 120 (mouse adapted virus)	6/6	6/6	5/6	1/6	0/6

Adult mouse mortalities from control mosquitoes:

Lot 172 (unmodified virus)	3/6	3/5	4/5	3/6
Lot 176 (mouse adapted virus)	5/6	3/6	5/6	

Transmission to baby mice, 13 days and more after infectious meal:
Lot 172 (unmodified virus): 7 fed, 4 transmitted
Lot 176 (mouse adapted virus): 7 fed, none transmitted

Recovery of virus by mouse inoculation:
Lot 172 (unmodified virus): 18 of 20 mosquitoes showed virus (90 per cent)
Lot 176 (mouse adapted virus): 1 of 12 showed virus (8 per cent)

The problem here is to decide how much the difference in the behavior of the 2 mosquito groups is due to dosage ingested, and how much to difference in virus strain. The 2 titrations show nicely the difference in behavior between unmodified and modified virus: if we had an objective method of measurement, it would probably be found that Saimiri 108 was circulating at least 10 times as much virus as Saimiri 120; and the difference falls on the dividing line between our categories of "small" and "moderate" amounts of virus, which seems to be very significant in governing haemagogus infection, as pointed out in the preceding section.

In the 3rd experiment, 161 haemagogus fed on a saimiri infected with modified Rodas virus (6 brain passages). Serum of the source monkey infected 4/6 adult mice in the $1:10^3$ dilution, 0/6 in the $1:10^4$ dilution. It thus again was on the borderline between the categories of "small" and "moderate" virus amounts. In this experiment we attempted to follow the history of the virus by the technique used in the temperature experiments, testing 5 separate mos-

quitoes daily for virus on the first 10 days, and on alternate days thereafter. The virus disappeared rapidly, only 1 mosquito being positive after 24 hours. After the 5th day, virus was recovered from 9 of 57 mosquitoes (16 per cent), positive mosquitoes showing a completely random distribution in time. Twenty-one attempts at transmission by bite to baby mice were made between the 12th and 23rd day. Two mosquitoes transmitted, both on the 21st day; both showed considerable virus on mouse inoculation (infecting 6/6 adult mice). It is interesting that the virus continued to show enhanced neurotropic properties even after monkey and mosquito passage, since the incubation period in test mice was generally much shorter, and mortality more regular, than in the case of unmodified virus.

From these 3 experiments one conclusion can surely be drawn: that it would be very difficult if not impossible to establish cyclic transmissions with haemagogus mosquitoes using a strain of virus that had been passaged several times through mouse brain. Saimiri monkeys infected with such virus show relatively low titers of virus in circulation (even though the infections are usually fatal). With the evidence at hand, we cannot say how much the low infection rate of haemagogus is due to this dosage factor, and how much to modification of the adaptive properties of the virus that enable it to invade the mosquito host, though we have the impression that both factors are probably involved.

VI. VIRUS TRANSMISSION

Transmission experiments with yellow fever (and other viruses) have generally been made by allowing a group of mosquitoes to feed on a susceptible host, so that there is no way of knowing how many of the mosquitoes were actually infective. The susceptibility of baby mice to subcutaneous inoculation provides an animal that can be used for testing transmission by individual mosquitoes, as pointed out by Bugher (1941). Our first experiments (Bates and Roca-García, 1945a, table 2) indicated that there might be considerable variability in the infectiveness of individual haemagogus mosquitoes, and we consequently planned a more detailed study of this phenomenon. A series of parallel tests indicated that the susceptibility of baby mice to our strains of virus by the subcutaneous and intracerebral inoculation routes was closely similar, so that it seems probable that these mice are sensitive indicators of transmission. The chief difficulty in handling baby mice is that sick individuals are sometimes eaten by the mother. We have had no losses except where there has been strong reason to presume that the baby was infected; nevertheless such cases have not been counted as "transmissions" in crucial experiments. In the vast majority of cases, including all crucial experiments (e.g., defining minimum incubation periods), the sick baby mouse has been recovered and brain material passaged to confirm the nature of the infectious agent.

As was pointed out in the section on "dosage" above, the amount of virus originally ingested by the mosquitoes has considerable influence on the mosquito incubation period. Studies of the effect of temperature on the time required for transmission are thus most satisfactory if carried out with parallel

lots of mosquitoes infected on a given host at the same time. Our best experiment of this sort was based on a group of haemagogus that fed on Aotus 15 at a time when considerable virus was in circulation (serum killing 4/5 adult mice in the dilution $1:10^6$, the highest tested). These mosquitoes were divided at random into 5 lots of 44 mosquitoes each: 3 lots were kept at constant temperatures of 25°, 30° and 35° respectively; 1 for 20 hours daily at 25° and 4 hours daily at 30°; and the last for 20 hours daily at 25° and 4 hours daily at 35°. The results are summarized in table 9.

In experiments in which a group of mosquitoes feed on a susceptible host, the first transmission would occur when the first mosquito became infective: even though 20 mosquitoes feed, it may be that only one is infective at that time. There seems, in fact, to be considerable individual variation in the time required for a mosquito to become infective, and in all of our experiments, we have observed that the longer the elapsed time, the higher the proportion of infective mosquitoes. One case will suffice to illustrate the phenomenon. The haema-

TABLE 9

Transmission to baby mice by haemagogus (infected on Aotus 15 and kept under different temperature conditions)

LOT NUMBER	TEMPERATURE	TOTAL NUMBER OF FEEDINGS	DAY OF FIRST FEEDING	FIRST TRANSMISSION	TOTAL NUMBER OF TRANSMISSIONS
				days	
271	25°	58	15	28	4
272	25°–30°	59	10	23	14
273	25°–35°	54	10	12	12
274	30°	18	10	10	13
275	35°	4	6	none	

gogus of lot 277, infected on Oedipomidas 3 and maintained at 25° for 20 hours daily and 35° for 4 hours daily, were kept for 60 days, during which time 84 tests of transmission were made with baby mice. The results, grouped in 5-day periods, are summarized in table 10. The first transmission occurred on the 12th day after the infectious meal, but the majority of the mosquitoes did not transmit until the 20th day or later. In this case, in a parallel lot kept at a constant temperature of 30°, the first transmission was also on the 12th day.

We kept records on individual mosquitoes in all of these experiments, so that it is possible to follow the history of a given individual. The selection of the mosquitoes for feeding tests was generally random—the mosquitoes frequently showed no desire to bite, and they would be tested with a given baby mouse until an individual was found that would insert its proboscis. If a given mosquito probed a baby mouse, the act was regarded as a "transmission attempt" regardless of whether blood was drawn or not. Thus transmission records are frequently available on a given individual mosquito over considerable periods of time. Once an individual becomes infective, it remains infective for life. Thus mosquito no. 39 of lot 272 failed to transmit at 12 and 21 days; but it

transmitted at 23, 24, 25, 26, 28 and 32 days. Mosquito no. 18 of lot 277 transmitted at 12, 24, 32, 33, 34, 36 and 37 days—every occasion on which it was tested. Very rarely a mosquito that had once transmitted failed to infect a baby mouse on some subsequent occasion: thus mosquito no. 6 of lot 277 failed to transmit at 16, 23, 26, 29 and 32 days, but transmitted at 34, 36, 37, 48 and 50 days; at 51 days it is recorded as biting a baby mouse without transmitting. We can only find 2 other such instances in the many hundreds of transmission attempts.

VII. DISCUSSION

From the experiments reported in the present paper it appears that the history of the yellow fever virus in the mosquito falls into two periods—a period of virus loss and a period of virus gain. This is in accord with what would be expected on theoretical grounds, and with various previously published experiments, notably those of Whitman (1937). Whether the mosquito becomes infected or not apparently depends on the events of the period of virus loss, which are presumably centered in the gut of the mosquito: it is probably a question of whether the virus can become established in the mosquito tissue (i.e., penetrate the gut wall) before it dies or has been eliminated. With a given mosquito strain and a given virus strain, the course of events in the period of virus loss seems to be governed by two variable factors: the amount of virus originally ingested by the mosquito and the environmental temperature. The more virus, the better the chance that some will get established in the mosquito tissues. The higher the temperature (within the range tested), the faster the course of events; and apparently also, the better the chance that some virus will get established.

The period of virus loss apparently lasts for 2 days at 30°, 3–4 days at 25° and 5 days at 20°. The virus particles in the gut would be in a non-living suspension, so that there would presumably be no multiplication; the loss in virus during this period would thus seem to reflect the rate of death of virus particles at these temperatures and in this medium. It is curious, under these circumstances, that the proportion of mosquitoes becoming infected with a given dosage and given virus strain should depend directly on temperature. Evidence on this point was presented in a previous paper (Bates and Roca-García, 1945a), and later experiments, especially those involving moderate virus dosages, show the same phenomenon. The phenomenon is not confined to yellow fever virus and haemagogus mosquitoes. Milzer (1942) found that *Aëdes agypti* did not become infected with the virus of lymphocytic choriomeningitis at temperatures below 26°, and it seems likely that many of the contradictory results in published insect-virus experiments stem from differences in the temperature factor. The increased likelihood of infection at higher temperatures may be a property of the mosquito, the virus or both: it may reflect an increased permeability of the mosquito gut at higher temperatures, or an increase in some activity property of the virus particles themselves.

In the period of virus gain, the relationship between rate of virus multiplica-

tion and temperature is surely simply another example of the influence of temperature on the speed of biological processes, but the data are too incomplete to warrant any detailed analysis from this point of view. It seems to us that infectiveness (ability to transmit) in the mosquito may be threshold phenomenon, dependent on the level of concentration of virus in the mosquito. Such evidence as there is suggests that yellow fever and other viruses show no tissue specificity in the mosquito host (Davis and Shannon, 1930; Merrill and Ten-Broeck, 1934), though this is a problem that has hardly been investigated in sufficient detail. The appearance of virus in the salivary secretion of the mosquito might then be dependent simply on the total virus content of the insect. This would explain the great variability in the "incubation period" (time between the infectious meal and the first infective meal) in individual mosquitoes of the same lot (e.g., the data in table 10).

TABLE 10

Transmission by bite to baby mice (haemagogus of Lot 277, infected on Oedipomidas 3 and maintained at 25°–35°C.)

DAYS	NUMBER OF FEEDINGS	NUMBER OF TRANSMISSIONS	PER CENT TRANSMITTING
11–15	20	3	15
16–20	17	2	13
21–25	12	7	58
26–30	11	6	55
31–35	12	10	83
36–60	12	11	92

The titrations of virus content of *Aëdes aegypti* reported by Whitman (1937) seemed to show that the amount of virus in the mosquito did not increase indefinitely—that after an initial period of regular increase it was subject to considerable and irregular variation. If this phenomenon occurs in haemagogus, it would not be demonstrable from our experiments, since we did not make many titrations with long-infected mosquitoes.

At 20° there seems to be no demonstrable increase in the virus content of the mosquito over a period as long as 22 days (data in table 5) and it is likely that mosquitoes would never become infective at this temperature. At the other extreme, at a constant temperature of 35°, experiments were not satisfactory because of the high mortality of the mosquitoes, the majority dying within the first few days: it was also difficult to induce mosquitoes kept at this temperature to feed. Twenty-eight transmission attempts were made with 3 different lots, from 5 to 12 days after infection, with a possible transmission once at 12 days (not confirmed by passage). In some cases mosquitoes failed to transmit after 10 days at 35°, when transmission was obtained from parallel lots kept at 30°. At constant temperatures of 25° and 30° the minimum incubation periods observed have been 28 days and 10 days respectively. These results, it may be noted, differ considerably from those usually reported for *Aëdes aegypti* and the Asibi strain of virus (Davis, 1932), being much longer. Davis also was able to

carry out experiments at temperatures above 30°, obtaining transmission after 4 days at 36°. From our data it is impossible to determine how much the lengthening of the incubation period is a property of the mosquito species and how much a property of the virus strain, though probably both are involved, as was pointed out in our first article.

We were greatly interested in carrying out experiments at varying temperatures, since temperature conditions in the forest canopy, where haemagogus are found most abundantly, are subject to wide diurnal fluctuations. It is interesting in this connection that virus development in mosquitoes kept for 20 hours daily at 25° and 4 hours daily at 35° was almost as rapid as in mosquitoes kept at a constant temperature of 30°. The difference between 4 hours daily exposure to 30° and 4 hours daily exposure to 35° is particularly striking (table 9; other experiments gave similar results). The difference in "mean temperature" between these is slight, since the daily mean calculated by hours in the one case would be 25.8° and in the other 26.6°. The accelerating effect on virus development must be due to the short daily exposure to the (for a mosquito) very high temperature of 35°.

We have made a number of experiments designed to study the effect of various temperature conditions on the longevity, speed of development and physiology of haemagogus mosquitoes. These will be written up separately. Of interest in the present connection is the fact that the 20 hours daily exposure to 25° and 4 hours daily shift to 35° seems to be very favorable to the mosquitoes. The longevity is greater than at a constant temperature of 30°; and the per cent of females laying eggs and the number of eggs laid per female are both greater than at 30°, taking these as indexes of the favorableness of temperature conditions for physiological processes in the mosquito.

In our first article (Bates and Roca-García, 1945a) we pointed out the relation between laboratory findings of the effect of temperature on virus development, and field findings of relatively high diurnal temperatures in the canopy zone of the forest. The favorable effect of short daily exposure to 35° is an even more striking example of this relation. Shade temperatures of 35° are rarely found in the Villavicencio area, but it is a common observation that haemagogus mosquitoes are to a surprising degree sun-loving insects. They are apt to bite commonly even in the forest floor zone in open spots where the sun reaches the ground; and the canopy zone, where they are abundant, contains considerable areas of sunlight. Various investigators have found that the body temperature of an insect rises rapidly in sunlight. For example, Wigglesworth (1939, p. 359) in his summary of the literature on this subject, quotes Strelnikow, who found that the temperature of *Bombus* rose from 28.7° in the shade to 41.6° in the sun in the course of 5 minutes. We have no data on the possible daily body temperatures of haemagogus mosquitoes in their natural environment, but it does not seem unlikely that they would be exposed for short daily periods to relatively high temperatures. Results with the 25°–35° alternation are certainly much more likely to approximate natural conditions than results with a constant temperature of 30°, which can only have a theoretical interest.

VIII. SUMMARY

1. The effect of temperature on virus development in haemagogus mosquitoes was studied by the inoculation in mouse groups of individual mosquitoes at regular intervals after the infectious meal and by the titration of pools of mosquitoes. It was found that there was an initial period of virus loss, followed by a period of virus gain, the rates in both cases depending on the temperature. The period of virus loss lasted for 5 days at 20°, 3–4 days at 25° and 2 days at 30°. At 20° the level of virus seemed to remain stable after the period of loss, there being no demonstrable increase over a 22 day period. At higher temperatures the rate of gain seemed to be a direct function of the environmental temperature.

2. The percentage of mosquitoes becoming infected and the length of the incubation period seemed also to be a function of the amount of virus ingested with the infectious meal. On the basis of titer of virus in circulation at the time of feeding, experiments can be divided into 4 arbitrary categories: trace of virus (serum of source animal not infecting adult mice in dilutions greater than 1:10); small amount of virus (no infections in dilutions above $1:10^3$); moderate amount of virus (no infections in dilutions above $1:10^5$) and large amount of virus (infections in dilution of $1:10^6$ or more). In the first category, virus has in no case been recovered from haemagogus; in the second, occasional individuals become infected; in the third, the majority of the mosquitoes show virus; in the fourth, virus is regularly recovered from 90 per cent or more of the mosquitoes. The minimum incubation period after the ingestion of a "moderate amount of virus" is 13 days at 30°. This may be shortened to 10 days where a "large amount of virus" has been ingested. There is some evidence that infection at a given temperature and given virus dosage depends in part on the characteristics of the individual mosquito.

3. Experiments were undertaken with pantropic virus strains modified by serial passage (6 consecutive passages) in mice. It was difficult to infect haemagogus on saimiri monkeys inoculated with these modified virus strains; with the evidence at hand it is impossible to decide how much this was due to the lower titers of virus circulated by such monkeys, and how much to possible modification of the ability of the virus particles to invade mosquito tissue.

4. Attempts were made to define the incubation period in mosquitoes by large numbers of tests for transmission with individual mosquitoes using 3-day-old mice as test animals. It was found that there was considerable variation among individual mosquitoes of the same lot in the time at which they became infective, but that once a mosquito became infective it remained so for life. The minimum incubation period was found to be 28 days at 25°; 23 days in mosquitoes kept for 20 hours daily at 25° and 4 hours daily at 30°; 12 days for a similar alternation of 25°–35°; and 10 days at a constant temperature of 30°. Results were unsatisfactory at a constant temperature of 35°, but no transmissions were obtained in 28 attempts at periods between 5 and 12 days.

5. The very favorable results obtained with mosquitoes alternated between 25° (20 hours) and 35° (4 hours) suggest that relatively short exposures to high temperatures in nature may greatly accelerate virus development.

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