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Twelve Isolations of Zika Virus from *Aedes (Stegomyia) africanus* (Theobald) taken in and above a Uganda Forest *

A. J. HADDOW, M. C. WILLIAMS, J. P. WOODALL, D. I. H. SIMPSON & L. K. H. GOMA

In continuation of a series of studies of arboreal mosquitos as virus vectors in Uganda, 12 strains of Zika virus and one strain of another Group B arbovirus were isolated between November 1961 and June 1963 from pools of Aedes (Stegomyia) africanus *caught on a 120-foot (36.5-m) tower in Zika forest. For five strains it is known at what height the mosquitos were caught : one was from mosquitos taken at ground level, and the other four were from mosquitos taken in or above the upper canopy after sunset. No small mammal trapped in the forest either on the ground or in the trees showed serum antibody for Zika virus.*

These findings suggest that in Zika forest, A. (S.) africanus *becomes infected from a virus reservoir that is probably not among the small animals tested and that infected mosquitos are liable to be spread widely beyond the forest by convection currents above the tree-tops in the first two or three hours after sunset.*

INTRODUCTION AND NARRATIVE

The series of virus isolations described below represents the most recent advance in an investigation of arboreal mosquitos as virus vectors begun in Uganda in 1944. As the epidemiological implications of the work seem to us to be of some importance, an introductory discussion of its various stages is desirable.

The first definite record of biting activity by mosquitos at high levels in tropical forest appears to be that of Allee (1926) and refers to Panama. In 1929 the Oxford University expedition to British Guiana noted both biting activity and the presence of larvae in the very high canopy there, and used trap-containers to obtain the latter. Hingston (1932), in his book on this expedition, says " I have little doubt that the roof of an equatorial forest is a rich breeding ground for myriads of mosquitos ". In Africa the first definite findings refer to work carried out in Tanganyika in 1941, where Harris (1942) showed that both *Aedes (Stegomyia) aegypti* (L.) and *A. (S.) simpsoni* (Theobald) will oviposit in trap bamboo containers as much as 60 feet (18 m) above ground. *A. (S.) simpsoni* had already been incriminated as a yellow fever vector by that time (Mahaffy et al., 1942). Oddly enough these observations attracted little attention and it was only when Dr Jorge Boshell rediscovered this arboreal trend in a suspected vector of yellow fever in the South American forests that the urgent need for study of the upper forest levels was recognized. Intensive work in South America and in Africa followed, the first detailed study of the vertical distribution and biting-cycles of sylvan mosquitos in Africa being that of Haddow et al. (1947).

After some years' work in Africa, repeated isolations of yellow fever virus from sentinel rhesus monkeys confined in the forest canopy in Uganda, and from *Aedes (S.) africanus* (Theobald) taken at this level (Haddow et al., 1948; Smithburn et al., 1949) confirmed the importance of the canopy and of this arboreal species. Since that time there have been frequent isolations of arboviruses from arboreal sylvan mosquitos, and particularly from *A. (S.) africanus* which, in Uganda at least, appears to be a most important vector. Isolations from this mosquito were summarized by Haddow (1961) and at that time were as follows:

* From the East African Virus Research Institute, Entebbe, Uganda. This study was carried out with the technical assistance of D. F. Santos, M. Lule and Y. Ssenkubuge.

Ntaya virus	1 possible ?
Yellow fever virus	4 definite, 2 presumptive
Zika virus	3
Rift Valley fever virus (Lunyo strain)	1
Chikungunya virus	1

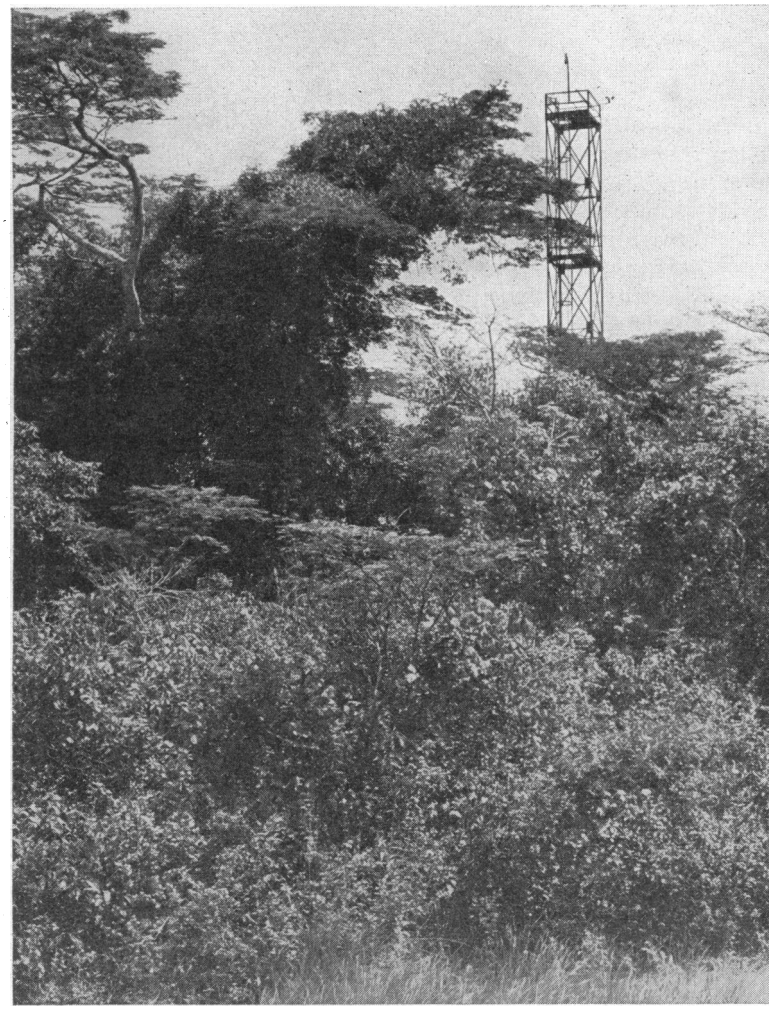
Since that time there have been five further isolations of chikungunya virus from *A. (S.) africanus* (Haddow et al., 1961) and 12 further isolations of Zika virus, which form the subject of this paper. Thus at the time of writing there have been 26 definite isolations of virus from *A. (S.) africanus*, and three other possible isolations.

During a great part of this entire period work had to be confined to ground level, the forest understorey and the canopy, as there was no way of working effectively above the latter. Catches made in a semi-emergent tree, at 82 feet (25 m) had, however, shown that mosquitos occur quite abundantly above the main canopy, and the zone above the forest was considered to be potentially of great importance for the following reasons. In studies on yellow fever epidemiology it had been noted that among the monkeys of small isolated forests the age-incidence of specimens with protective serum was such as to suggest an endemic state, whereas the number of monkeys in such forests was invariably too small for the maintenance of endemic yellow fever. This suggested either that an unknown cycle of infection might be occurring, or else that mosquitos from some larger forest, where endemicity was possible, were dispersing widely, yellow fever virus thus being re-introduced periodically among the isolated monkey communities of small forests. Support was given to this view by the following observations. In April 1943 the prevalence of protective sera among 33 monkeys from Bukasa Island in the Sese Archipelago, Lake Victoria, was only 3%. In December of the same year another good sample (36 monkeys) was obtained there, and it was found that the prevalence of yellow fever protection had risen to 89%. Quite clearly an enzootic had occurred among the monkeys on this island. Fully 20 miles (or over 30 km) from Bukasa, across the open waters of the lake, lies Kome Island, where intermittent collections between April 1940 and March 1943 had yielded a sample of 53 monkeys, not one being protective. New collections in April-May 1944 showed, however, that the virus had been active, 7 of 31 monkeys (23%) showing yellow fever antibodies. Immune specimens were also present in a small sample collected some years later, in 1949. The sudden appearance of virus activity in Kome, just at the time of the Bukasa enzootic, suggested that virus had been introduced from the latter and, as there was no evidence of enhanced yellow fever activity in man on either island, wind-borne mosquitos seemed to provide the most likely method of virus transport. This subject has been discussed in more detail elsewhere (Haddow et al., 1951).

Diffusion of sylvan mosquitos into banana plantations and other vegetation round the forest was known to occur at ground level (Haddow, 1945). For wide dispersal, however, it seemed more likely that the mosquitos must leave the forest at a much higher level, where wind-flow is less impeded by belts of vegetation, etc. In this connexion a very important observation was made by Wellington (1945), who pointed out that as forest is darker than the surrounding landscape there is (relatively) very active radiation of heat from the canopy after sunset, " thermal " convection currents tending to develop. He suggested that the combined effects of convection and wind might under such conditions transport small insects over considerable distances. This is, in fact, known to occur in aphids (see, for example, Johnson, 1953), while Hocking (1953) has recorded, in his useful summary of published information on this subject, mosquito flights of up to 177 km (about 110 miles). Altogether Hocking records three flights of more than 145 km (90 miles) and 24 in the range of 15-65 km (10-40 miles).

There was thus a very strong case for intensive work in the zone above the forest canopy, but the necessary equipment—a steel tower 120 feet (36.5 m) high, with various platforms for mosquito collection and meteorological work—could not be obtained until 1958, when it was erected with the help of funds generously provided by the World Health Organization. This tower is shown in Fig. 1. The first forest in which it was set up (Mpanga forest in central Uganda) was not entirely satisfactory, as the species in which we are particularly interested (notably *A. (S.) africanus*) were unaccountably scarce as adults, though larvae were present in large numbers. A great deal of work was, however, carried out, and it was shown that both males and females of many species do occur above the canopy, mainly by night. Thus of 27 species or groups recorded, 16 yielded specimens from the air above the canopy. The greatest activity at this level was in the hour after sunset (just when thermals may develop) though there was also a lesser burst of activity at the upper levels just before sunrise. Male swarms were

FIG. 1
STEEL TOWER FOR COLLECTION OF MOSQUITOS AT LEVELS TO ABOVE FOREST CANOPY



Photograph by Duncan Whitfield, EACSO.

also shown to occur above the top of the tower at these times. This work has been discussed elsewhere in a series of papers (Haddow et al., 1960). Mpanga forest was unproductive where virus work was concerned. Thus in forty 24-hour catches on the tower, made simultaneously at ground level, 30, 60, 90 and 120 feet (9, 18, 27.5 and 36.5 m), almost 13 000 mosquitos and tabanids (horseflies) were taken and practically all of these were inoculated into mice, without a single virus isolation being made.

It was decided, therefore, that the tower should be moved to Zika forest near Entebbe, a well-known field station of the East African Virus Research Institute, abounding in tree-hole breeders and other mosquitos (between 80 and 90 species have so far been recorded there, and the fauna of birds, rodents, insectivores, bats and viverrids is exceptionally large and varied). This forest had already yielded the original strains of Zika virus in 1947-48, the first coming from a sentinel monkey in the canopy, and

the second from *A. (S.) africanus* taken near the sentinel station (Dick et al., 1952). A strain of chikungunya virus had also been isolated there (again from *A. (S.) africanus*) by Weinbren et al. (1958); of the pool of 78 specimens from which this strain was isolated 74 came from the forest canopy at 65 feet (nearly 20 m).

Zika is a small isolated lake-shore forest, under one square mile (2.5 km²) in extent. It is particularly suitable for mosquito work as, within a narrow compass, it combines hill-slope forest and very wet swamp-forest, the latter in turn giving way to open swamp. In addition, it lies very near a large mission and its group of schools, the inmates being under constant surveillance for signs of virus activity. The forest itself has been described in detail by Buxton (1952) while Corbet (1964) has shown, by means of a transect, the relationship of the tower to the forest.

At Mpanga the platforms for mosquito catching were placed at 30-foot (9-m) intervals, but at Zika it was decided that this vertical interval should be reduced, the present arrangement being:

120 feet (36.5 m) :	platform completely exposed; there is only one tree of this height within 100 yards (or about 90 m) of the tower;
100 feet (30.5 m) :	also very exposed; above the level of the tallest emergent trees near the tower;
80 feet (24 m) :	in open air above the canopy, but sheltered to some extent by nearby emergent and semi-emergent trees;
60 feet (18 m) :	in a leafy part of the upper canopy;
40 feet (12 m) :	at the lower edge of the main canopy;
20 feet (6 m) :	clearly above the shrub layer, which is very dense at Zika and reaches 12-15 feet (3.5-4.5 m); there is no real understorey in this forest;
Ground level :	heavily shaded by the dense shrub layer.

There are thus three platforms in the open air above the foliage of the canopy.

An important part of the earlier work on tree platforms and of the present studies on the tower has been the investigation of the daily vertical migrations of some mosquitos. There are a few in which biting is practically confined to a single level but many, which bite in fair numbers at several levels, give very clear evidence of an upward movement in the evening and a downward movement in the morning. This trend was first noted by Mattingly (1949). It was later very clearly shown in *Aedes (Finlaya) ingrami* Edwards (Haddow, 1954), *A. (S.) africanus* (Haddow, 1961) and *Mansonia (Coquillettidia) fuscopennata* (Theobald) (Haddow et al., 1960). In *A. (S.) africanus* the picture is particularly clear, biting during the day being almost exclusively at ground level, while at all other levels it occurs mainly after sunset (Fig. 2). Thus the vertical distribution by day is entirely different from that by night (Fig. 3). Such movements as this are considered to be most important epidemiologically, as they form a link between the fauna of the upper forest layers on the one hand and man and his domestic animals on the other. In addition, Dr Philip S. Corbet has shown, by means of painstaking and prolonged study, that the highest concentrations of parous females (i.e., those which have taken at least one blood meal previously and which are thus potential vectors) are to be found by night at the highest levels, at least in some species (Haddow et al., 1960; Corbet, 1962). This fact emphasizes the desirability of intensive work in and above the canopy.

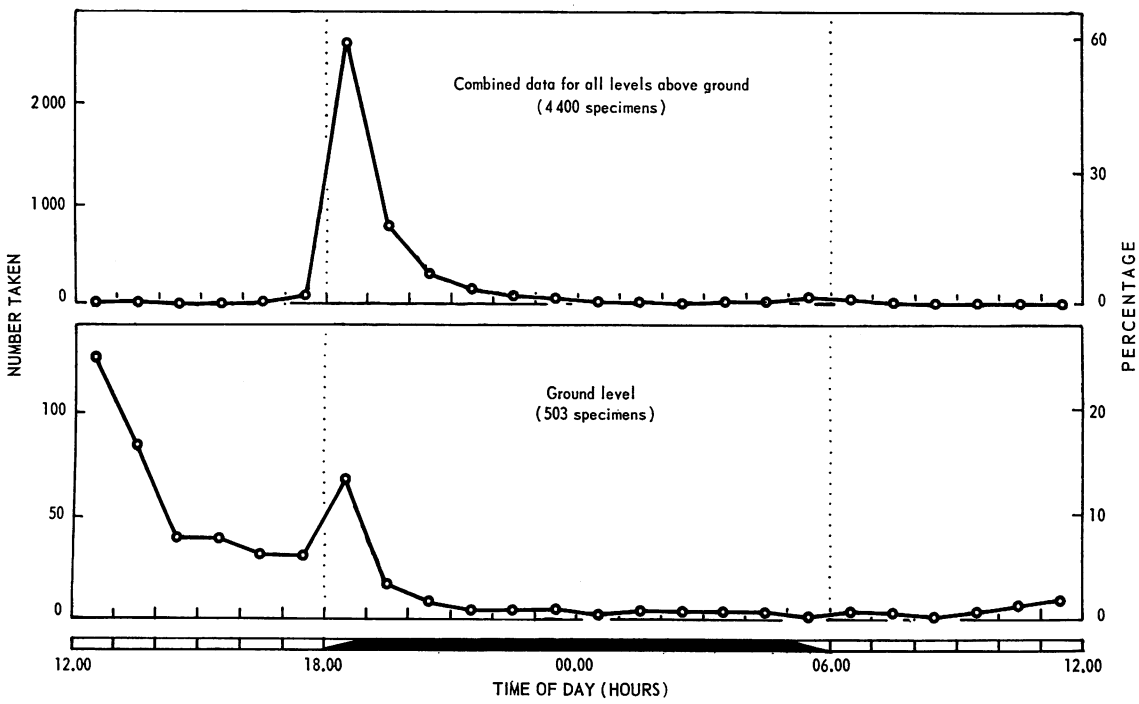
When work was begun on the tower at Zika in 1961, large numbers of *A. (S.) africanus* were taken, some as high as 120 feet (36.5 m). Before 2000 had been processed, five strains of chikungunya virus had been obtained from this species (Haddow et al., 1960) and five others had been isolated from Institute employees working on the tower (Knight & Williams, 1961). At this stage, therefore, Zika forest had yielded 11 strains of chikungunya virus and two of Zika virus. The latter agent, it may be added, has also been isolated twice from *A. (S.) africanus* taken in Lunyo forest, between Zika and Entebbe (Weinbren & Williams, 1958), while Smithburn (1952) has demonstrated antibodies in man in various parts of Uganda. The course of an infection in man has been described by Simpson (1964).

Following the chikungunya isolations, work had to be suspended for some time, but was taken up once more in November 1961. The catching programme from that time until June 1963 was as follows:

1. *21 November 1961–18 May 1962.* A series of 26 "sunset catches". These covered the 4-hour period 16.00-20.00 hours (our catches are always adjusted so that 18.00 corresponds to the minute of sunset; as the tower is almost on the equator, night is of virtually

FIG. 2

BITING-CYCLE OF *AEDES (S.) AFRICANUS* IN FIFTY 24-HOUR CATCHES ON THE TOWER AT ZIKA [a]



[a] The scales have been adjusted so that the greatest ordinates in the two graphs are of the same height.

constant duration throughout the year). These catches continued a series begun earlier in 1961.

2. *23 May–17 December 1962.* Thirty-eight 24-hour catches, completing a series of 50, begun in 1961.

3. *13 March–30 May 1963.* Twenty further sunset catches, completing a series of 50 in all.

4. *20 March–26 June 1963.* Twenty-five "sunrise catches", covering the period 04.00-08.00, part of a projected series of 50. These catches resemble the sunset catches. The clock is set so that 06.00 corresponds to the minute of sunrise.

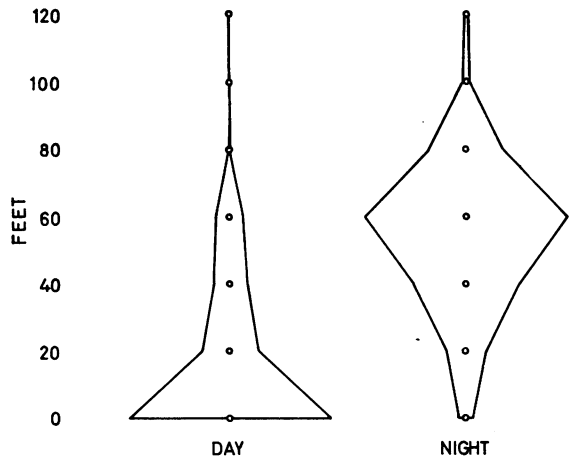
5. *6 June–26 June 1963.* A series of six routine catches, to collect mosquitos for inoculation, covering the period 18.00-20.00 hours.

In all these series man was the bait, and in almost all catches work went on simultaneously at all seven levels.

Not all mosquitos taken were inoculated. When the work began one species representative of each level and active in the crepuscular period was

FIG. 3

VERTICAL DISTRIBUTION OF *AEDES (S.) AFRICANUS* ON THE TOWER AT ZIKA BY DAY AND BY NIGHT [a]



[a] By day 605 specimens were taken, and by night 4298. The scales have been adjusted so that the maximum width of each diagram is the same.

chosen. These were: above the canopy, *Mansonia (C.) aurites* (Theobald); the canopy, *A. (S.) africanus* (and the arboreal tabanid *Chrysops centurionis* (Austen)); the zone between canopy and shrub layer, *A. (F.) ingrami*; ground level, *Culex (Culex) annulioris* Theobald. Towards the end of the work the list had been increased by the inclusion of all other species in a large mixed group. A ground-haunting tabanid, *Chrysops funebris* Austen occurred in small numbers, and was inoculated separately. The species inoculated were as follows:

Species in order of frequency	*No. inoculated into mice*
Mansonia (Coquillettidia) aurites (Theobald)	19 637
Aedes (Stegomyia) africanus (Theobald)	8 125
Mansonia (Coquillettidia) fuscopennata (Theobald)	7 113
Culex (Culex) annulioris Theobald	3 979
Mansonia (Mansonioides) uniformis (Theobald)	3 859
Mansonia (Mansonioides) africana (Theobald)	3 241
Aedes (Finlaya) ingrami Edwards	1 513
Aedes (Stegomyia) apicoargenteus (Theobald)	1 048
Anopheles (Anopheles) implexus (Grünberg)	277
Culex spp. indet.	254
Anopheles (Anopheles) paludis (Theobald)	246
Chrysops centurionis Austen	205
Mansonia (Coquillettidia) pseudoconopas (Theobald)	78
Mansonia (Coquillettidia) metallica (Theobald)	76
Mansonia (Coquillettidia) maculipennis (Theobald)	64
Eretmapodites oidipodeios Graham group	49
Culex (Culex) poicilipes Theobald	45
Mansonia (Coquillettidia) fraseri (Theobald)	37
Eretmapodites chrysogaster Graham group	29
Aedes (Mucidus) nigerrimus (Theobald)	26
Aedes (Aedimorphus) cumminsii (Theobald)	13
Aedes (Aedimorphus) tarsalis (Newstead) group	10
Anopheles (Anopheles) obscurus (Grünberg)	5
Culex (Neoculex) insignis (Carter) group	5
Hodgesia cyptopus Theobald	5
Aedes (Aedimorphus) domesticus (Theobald) group	4
Eretmapodites quinquevittatus Theobald	4
Aedes (Mucidus) grahamii (Theobald)	3
Chrysops funebris Austen	3
Anopheles (Cellia) moucheti Evans	2
Aedes (Finlaya) longipalpis (Grünberg)	1
Aedes (Neomelaniconion) circumluteolus (Theobald)	1
Anopheles (Cellia) marshallii (Theobald)	1
Culex (Culiciomyia) macfiei Edwards	1
Culex (Culex) moucheti Evans	1
Eretmapodites sp. indet.	1
Mansonia (Coquillettidia) versicolor (Edwards)	1
Total	49 962

The names are those given in the latest world catalogue (Stone, Knight & Starcke, 1959).

The mosquitos and tabanids were divided into the following separate pools:

Species	*No. of pools*
A. (S.) africanus	688
M. (C.) aurites	110
Chrysops centurionis	57
Mixed species and *C. funebris*	57
C. annulioris	52
A. (F.) ingrami	51
Total	1 015

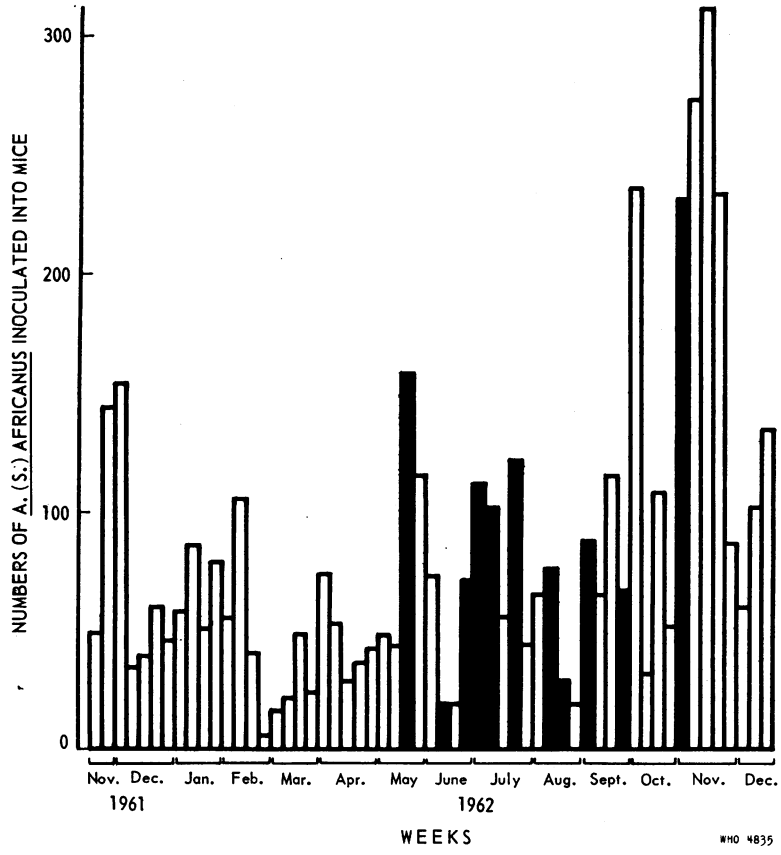
During the course of the work a strain of another Group B arbovirus, Usutu, was isolated from *M. aurites*, a mosquito which feeds preferentially on birds (Williams et al., 1958; McClelland & Weitz, 1960) but which will also attack man readily. It was unusually abundant during the year concerned. The isolation of this strain (MP 1626) occurred in October 1962, and is discussed elsewhere (Williams et al., 1964). All the other strains were Zika virus, and came from *A. (S.)* africanus. Eleven of these were obtained during the catch programme covering the period November 1961 to December 1962 and their occurrence is shown diagrammatically in Fig. 4. The remaining strain was obtained some months later.

The first strain (MP 1399) came from a pool collected on 23-24 May 1962, and the next two (MP 1416 and MP 1429) from pools collected on 14-15 and 27-28 June. Our plan had been that, should virus activity on the tower become apparent, the mosquitos would be subdivided by level, and if possible by fairly short time-intervals also, in an attempt to find whether there was a level or time at which transmission seemed particularly active. Most unfortunately, the present series of isolations coincided with the first isolation of a virus from a biopsy in a case of Burkitt's lymphoma syndrome (Burkitt, 1962). It was subsequently found that this was merely a strain of herpes virus, but at the time all available mice were being used for its study, and it did not seem justifiable to divert additional groups of mice for mosquito inoculation.

Two further strains (MP 1439 and MP 1446) were isolated from mosquitos taken on 4-5 and 11-12 July 1962. Another (MP 1473) was obtained from a pool made up of *A. (S.) africanus* taken in a 24-hour catch on 25-26 July and a sunset catch (at 60 feet—18 m—only) on 26 July. At this stage five strains of virus had been isolated from 10 consecutive pools of *A. (S.) africanus*, taken at inter-

FIG. 4

NUMBERS OF *AEDES (S.) AFRICANUS* INOCULATED INTO MICE WEEKLY, FROM WEEK BEGINNING 19 NOVEMBER 1961 TO WEEK ENDING 22 DECEMBER 1962 [a]



[a] The weeks in which virus was isolated are shown in black.

vals of approximately one week. It was clear that very active transmission was going on and that, unless a unique opportunity was to be lost, more mice must be allocated, to permit subdivision of the *A. (S.) africanus* by time and level. This was done, though in the first two catches only partial subdivision was possible.

A catch made on 15-16 August yielded the next strain (MP 1501). In this catch mosquitos from above the canopy were pooled (there being none from 120 feet—36.5 m). Similarly those from the canopy (60 and 40 feet—18 m and 12 m) were pooled, as were those from 20 feet (6 m) and ground level. The virus came from a pool of nine (one from 100 feet—30.5 m—and eight from 80 feet—24 m). That from 100 feet was taken in the period 20.00-21.00 hours, and those from 80 feet were made up of six taken between 18.00 and 19.00 hours and two taken between 19.00 and 20.00 hours. The infected pool thus came entirely from above the canopy, in the 80-100-foot zone, within the three-hour period following sunset, i.e. 18.00-21.00 hours.

MP 1510, the next strain, came from a catch made on 20-21 August. This catch, totalling 28 *A. (S.) africanus*, unfortunately had to be inoculated *en bloc*, mice again being in short supply. There is thus no information as to the time or level at which infected specimens were taken. In all subsequent catches,

however, complete subdivision by levels has been the rule.

MP 1521 came from a catch made on 6-7 September, the isolation being from a single *A. (S.) africanus* taken at 100 feet in the hour 19.00-20.00 (i.e., the second hour after sunset).

MP 1582 was isolated from a catch made on 25-26 September. It came from a pool of two *A. (S.) africanus* taken at 80 feet, in the hour 19.00-20.00.

A considerable period followed without further isolations, but one more (MP 1751) came from a catch made on 1-2 November, from an unusually large pool of *A. (S.) africanus* taken at ground level. The pool consisted of 50 specimens and the interesting point is that these were taken during the afternoon. As noted above, biting by *A. (S.) africanus* during the daylight hours is largely confined to ground level. No further isolations were made before the series was wound up in mid-December.

Thus of four isolations in which the level was known, three came from above the canopy. This is a striking fact when it is remembered that only a small proportion of the total yield of *A. (S.) africanus* comes from the high platforms. From the time when subdivision by levels was begun until the end of the series (excluding the one catch which was pooled as a whole and from which MP 1510 was obtained), the following numbers of *A. (S.) africanus* were inoculated:

Level	*No. taken*	*Percentage*
120 feet; 36.5 m	24	1
100 feet; 30.5 m	45	2
80 feet; 24 m	398	17
60 feet; 18 m	964	41
40 feet; 12 m	447	19
20 feet; 6 m	176	8
Ground level	282	12
Total	2 336	100

Thus the levels above the canopy, which yielded three strains, made up only 20% of the total. If the figure be restricted to the known infected levels (80 and 100 feet) and to the period within which the infected specimens were taken (18.00-21.00 hours), it is found that there were only 32 from 100 feet and 344 from 80 feet—376 in all, or 16% of the total sample.

When work was taken up again in March 1963 (see above), sunset catches were subdivided into two periods, 16.00-18.00 and 18.00-20.00 hours, for inoculation purposes. A similar procedure was followed in the case of sunrise catches. Routine catches, which covered the period 18.00-20.00 hours, required subdivision by level only. Virus activity was much more restricted, only one strain (MP 4039) being isolated. This came from a catch made on 22 May, the pool consisting of 146 *A. (S.) africanus* taken at 60 feet, in the period 18.00-20.00 hours (i.e., in the top of the canopy, in the two hours after sunset).

The main features of this series of isolations are summarized in Table 1.

MATERIALS AND METHODS

The laboratory procedure followed in isolating, reisolating and identifying these strains will now be described.

(*a*) Identified mosquitos were chilled at approximately −20°C for periods ranging from two to 48 hours before preparation for inoculation.

(*b*) Suspensions of the mosquitos were made by grinding in pestles and mortars with a small quantity of glass powder, using 0.75% bovine plasma albumin (Armour Fraction V) in phosphate-buffered saline pH 7.4 (BPA). Penicillin (200 units/ml) and streptomycin (0.01 g/ml) were added. The suspensions were centrifuged lightly at 4°C for 5 minutes and the supernatant removed for inoculation.

(*c*) Newborn albino Swiss mice, descended from the stock of Carworth Farms, New York, were used for attempted isolation, each mouse being inoculated with 0.01 ml by each of three routes (intracerebral, intraperitoneal and subcutaneous).

(*d*) Mosquito pools were stored at −60°C until reisolation was attempted.

(*e*) Inoculated mice were examined daily for 21 days, suspensions in BPA of brains from sick mice being passaged by the intracerebral route.

(*f*) Neutralization tests were made with tenfold dilutions, in BPA, of virus against a constant dilution of antiserum. Serum virus mixtures were incubated at 37°C for one hour before intracerebral inoculation into 1-3-day-old mice.

(*g*) Haemagglutination-inhibition (HI) was done by the method of Clarke & Casals (1958) using Arcton-extracted antigens (Porterfield & Rowe, 1960).

(*h*) Virus titres were calculated by the method of Reed & Muench and are expressed as the reciprocal of the $\log_{10}$ dilution which killed 50% of the mice inoculated.

TABLE 1

DATES, LEVELS AND TIMES OF CAPTURES IN ZIKA FOREST OF POOLS OF *AEDES (S.) AFRICANUS* FROM WHICH 12 STRAINS OF ZIKA VIRUS WERE ISOLATED

Date	Strain designation	No. of *A. (S.) africanus* in pool	Level	Time (hours)
23-24 May 1962	MP 1399	159	—	—
14-15 June 1962	MP 1416	17	—	—
27-28 June 1962	MP 1429	71	—	—
4-5 July 1962	MP 1439	112	—	—
11-12 July 1962	MP 1446	102	—	—
25-26 July 1962	MP 1473	121	—	—
15 August 1962	MP 1501	9	80 & 100 feet; 24 & 30.5 m	18.00-21.00
20-21 August 1962	MP 1510	28	—	—
6 September 1962	MP 1521	1	100 feet; 30.5 m	19.00-20.00
25 September 1962	MP 1582	2	80 feet; 24 m	19.00-20.00
1 November 1962	MP 1751	50	Ground	Afternoon
22 May 1963	MP 4039	146	60 feet; 18 m	18.00-20.00

RESULTS

Isolation and reisolation

Newborn mice receiving inocula of the 12 *A. (S.) africanus* pools mentioned in Table 1 were first seen to be sick between the sixth and ninth days after inoculation. First passages with mouse brain material gave titres in newborn mice ranging from 6.0 to 8.0 log LD_{50}. Reisolation was successful in all cases except the first (MP 1399). Estimates of the number of infectious virus particles in the mosquito pools were obtained by titration; the titres may be seen in Table 2. It is perhaps of interest to note that the first two pools (MP 1399, 1416) and the last two pools (MP 1751, 4039) contained the smallest amounts of virus.

Identification

Using 1st-3rd-passage mouse brain, all original and reisolate strains gave haemagglutinins with an optimal activity around pH 6.0 and end-points ranging from 1 : 80 to 1 : 2560. Screening by HI against a range of our stock antisera showed that all strains were arboviruses belonging to Casals' Group B. Next an antigen derived from each pool (either from original or reisolation material) was tested against 12 different Group B antisera prepared with the following viruses: dengue (Hawaiian) received from Dr Smithburn while he was working at the South African Institute for Medical Research; Entebbe bat salivary gland virus (IL 30) (Lumsden et al., 1961); H 336 (Smithburn et al., 1959); MP 1626 (Williams et al., 1964); Ntaya (Smithburn & Haddow, 1951); Spondweni (AR 94) (Kokernot et al., 1957); Uganda S (Dick & Haddow, 1952); Usutu (AR 1776), received from B. M. McIntosh, South African Institute for Medical Research; Wesselsbron (H 177) (Smithburn et al., 1957); West Nile (B 956) (Smithburn et al., 1940); yellow fever (French neurotropic); and Zika (Dick et al., 1952).

The results of HI screening may be seen in Table 3. All strains gave a pattern of inhibition similar to Zika virus. The MP 1510 antigen gave a similar pattern but higher titres; this is considered to be a strain variation.

In order to confirm the identification of all strains as Zika virus by another method, the following neutralization tests were carried out. Initially strain MP 1429 was picked out as the type strain for the outbreak, antisera were prepared in mice and in guinea-pigs with early-passage mouse brain material, and a cross-neutralization test was made with Zika virus and its homologous antiserum. The following results were obtained:

Virus	Titre in BPA	Neutralization index Antiserum MP 1429 (in mice)	Zika
MP 1429, passage 1	6.8	1.3	2.4
Zika (MR 766), passage 19	8.2	1.8	2.9

The results indicate a very close relationship between MP 1429 and Zika virus, no significant difference occurring.

In view of the fact that a strain of virus isolated in West Africa by Macnamara (1954) and identified as Zika virus has been shown to be close to if not identical with Spondweni virus under the strain name of Chuku (D. H. Clarke—personal communication), a cross-neutralization test was made with MP 1429, Zika and Spondweni viruses and their antisera with the following results:

Virus	Titre in BPA	Neutralization index Antiserum Spondweni	Zika	MP 1429 (in guinea-pigs)
Spondweni (AR 94), passage 6	7.9	1.6	0	1.6
Zika (MR 766), passage 19	7.5	0	1.8	3.0
MP 1429, passage 1	7.1	0.8	1.6	2.4

These results show the difference between Zika and Spondweni viruses clearly. When, however, the results with MP 1429 are considered, again the neutralization indices obtained with Zika indicate no significant difference, but, on the other hand, there is a definite cross-relationship with Spondweni virus. It is not known whether the difference between Zika and MP 1429 viruses is (1) a real strain difference; (2) related to the passage level of the two strains (19 and 1 respectively) as had previously been suggested by Dr R. M. Taylor (personal communication); or (3) associated with the fact that the MP 1429 antiserum in this test was prepared in guinea-pigs whereas for the Zika and Spondweni antisera mice were used. Nevertheless, in view of the clear-cut results obtained with the HI test (Table 3), we are of the opinion that MP 1429 is so closely related to Zika virus that it does not warrant designation as a new virus.

Finally, in order to determine the relationship of MP 1429 to the other new isolates in this series, they were compared in a series of neutralization tests with MP 1429 antiserum. In Table 2 it may be seen that all strains were neutralized to a significant degree by MP 1429 antiserum.

During the course of the investigation, trapping of small mammals has been carried out both at ground level and on the old tree platforms of Zika No. IV station (there is one at 38 feet—11.5 m—and one at 65 feet —20 m—and the station is about 50 yards—45 m—from the tower).

The trapped animals were as follows:

1 Potto—*Perodicticus potto* (P. L. S. Müller)
3 Palm civets—*Nandinia binotata* (Reinwardt)
4 Squirrels—*Heliosciurus rufobrachium* Waterhouse
2 Tree rats—*Thamnomys rutilans* Dollman
7 Giant pouched rats—*Cricetomys gambianus* Wroughton
8 Field rats—*Arvicanthis abyssinicus* (Rüppell)

All these mammals were tested by HI against Zika antigen. No Zika antibodies were demonstrated.

DISCUSSION

This series of isolations proves for the first time the truth of the hypothesis already discussed in the Introduction, that during a period of arbovirus activity in a tropical forest infected mosquitos may be found flying above the forest canopy. The series again emphasizes the pre-eminence of *Aedes (S.) africanus* in relation to arboviruses in the Uganda forests. The fact that the infected mosquitos were collected above the forest canopy during the post-sunset period, at a time when convection currents might be expected to disseminate the insects over considerable distances, is considered to be of great epidemiological significance. The need for further study of wind and the dispersal of mosquitos above the forest canopy is obvious.

Zika virus is apparently enzootic in Zika forest, and the evidence so far suggests that *A. (S.) africanus* is the principal (if not the only) vector and that forest-dwelling monkeys and man are on occasions involved. This important mosquito appears to be fairly catholic in its tastes, for it is known to have a greater preference for monkeys than man (Haddow & Dick, 1948) and also to come to rodent, avian and reptilian bait (Corbet & Ssenkubuge, 1962; Williams, 1963). In view of these feeding habits and of the repeated isolations of Zika virus, indicating a considerable virus activity, it is perhaps surprising that none of the small sample of sera from mammals contained HI antibodies to Zika virus. To obtain a knowledge of potential hosts and antibody responses, susceptibility trials are required with the vertebrates occurring in Zika forest.

The virus identification studies raise the problem of the importance of minor strain differences. We have taken the view that although strain differences have been noted they are not of a sufficient order to justify separation from the type strain of Zika virus

TABLE 2

TITRES AND NEUTRALIZATION INDICES (NI) OF 12 ZIKA VIRUS STRAINS

Virus strain [a]	Virus titre of pool		Neutralization tests with MP 1429 antiserum							
	Original inoculation	Reisolation	Titre	NI	Titre	NI	Titre	NI	Titre	NI
MP 1399	3/10 undil.	Nil	4.8	⩾2.2						
MP 1416	⩾1.8	1.1	6.5	2.4						
MP 1429	⩾2.5	3.2	7.2	3.0	7.8	4.7	6.7	2.5	7.5	3.0
MP 1439	⩾2.1	2.3	5.8	⩾2.5						
MP 1446	⩾1.5	1.3	5.8	1.8						
MP 1473	⩾2.5	⩾3.3			6.8	4.4				
MP 1501	⩾2.5	⩾3.7					7.4	3.6		
MP 1510	⩾2.5	⩾3.7					6.8	⩾4.0		
MP 1521	⩾1.6	2.1					5.7	2.1		
MP 1582	⩾1.0	3.9					6.6	3.4		
MP 1751	⩾1.0	1.0					7.0	⩾4.2		
MP 4039	3/9 undil.	6/41 undil.							7.1	2.2

[a] For dates of collection see Table 1.

TABLE 3

TITRES [a] OBTAINED IN HI TESTS USING REFERENCE ANTISERA AND DIFFERENT VIRUS STRAINS

Antiserum	Homologous titre	Virus													
		Spondweni	Zika	MP 1399	MP 1416	MP 1429	MP 1439	MP 1446	MP 1473	MP 1501	MP 1510	MP 1521	MP 1582	MP 1751	MP 4039
Dengue 1	2	.	.	.	.	.	.	.	1	.	3	.	.	.	.
Entebbe bat	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H 336	8+	4	4	4	4	4	5	4	5	5	7	4	4	4	4
MP 1626	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ntaya	8+	2	.	.	.	.	.	.	.	.	.	.	.	.	.
Spondweni	5	5	2	2	2	1	2	2	3	2	4	2	2	2	2
Uganda S	7	.	.	.	.	.	.	.	1	.	3	.	1	.	1
Usutu	8+	4	4	4	4	4	5	4	5	4	7	4	4	4	4
Wesselsbron	5	.	.	.	.	.	.	.	.	.	2	.	.	.	.
West Nile	4	.	.	.	.	.	.	.	.	.	1	.	.	.	.
Yellow fever	4	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Zika	5	1	5	6	5	5	6	5	7	6	8	5	5	5	6

[a] Titres expressed as follows:
. = No inhibition with serum dilution of 1/20.
1 = Inhibition with serum dilution of 1/20.
2-8 = Inhibition with serum dilutions of 1/40-1/2560.
8+ = End-point not reached with serum dilution of 1/2560.

isolated in the same forest years earlier. Clearly in this matter there is a need for more fundamental studies on antigenic changes, particularly those occurring during early passage series. The present work also indicates that the HI test may be a better tool for the separation of early-passage Zika virus strains from Spondweni virus than the neutralization test.

Considering the virus titres found in the mosquito pools (Table 2) it has already been noted that the first two and the last two pools were lower in titre than the others. Such a result may well be fortuitous. It may, however, be of significance in relation to the reservoir mechanism, and it will therefore be of interest to see whether studies on other arbovirus outbreaks give similar results.

RÉSUMÉ

On a isolé douze souches de virus Zika ainsi qu'une souche d'un autre arbovirus du Groupe B chez des moustiques capturés sur une tour d'acier de 36,60 m située dans la forêt de Zika près d'Entebbé (Ouganda). Les captures ont été effectuées sur la tour à 6 niveaux différents, tous les 6,10 m, de novembre 1961 à juin 1963. Les souches de virus de Zika ont été isolées sur des souriceaux; elles provenaient toutes de moustiques *Aedes (Stegomyia) africanus* (Theobald) capturés entre mai et novembre 1962 — sauf pour l'une d'elles, isolée en mai 1963 chez des moustiques de la même espèce. Après les premiers isolements, les captures ont été classées selon l'heure et le niveau au-dessus du sol. L'on a ainsi pu, pour 5 souches, répartir les moustiques en 5 groupes: 50 capturés au niveau du sol au cours de l'après-midi; 146 capturés à 18,30 m (en pleine futaie) entre 18 h. et 20 h.; 2 capturés à 24,40 m (au-dessus de la futaie) entre 19 h. et 20 h.; 9 capturés à 24,40 m et à 30,50 m entre 18 h. et 21 h.; 1 capturé à 30,50 m entre 19 h. et 20 h. Ainsi 3 des 5 souches provenant de moustiques se trouvant à des niveaux connus ont été isolées chez des insectes capturés au-dessus de la cime des arbres dans les 3 heures suivant le coucher du soleil (18 h.). Ceux-ci ne représentent que 16% des *Aedes africanus* obtenus. On peut donc penser que les vecteurs infectés se trouvent dans une zone relativement réduite.

Les virus ont été identifiés par les épreuves d'inhibition de l'hémagglutination et de neutralisation. Celle-ci a montré que toutes les souches étaient analogues et apparentées à la fois au virus Zika et au virus Spondweni. Les épreuves d'inhibition de l'hémagglutination ont mis en évidence leur identité avec le virus Zika et les ont nettement séparées du virus Spondweni. Vingt-cinq petits mammifères ont été capturés au sol et dans les arbres; l'on n'a pu mettre en évidence chez eux, par l'épreuve d'inhibition de l'hémagglutination, d'anticorps Zika.

Ces observations incitent à penser que dans la forêt de Zika *A. africanus* s'infecte à partir d'un réservoir de virus ne faisant probablement pas partie des petits animaux testés et que les moustiques infectés peuvent se répandre largement au-delà de la forêt grâce aux courants de convection qui se forment au-dessus de la cime des arbres dans les 2 ou 3 heures suivant le coucher de soleil.

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