A STUDY OF THE PRINCIPAL MOSQUITO SPECIES IN THE HIGHVELD REGION OF SOUTH AFRICA TO ASSESS THEIR RELATIVE VECTORIAL IMPORTANCE IN THE TRANSMISSION OF WEST NILE AND SINDBIS VIRUSES

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T R A C T

The first chapter reviews our knowledge of the ecology and epidemiology of West Nile (WN) and Sindbis (SIN) viruses and those following detail studies carried out to obtain the answers to three main questions. Firstly, the relative capability and significance of Culex (Culex) univittatus Theobald as a vector had to be fully established. Although previous studies had shown that it is almost certainly the maintenance vector in feral transmission cycles, further work was called for to settle this question. Secondly, the identity of the mosquito vector or vectors transmitting each virus to man needed to be established and, thirdly, did the over-wintering mechanism of the viruses reside in the mosquito vector? In endeavouring to answer these questions, the investigations were confined mainly to the six most prevalent mosquito species in the Highveld region of South Africa. These are Culex univittatus, Culex (Culex) theileri Theobald, Culex (Culex) fatigans Wiedemann, Culex (Culex) pipiens Linnaeus, Aedes (Neomelaniconion) unicentatus McIntosh and Aedes (Aedimorphus) dentatus (Theobald).

Answers to the first two questions came from field studies of the feeding habits of mosquitoes and from laboratory transmission experiments designed to determine the vector capability of the different species, together with a previous knowledge of the infectivity levels in feral Highveld mosquito populations. Studies of feeding habits included the investigation of preference for man and birds, vertical distribution during feeding and endophagy undertaken by a variety of mosquito collecting methods. In the laboratory experiments, the susceptibility of each species to infection and its ability to transmit was tested with each virus. For this purpose laboratory colonies of each Culex species were established, whereas the aedine species were reared from field-collected females. It is concluded that C. univittatus is maintaining both WN and SIN viruses in wild birds on the Highveld and that this species is also the main vector transmitting each virus to man.

It appears that none of the other common Highveld species is a vector of any significance, but C. theileri and C. fatigans could act in a minor capacity in infecting man with WN virus acquired by

feeding on birds. C. theileri and A. unidentatus might possibly fulfil the same role with SIN virus.

The following conclusions are also drawn from various laboratory transmission experiments. A blood-virus mixture used as the infecting meal can, in some cases, give a misleading assessment of vector capability. The demonstration of virus in the salivary glands of a mosquito can not be taken as an indication that it will transmit. A decrease in the concentration of virus in the infective feed can cause a significant lowering of the subsequent transmission rate.

As concerns the third main question, field studies were made on the winter biology of the four commonest Culex species through three winters. The indication was that low-level populations of immature and adult C. pipiens, C. theileri and C. univittatus are present during this season. They are active in relatively warm spells and quiescent when temperatures fall. Diapause does not seem to occur. The same is thought to apply to C. fatigans, although evidence was obtained only for the occurrence of the immature stages. The adult mosquitoes trapped in the 1972 autumn-winter-spring series were dissected for parity determinations. In preparation for this, the applicability of the ovarian tracheation technique for determining parity was evaluated on the four Culex species and found suitable. From the field observations it is concluded that C. univittatus is unlikely to carry WN and SIN viruses through the Highveld winter, either as an infected quiescent overwintering adult or by transmitting the virus during warm winter spells.

I, Peter Graham Jupp, hereby declare that the work on which this thesis is based was carried out in my capacity as entomologist to the Arbovirus Research Unit at the South African Institute for Medical Research, Johannesburg. I further declare that this thesis is original except where stated in the acknowledgements and that neither the whole work nor any part of it has been, is being, or is to be submitted for a degree in any other university.

A C K N O W L E D G E M E N T S

This study formed part of the investigations I have been engaged in as entomologist to the Arbovirus Research Unit, South African Institute for Medical Research, Johannesburg. I would like to thank Professor J.H.S. Gear, Director of the Poliomyelitis Research Foundation and formerly Director of the South African Institute for Medical Research, for permission to undertake this work for submission as a thesis and for acting as my supervisor on behalf of the University of the Witwatersrand. I am particularly indebted to my colleague, Dr. B.M. McIntosh, Head of the Arbovirus Unit, for his continual encouragement and advice since I started this study, as well as for his valuable participation in the planning of the first transmission experiments in which Culex fatigans and Culex univittatus fed on blood-virus mixtures. Dr. McIntosh also kindly read the draft manuscript and afterwards made helpful suggestions. Various other members of the Arbovirus unit, both past and present, rendered technical assistance at different times and I would like to thank Mrs. D.U. Hanmer (née Dickinson), Mrs. B.M. Hawkins (formerly Mrs. B.M. Gutteling), Mrs. W. Mouton, Mrs. G.M. Keenahan, Mrs. I. Dos Santos and Dr. E. Anderson for carrying out essential laboratory tests, Messrs. J.J. Taljaard, J. de Sousa, A. Walters and J. Molala for their assistance with the fieldwork, and Mr. J. Kgasago for his help in the insectary. I would also like to express my gratitude to the Chief City Engineer, Johannesburg and Messrs. A.C. Cilliers and H.J. Stoop for allowing me to work at Olifantsvlei and on farms at Lake Chrissie respectively, and the farm managers at Olifantsvlei for their willing co-operation: to Messrs. Andersons Chick Sales and Messrs. Gaymans Chick Sales, both in Sandton near Johannesburg, for their generous donations of chicks for the transmission experiments: to Dr. A. Joosting for his willing help with statistical analysis of some of the experimental data: to Dr. J.H. Mason for his kind assistance in checking the manuscript: to the Library Staff of the South African Institute for Medical Research and Mrs. Main of the Poliomyelitis Research Foundation for obtaining references and checking the bibliography: and to Mrs. A. Loedecke for kindly typing the manuscript and giving much help with its presentation.

PUBLICATIONS ARISING OUT OF THESIS

Nearly all the work on which this thesis is based has either been published already or is expected to appear in the journals soon. There are ten papers altogether, eight have been published, one has been submitted for publication and the last is in preparation. A list of these follows:

JUPP, P.G. (1969). Preliminary studies on the overwintering stages of *Culex* mosquitoes (Diptera: Culicidae) in the highveld region of South Africa. *J. ent. Soc. sth. Afr.* 32: 91-98.

JUPP, P.G. & McINTOSH, B.M. (1970). Quantitative experiments on the vector capability of *Culex (Culex) pipiens fatigans* Wiedemann with West Nile and Sindbis viruses. *J. med. Ent.* 7: 353-356.

JUPP, P.G. & McINTOSH, B.M. (1970). Quantitative experiments on the vector capability of *Culex (Culex) univittatus* Theobald with West Nile and Sindbis viruses. *J. med. Ent.* 7: 371-373.

JUPP, P.G. (1971). The laboratory colonization of *Culex (Culex) theileri* Theobald and *Aedes (Dicromyia) furcifer* (Edwards) (Diptera: Culicidae). *J. ent. Soc. sth. Afr.* 34: 191-193.

JUPP, P.G., McINTOSH, B.M. & DICKINSON, D.B. (1972). Quantitative experiments on the vector capability of *Culex (Culex) theileri* Theobald with West Nile and Sindbis viruses. *J. med. Ent.* 9: 393-395.

JUPP, P.G. (1973). Distinguishing nulliparous from parous females by the ovarian tracheation technique in four South African species of *Culex* (Diptera: Culicidae). *J. ent. Soc. sth. Afr.* 36: 271-273.

JUPP, P.G. (1973). Field studies on the feeding habits of mosquitoes in the highveld region of South Africa. *S. Afr. J. med. Sci.* 38: 69-83.

JUPP, P.G. (1974). Laboratory studies on the transmission of West Nile virus by *Culex (Culex) univittatus* Theobald; factors influencing the transmission rate. *J. med. Ent.* 11: 455-458.

JUPP, P.G. Further studies on the overwintering stages of Culex mosquitoes (Diptera: Culicidae) in the highveld region of South Africa. Submitted for publication to the J. ent. Soc. sth. Afr.

JUPP, P.G. The susceptibility of four South African species of Culex (Diptera: Culicidae) to west Nile and Sindbis viruses with a comparison of the results obtained using two different infecting methods. In preparation.

C O N T E N T S

T A B L E S

F I G U R E

CHAPTER 1

GENERAL INTRODUCTION

On the basis of antigenic relationships Sindbis (SIN) virus is classified in the A group of arboviruses (arthropod-borne viruses) and West Nile (WN) in the B group. SIN virus was first isolated from mosquitoes in Egypt in 1952 (Taylor et al. 1955), but it was not until 1961 that it was implicated as a cause of disease in man when, in Uganda, it was isolated from the blood of five persons with fever, headache and generalized muscular pain (EAVRI, 1962). Later a more severe illness, with skin lesions and arthralgia in addition, was reported in people living in the South African Highveld (Malherbe et al. 1963; McIntosh et al. 1964) and also in a child in Australia who had fever and skin lesions (Doherty et al. 1969).

WN virus was initially isolated from the blood of a patient in Uganda (Smithburn et al. 1940) and subsequently from the blood of three children in Egypt (Melnick et al. 1951). The studies of Taylor et al. (1956) have shown the virus to be the cause of an endemic disease in Egypt and those of other investigators (Bernkopf et al. 1953; Goldblum et al. 1954; Marberg et al. 1956; Goldblum, 1959) of epidemic disease in Israel.

SIN virus has also been isolated from arthropods and/or birds in the Central African Republic, Cameroun, Israel, Australia, Borneo, Malaya, India, the Phillipines and Czechoslovakia. WN virus is also widespread in Africa and the Middle East and isolations have been reported from Egypt, South Africa, Uganda, the Congo, the Central African Republic and Mocambique in Africa and from Israel, India, Pakistan, France, Portugal and the U.S.S.R.

With both viruses, slight antigenic differences have been reported among strains from different countries (Hammam et al. 1965; Theiler and Downs, 1973). Information on SIN virus was last reviewed in 1973 and that on WN virus in 1972 (Catalogue of Arboviruses). The two viruses have also been included in various reviews on the arboviruses (Casals and Reeves, 1965; Coret et al. 1968; McIntosh and Gear, 1974). In an account of the mosquito vectors of arboviruses in Southern Africa, McIntosh (1974) summarized the state of

our knowledge up to the end of 1970 on the vectors of WN and SIN viruses in this region.

SINDBIS VIRUS

The results of investigations on SIN virus carried out in various countries show that it is an avian virus maintained in a feral cycle between birds and ornithophilic mosquitoes. Transmission to man and domestic animals appears to be incidental. All the available information on the isolation of the virus from man, bird and arthropod is given in Table 1. Virus has been recovered from man only in a few instances, probably because the viraemia lasts only for a few days. Thus, human infection is usually diagnosed retrospectively from the antibody response. Isolations have also been made from six species of bird, 19 species of ornithophilic mosquito, mainly of the genus Culex, and 10 species of mosquito not considered ornithophilic. Single isolations have been obtained from the tropical fowl mite, two species of tick and one species of Culicoides. The virus has been transmitted successfully in the laboratory by five different mosquito species.

In the five countries where SIN virus has been isolated from birds, as well as in the U.S.S.R. (Berezin et al. 1968), antibody against this virus has been detected in wild avian populations. McIntosh et al. (1969) showed that inoculation of virus into wild birds produced high viraemia without signs of illness in most species. Subsequently antibody response was determined with the neutralization and haemagglutination-inhibition tests and it was found that the response of some birds was poor, a transient or negative response being produced, and it is apparent that antibody surveys on wild avian populations will only identify a proportion of birds previously infected. Domestic chickens exhibit a low viraemia but often produce antibody except in the very young, so that they are probably not an important source of infection for mosquitoes. Domestic pigeons exposed in South Africa as virus sentinels are frequently infected, with Culex univittatus implicated as the vector responsible for most of these infections (ARU: Arbovirus Research Unit, South African Institute for Medical Research, Johannesburg; unpublished).

TABLE 1. ISOLATIONS OF SINDBIS VIRUS

ARTHROPOD/VERTEBRATE	NO. ISOLATIONS	COUNTRY	REFERENCE
ORNITHOPHILIC MOSQUITOES			
Culex univittatus	7	Egypt	Taylor et al, 1955
C. antennatus	4	"	" " " "
C. univittatus	43	S.A.; Transvaal highveld	McIntosh et al, 1967; ARU[a] unpublished.
	28	S.A.; O.F.S.	ARU, unpublished.
	8	" ; Cape	" "
C. theileri	1	" ; Transvaal highveld	McIntosh et al, 1967
	5	" ; O.F.S.	ARU, unpublished
	4	" ; Cape	" "
C. pipiens	3	" ; Transvaal highveld	McIntosh et al, 1967
	1	" ; O.F.S.	ARU, unpublished
C. neavei [b]	4	" ; Natal	Worth et al, 1961; McIntosh et al, 1972
C. annulioris) C. tigripes) pool	1	" ; Transvaal highveld	Weinbren et al, 1956
C. tigripes	1	" ; Natal	ARU, unpublished
Mansonia africana	1	" ; "	Worth et al, 1961
C. univittatus	1	Israel	Nir et al, 1972
C. pipiens molestus	1	"	" " " "
C. telesilla	2	Central African Republic	Ann Rept Inst. Pasteur de Dakar, 1972
C. weshei	1	" " "	" " " " " " " "
C. decens	1	" " "	" " " " " " " "
C. tigripes	1	" " "	" " " " " " " "

TABLE 1. ISOLATIONS OF SINDBIS VIRUS (CONT.)

ARTHROPOD/VERTEBRATE	NO. ISOLATIONS	COUNTRY	REFERENCE
C. cecens	2	Cameroun	Ann Rept Inst. Pasteur de Dakar, 1972
C. pseudovishnui	11	Sarawak	Simpson et al, 1970
C. tritaeniorhynchus	1	"	" " " "
	1	Malaya	Catalogue of arboviruses, 1973
C. bitaeniorhynchus	1	Philippines	Rudnick et al, 1962
C. pipiens fatigans	1	"	Lockard, 1968
C. annulirostris	5	Australia	Doherty et al, 1963
Aedes africanus	3	Cameroun	Ann Rept Inst. Pasteur de Dakar, 1972
Mansonia africana	2	"	" " " " " " " "
Coquillettidia fuscopennata [c]	1	Uganda	Woodall et al, 1964
NON-ORNITHOPHILIC MOSQUITOES			
C. gelidus	3	Sarawak	Simpson et al, 1970
C. nebulosus	1	Cameroun	Ann Rept Inst. Pasteur de Dakar, 1972
M. annulifera	1	Sarawak	Simpson et al, 1970
Ae. cumminsii	1	S.A.; Natal	McIntosh et al, 1972
Ae. circumluteolus	2	" "	" " " "
Ae. simpsoni	1	Cameroun	Ann Rept Inst. Pasteur de Dakar, 1972
Anopheles pharoensis	1	Egypt	Taylor et al, 1955
An. listeri	1	S.A.; O.F.S.	ARU, unpublished
An. squamosus	1	Cameroun	Ann Rept Inst. Pasteur de Dakar, 1972
Eretmapodites aedipodius	1	"	" " " " " " " "

TABLE 1. ISOLATIONS OF SINDBIS VIRUS - (CONT.)

ARTHROPOD/VERTEBRATE	NO. ISOLATIONS	COUNTRY	REFERENCE
OTHER ARTHROPODS			
Ornithonyssus bursa [a] - Tropical fowl mite	1	India	Shah et al, 1960
Boophilus decoloratus	1	Cameroun	Ann Rept Inst. Pasteur de Dakar, 1972
Amblyomma variegatum	1	"	" " " " " " "
Culicoides spp	1	S.A.; O.F.S.	ARU, unpublished
BIRDS			
Crow (Corvus corone sardonius)	1	Egypt	Taylor et al, 1955
Masked weaver (Ploceus velatus)	1	S.A.; Transvaal highveld	McIntosh et al, 1968
Turtle dove (Streptopelia turtur)	1	Israel	Nir et al, 1967
Wagtail (Motacilla alba)	1	India	Shah et al, 1960
Myna (Gracula religiosa)	1	"	" " " "
Reed Warbler (Acrocephalus scirpaceus)	1	Czechoslovakia	Ernek et al, 1973
MISCELLANEOUS VERTEBRATES			
Sentinel hamster	1	S.A.; Natal	ARU, unpublished
MAN	5	Uganda	EAVRI, 1962
	2	S.A.; Transvaal highveld	McIntosh et al, 1964; ARU unpublished

TABLE 1. ISOLATIONS OF SINDBIS VIRUS - (CONT.)

ARTHROPOD/VERTEBRATE	NO. ISOLATIONS	COUNTRY	REFERENCE
MAN (cont.)	1	Australia	Doherty et al, 1969
	2	Egypt	Abdel - Wahab, 1970

S.A. : South Africa; O.F.S. : Orange Free State province.

a: Arbovirus unit, South African Institute for Medical Research, Johannesburg.

b: For first 3 of these isolations the species was referred to as C. univittatus.

c: Referred to as Mansonia fuscopennata in publication.

d: Coelionyssus burse in publication

In South Africa antibody has also been found in cattle, sheep and horses (McIntosh *et al*. 1962; Dickinson *et al*. 1965) but two calves inoculated with virus (ARU, unpublished) did not become viraemic. Antibody has not been found in the wild rodents of South Africa (ARU, unpublished), but viraemia did occur when *Arvicanthis abyssinicus* was inoculated with virus (LAVRI, 1964). Furthermore, in Australia Whitehead (1969) showed that domestic rabbits, marsupial mice and *Rattus norvegius* were refractory to infection. It would appear that domestic animals and rodents are not vertebrate maintenance hosts of the virus and that antibody, when present, probably reflects a previous 'dead-end' infection, the level of viraemia having been too low to permit the infection of further mosquitoes. SIN virus is widespread in South Africa but antibody surveys show that human infection has occurred most frequently in the Highveld, the inland plateau region (Weinbren, 1955; Kokernot *et al*. 1956; McIntosh *et al*. 1962).

WEST NILE VIRUS

Details of the isolation of WN virus from man, bird and anthropod are given in Table 2. It has been isolated from at least 12 ornithophilic and five non-ornithophilic mosquito species, two species of tick, five species of bird, one bat and from two horses. The ecolo- of this virus appears to be very similar to that of SIN virus wi' maintenance in birds by ornithophilic *Culex* mosquitoes. Thirte different mosquito species have been shown to transmit the viru the laboratory with a varying degree of efficiency.

WN virus is more important as a cause of human disease than SIN virus, clearly shown by the epidemics it has caused in Israel and more recently in South Africa. The clinical aspects of the disease have recently been summarized by McIntosh and Gear (1974). anu vary from a barely recognizable subclinical disease to one causing death. A fatal outcome is rare and occurs mainly in the aged as a sequel to encephalitis, whereas mild infections are most common in the young. As a rule, the symptoms shown are fever, headache, flushed face, sore throat, muscular and orbital pain, maculopapular rash and lymphadenopathy. Clinically the di:sase can be confused with infections caused by SIN, Chikungunya and rubella viruses. As with SIN

TABLE 2. ISOLATIONS OF WEST NILE VIRUS

ARTHROPOD/VERTEBRATE	NO. ISOLATIONS	COUNTRY	REFERENCE
ORNITHOPHILIC MOSQUITOES			
Culex univittatus	12	Egypt	Taylor et al, 1956
C. antennatus	5	"	" " " "
C. univittatus	48	S.A.; Transvaal highveld	McIntosh et al, 1967; ARU unpublished
	13	" ; O.F.S.	ARU unpublished
	33	" ; Cape	" "
C. theileri	1	S.A.; Transvaal highveld	McIntosh et al, 1967
	1	" ; O.F.S.	ARU, unpublished
	4	" ; Cape	" "
C. pipiens	1	" ; O.F.S.	" "
C. neavei	1	" ; Natal	" "
C. univittatus	7	Israel	Nir et al, 1968, 1972
C. pipiens molestus	1	"	Olejnik, 1952
C. modestus	2	France	Hannoun et al, 1964; Mouchet et al, 1970
C. fatigans	1	India	Pavri & Singh, 1965
C. vishnui complex	7	"	Work, 1971
C. weshei	1	Central African Republic	Ann Rept Inst. Pasteur, Bangui, 1969
Coquillettidia metallica [a]	1	Uganda	Woodall et al, 1961
Coq. microannulata	1	S.A.; Natal	ARU, unpublished

TABLE 2 ISOLATIONS OF WEST NILE VIRUS - (CONT.)

ARTHROPOD/VERTEBRATE	NO. ISOLATIONS	COUNTRY	REFERENCE
NON-ORNITHOPHILIC MOSQUITOES			
Aedes circumluteolus	1	S.A.; Natal	ARU, unpublished
Ae. cabalius/juppi	1	" ; U.F.S.	" "
Anopheles coustani	1	Israel	Nir et al, 1968
An. subpictus	1	India	Work, 1971
An. maculipennis	1	Portugal	Filipe, 1972
OTHER ARTHROPODS - TICKS			
Argas reflexus hermanni	2	Egypt	Schmidt & Rufaec, 1964
Hyalomma plumbeum plumbeum	>2	U.S.S.R.	Shalunova et al, 1968; Chumakov et al, 1968
BIRDS			
Pigeon (Columba livea)	2	Egypt	Taylor et al, 1956
Crow (Corvus corone sardonius)	1	"	" " " "
Warbler (Sylvietta rufuscens)	1	S.A.; Natal	Kokernot & McIntosh, 1959
Sentinel pigeon	1	" ; Transvaal highveld	ARU, unpublished
Turtledoves (Streptopelia turtur)	4	Israel	Nir et al, 1967, 1972
Wagtail (Motacilla alba)	1	"	" " " , 1972

TABLE 2 ISOLATIONS OF WEST NILE VIRUS - (CONT.)

ARTHROPOD/VERTEBRATE	NO. ISOLATIONS	COUNTRY	REFERENCE
MISCELLANEOUS VERTEBRATES			
Bat (Rousettus leschenaulti)	1	India	Paul et al, 1970 [1]
Sentinel Hamster	2	Mocambique	ARU, unpublished
Horse	2	Egypt & France	Schmidt & El Mansoury, 1963, Panthier et al, 1966
MAN	3	Uganda	Smithburn, 1940; Woodall et al, 1961; EAVRI, 1963
	Many	Egypt	Melnick et al, 1951; Taylor et al, 1956
	Many	Israel	Marlberg et al, 1956, Spigland et al, 1958
	13	S.A.	Kokernot & McIntosh, 1959, ARU unpublished
	1	Congo	Lucasse, 1963
	2	Pakistan	Burney & Munir, 1966
	1	India	Paul et al, 1970 [2]
	2	France	Hannoun et al, 1964

a: Referred to as Hansonia metallica in publication

virus infection, the isolation of the virus from human blood is difficult because of the low level viraemia but diagnosis can also be made retrospectively from the antibody response.

Antibody has been found in a number of avian species in the three countries where virus has been isolated from birds. McIntosh et al. (1969) showed that after the inoculation of virus into wild birds almost all species tested developed viraemia, showing that they could play an important role in transmission cycles. Sentinel fowls and sentinel pigeons exposed in South Africa are frequently infected and, as with SIN virus, there is good evidence that C. univittatus is mainly responsible for these infections.

In Egypt [illegible] & El Ramsoury, 1963) and in France (Panthier et al. 1966) [illegible] has been isolated from horses which died from encephalo[illegible]itis. In Egypt high antibody rates were also found in horses but experimental infection of horses and donkeys indicated that these animals were unlikely to infect mosquitoes to any extent because of low viraemia. In South Africa, antibody is common in cattle (McIntosh et al. 1962; Dickinson et al. 1965) but, experimentally, the injection of virus into calves failed to produce viraemia (ARU unpublished). Several species of African rodents have been tested for susceptibility but in only a species of Aethomys (McIntosh, 1961) and in Arvicanthis abyssinicus (EAVRI, 1964) was circulating virus found. As with SIN virus, cattle and rodents are probably not important vertebrate hosts. The distribution of WN virus in South Africa is very similar to that of SIN virus as was revealed in the same antibody surveys.

ECOLOGICAL STUDIES OF THE VIRUSES

The ecology of both viruses has received the most attention in Egypt, Israel and South Africa. The only other instance where an extensive ecological study has been undertaken is with WN virus in the Camargue region of France.

Positive observations relating to SIN virus in both Egypt and Israel are fewer than for WN virus, because in Egypt, the studies of Taylor et al. (1955) were preliminary, whereas in Israel SIN virus is much less prevalent than WN virus.

The studies of Taylor et al. (1956) showed that WN virus is widely distributed along the Egyptian Nile and in Southern Sudan, and that it is endemic in the West Nile Delta, where it causes essentially a childhood disease with annual peaks of transmission during mid summer when virus was isolated often from febrile children. Field observations indicated that the virus is maintained in wild birds by the ornithophilic mosquito, C. univittatus. Experiments (Hurlbut, 1956) showed that both this mosquito and Culex pipiens could transmit virus. Other observations by Taylor et al. (1956) suggested that the virus might overwinter in the West Nile Delta through a process of retarded transmission by C. pipiens, a mosquito that remains active throughout the winter.

The investigations of SIN virus in Egypt by the same team of workers (Taylor et al. 1955) indicated that this virus has an ecology similar to that of WN virus involving wild avian species and C. univittatus. Laboratory transmission of SIN virus by this species of mosquito was also demonstrated. Fewer isolations of both viruses were made from Culex antennatus which suggests that this mosquito may play a secondary vectorial role in Egypt.

From the most recent studies in Israel (Nir et al. 1967, 1968, 1972; Margalit & Tahori, 1970) it is evident that WN virus is endemic in that country. It becomes active each summer and in some years quite extensive epidemics may occur among the human population. SIN virus seems to be less prevalent because surveys showed that only a small proportion of avian sera contained antibody against group A viruses. Furthermore, only three isolations of SIN virus have been made, from a turtle dove (Streptopelia turtor), from Culex pipiens molestus and from C. univittatus respectively. Serological surveys did not implicate any other vertebrates except birds as hosts of either virus.

In both the Nile Delta and Israel, C. univittatus has been incriminated as the arthropod maintenance host in the feral cycle of WN virus and as the vector responsible for transmitting the virus to man. The same probably applies to SIN virus. The mosquito referred to in both these countries as C. univittatus is readily attracted to both birds and man (Hurlbut & Weitz, 1956; Nir et al. 1968). However, in South Africa, this species, although strongly ornithophilic, is

only poorly anthropophilic (Jupp & McIntosh, 1967). Apart from this ecological difference, the Egyptian form of the mosquito differs morphologically from the typical form of C. univittatus which occurs in South Africa (Jupp, 1972). There are also differences which show up in the laboratory - the Egyptian form was reported to be stenogamous as it mated readily in small cages (Hurlbut & Weitz, 1955), whereas the South African Highveld form is eurygamous and requires a large cage and special lighting conditions before it will mate readily (Jupp & Brown, 1967). Thus the evidence suggests that the Egyptian form may be a species distinct from C. univittatus. The Israeli form of the mosquito is probably taxonomically the same as that from Egypt (Jupp, 1972; Mattingly, 1954). Typical C. univittatus from South Africa and the Egyptian-Israeli form appear to belong to a group of closely related allopatric species which also includes Culex neavei from the sub-tropical coastal lowlands of Natal in South Africa. C. univittatus is not found in the sub-tropical coastal plain (Jupp, 1971) which may explain why SIN virus is fairly common but WN virus rare in this region as C. neavei is probably an efficient vector of SIN virus but not of WN virus.

A common ecology for the two viruses gains additional support from the work by Ben-Porath and co-workers (1966) in Israel. Their serological survey of man showed that antibody to SIN virus was much more frequent in persons who also had antibody to WN virus.

It was of great interest when WN virus was found to be active in France in the Rhone delta because up to this time its distribution was thought confined to Africa, the Middle East and India. Thirteen human infections by WN virus were diagnosed between 1962 and 1964 (Panthier et al. 1966). The virus was recovered from two of these patients and two isolations were made from Culex modestus (Hannoun et al. 1964). An ecological study of this mosquito (Mouchet et al. 1970) showed that it has unusually catholic host preferences, feeding on man and a wide range of animals including birds. It is thought to be the only vector responsible for maintaining the virus.

The virus content of the 'acute phase' blood of infected persons has been ascertained in the case of WN virus and the highest titre recorded was 63 LD_{50}/0.03 ml of serum (Goldblum et al. 1957). The level of viraemia in patients infected with SIN virus has not been measured but also appears to be low. Because of this low viraemia with both viruses in human beings, a man-mosquito-man cycle has not

been thought epidemiologically important. However, there is evidence, in South Africa, that such a cycle might be of some importance with the transmission of WN virus.

In South Africa extensive investigations have shown a similar maintenance cycle to that in Egypt and Israel for the two viruses. Field investigations carried out in the Transvaal Highveld (McIntosh et al. 1967; Jupp & McIntosh, 1967), as well as in the Orange Free State and Northern Cape Province (ARU, unpublished) show that both viruses are maintained in feral transmission cycles between species of wild birds and C. univittatus, a largely ornithophilic mosquito. Studies on the response of common Highveld birds to inoculation with each virus (McIntosh et al. 1969) and the results of antibody surveys in these birds (McIntosh et al. 1966, 1969[2]) have provided the additional evidence that they are maintenance hosts of both viruses. The two viruses are widespread in South Africa and are enuemic in the Highveld region where there is regular summer infection of birds and C. univittatus and sporadic human infection. Although minor outbreaks of disease due to both viruses may occur in man, only one epidemic on a large scale has been recorded in South Africa. This was a recent epidemic caused by WN virus at Upington in the Northern Cape Province (ARU, unpublished). In South Africa, as in Egypt and Israel, it seems that most human infections would result from mosquitoes which had acquired virus from birds rather than from other humans, since both viruses circulate at high titres in the blood of avian species but at a low level in human blood and this suggests that human infection will largely occur in close temporal and special relationship to avian infection.

ARTHROPOD VECTORS OTHER THAN MOSQUITOES

As has already been emphasized no vertebrate animals other than birds seem to be of any significence as maintenance hosts in the feral cycles of either virus. The question arises as to whether the arthropod maintenance host is similarly confined to mosquitoes. The findings made in the various countries indicate that mosquitoes occupy the primary vectorial role but there is some evidence that ticks may play a minor part as will be discussed below. Isolations

from any other arthropods are rare but there are two such recoveries of SIN virus. The first was isolated from a pool of the tropical fowl mite, Ornithonyssus bursa (Shah et al. 1960). These mites were taken off domestic chickens in India but the isolation was probably due to virus present in the blood recently imbibed by the mites from a viraemic fowl. The second isolation was made from a pool of Culicoides species collected in South Africa (ARU, unpublished) and the presence of virus in these insects probably had a similar origin.

There have been several viral isolations from wild-caught ticks (Tables 1 and 2) but laboratory transmission tests have not yet been undertaken with the species concerned. Those transmission tests which have been reported were done on other species. Two strains of WN virus were recovered from the avian tick, Argas reflexus hermanni, collected in the Nile Delta (Schmidt & Mufeed, 1964). This collection was made in mid-winter and the species has a close association with birds known to be susceptible to WN virus. This led to the suggestion that A. reflexus hermanni might be an overwintering vector of the virus, but it is not known if this particular argasid can transmit it although Hurlbut (1956) showed that Argus persicus was insusceptible to infection by either WN or SIN virus. Several strains of WN virus have been recovered from Hyalomma plumbeum plumbeum (Shalunova et al. 1968; Chumakov et al. 1968) in the Astrakhan region of the U.S.S.R. As the nymphs of this tick may feed on birds, transmission tests would be a worthwhile undertaking. Experiments by Hurlbut (1956) with Ornithodorus species, presumably adults, and with WN virus demonstrated that O. savignyi and O. erraticus could sometimes become infected but could not transmit, whereas the nymphs of O. erraticus and O. coniceps transmitted the virus successfully (Vermeil et al. 1958, 1960). Individuals of O. coniceps in this test remained infected for ten months at 22°C. Other successful transmissions with WN virus have been reported for nymphal and adult O. moubata (Whitman & Aitken, 196[illegible]), although these may have been due rather to surface contamination with tick excreta than to infective bite. Vermeil and co-workers collected O. coniceps from a colony of gulls but apparently Ornithodorus species usually parasitize mammals including bats and only occasionally attack birds (Hoogstraal, 1956).

SCOPE OF INVESTIGATIONS FOR THESIS

The foregoing account is a summary of the present state of our knowledge of the ecology and epidemiology of WN and SIN viruses. As can be seen considerable attention has been given to the investigation of these viruses in South Africa. When the studies on which this thesis is based began, certain field observations relating primarily to the mosquito vectors had already been completed (McIntosh et al. 1967; Jupp & McIntosh, 1967; Andersen, 1967), together with field and laboratory studies on the avian hosts (McIntosh et al. 1966; 1969 [1 & 2]). It had emerged from this earlier work in the Highveld region that C. univittatus is almost certainly the maintenance vector while, according to ecological evidence alone, several other species might be involved as vectors but to a lesser degree. However, the relative capability and significance of C. univittatus and the other Highveld mosquito species as vectors was by no means fully understood. Hence it was considered necessary to know more of the ability of C. univittatus and the other ornithophilic species to participate in feral transmission of both viruses as well as to establish the identity of the vector or vectors transmitting infection to man. From the previous work it was unlikely that C. univittatus could be the main vector which transmits infection to man as its feeding habits did not suggest this. Thus other species came into consideration as link vectors which could transfer virus from infected birds in the feral cycles over to man. Such species would need suitable feeding habits and a high level of vector capability. As epidemics due to either virus have not occurred in South Africa until very recently, it has not been possible to make observations during such events so as to collect direct evidence as to the identity of the vector or vectors transmitting infection to man. Therefore it was hoped that from a study of feeding habits and vector capability of different species of mosquito, evidence might be gathered as to the identity of such vector or vectors. Furthermore, the mechanism by which the two viruses survive the Highveld winter to become active again in the summer remained unknown, so field studies were undertaken on the winter biology of Highveld Culex species to learn if the mosquito vector could be responsible for this.

Thus it can be seen that three different principal avenues of

investigation have been adopted in this thesis so as to make a broad approach to reaching the objectives of the study referred to above. These main lines of study were field observations on feeding habits, laboratory studies on vector capability and field studies on the winter biology of Highveld mosquitoes.

STUDY AREAS

The Highveld region of South Africa is outlined on the map in figure 1. It is the high inland plateau of undulating grassland, lying between 1200 and 1525 metres above sea level, which occupies the small country of Lesotho and the following portions of the Republic of South Africa : the Orange Free State province (except the south-west), the Transvaal province (except the northern and eastern bushveld) and a very small portion of the Cape province. The Highveld has a temperate climate and summer rains; the mean annual temperature is 13°C - 17°C and the mean annual rainfall 500 - 700 mm. During the cold dry winter at Olifantsvlei, one of the main study areas, mosquito activity falls to a very low level. Spring can be said to begin at the beginning of September when mean daily temperatures rise to about 16°C - 17°C and the mosquitoes start to increase in number in baited traps just before the onset of the summer rains. From the middle of May until the start of June mean daily temperatures fall to about 9°C - 14°C and mosquito activity drops to a low level. For the studies of feeding habits, mosquito collections were made during the summer months from December to March when mosquito density was high, while the studies of winter biology started in May and continued until the end of October.

The field observations were carried out in three different study areas on the Highveld, at Olifantsvlei, at Lake Chrissie and in the north-eastern suburbs of Johannesburg. The topography of the first two areas has been described in detail by McIntosh and his co-workers (1967), so only an outline will be given here.

Olifantsvlei is a cattle farm and effluent disposal area 1500 m. above sea level and situated about 21 km. south-west of Johannesburg. The part of Olifantsvlei where fieldwork was conducted is rural in nature and is well watered by effluent. This causes the accumulation

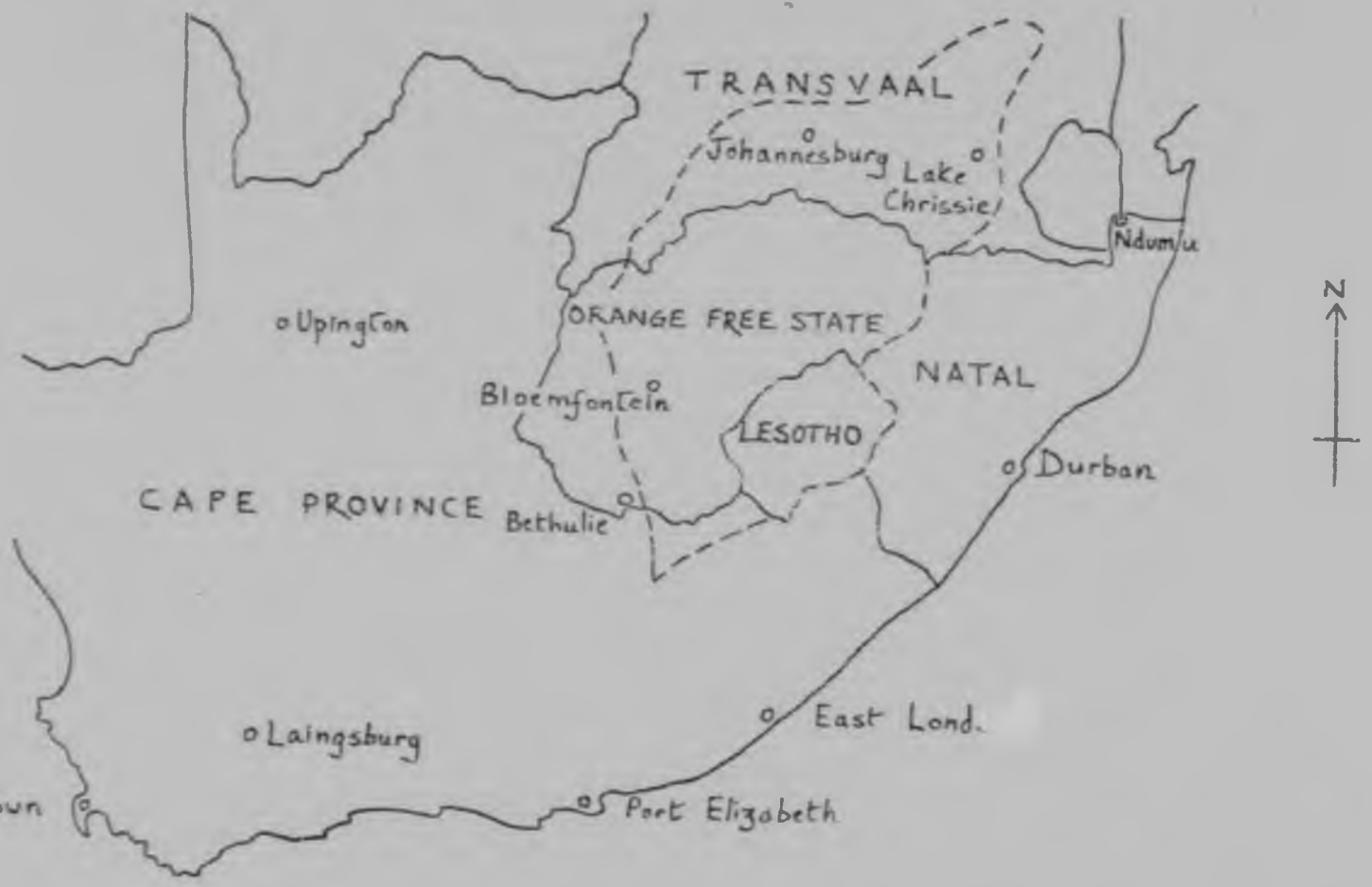

Fig. 1. South Africa with borders of the Highveld region shown by the dotted line.

of semi-permanent or permanent water in furrows and on grazing lands thus providing suitable mosquito breeding sites and attracting many birds. A prominent feature is the small perennial flowing Klip river into which surplus effluent is discharged. In the river bed are extensive reeds which provide shelter and roosting sites for numerous birds.

Lake Chrissie is fairly typical grassland country of the eastern Highveld. The sheep and cattle farms where observations were made are exceptionally well watered for Highveld, with several pans, some very large and permanent. Lake Chrissie was chosen as representative of a more undisturbed Highveld habitat, as compared with Olifantsvlei which is much wetter than typical Highveld country.

A series of collections of mosquitoes from inside houses was made in three suburbs located to the north-east of Johannesburg about 12 km. from the city centre. Some of the overwintering studies and one series of mosquito collections with baited traps were conducted at Rietfontein in the grounds of the Poliomyelitis Research Foundation. The last locality is adjacent to the other suburbs but has large grounds with cultivated fields and trees so that it is partly suburban and partly rural in character.

CHAPTER 2

FIELD STUDIES ON FEEDING HABITS

One aspect of the earlier studies of the ecology of WN and SIN viruses in the Highveld was the bionomics of the mosquito species occurring here (Jupp and McIntosh, 1967). Over a three-year study period data were collected on prevalence, including for some species observations of seasonal fluctuation, feeding preferences and other host-seeking behaviour. This information was used, in conjunction with evidence on viral infectivity of wild-caught mosquitoes collected in the parallel study (McIntosh et al. 1967), to assess the likely vectorial rôles of the various mosquitoes. The conclusion reached was that only five species were sufficiently prevalent to warrant their classification as vectors of epidemiological significance. These were Culex (Culex) univittatus Theobald, Culex (Culex) theileri Theobald, Aedes (Neomelaniconion) lineatopennis (Ludlow) (A. (N) unidentatus McIntosh), Aedes (Ochlerotatus) caballus (Theobald) (A. (O) juppi McIntosh) and Culex (Culex) pipiens Linnaeus. C. univittatus, it was concluded, is the primary wild cycle vector of both viruses. This species feeds mainly on wild birds and domestic fowls but has a low feeding preference for man. Thus it was thought unlikely that C. univittatus was the main transmitter of infection to man. Instead it was suggested that some, if not most, human infections were transmitted by C. theileri and/or A. lineatopennis (A. unidentatus), both of which feed on birds and man and will enter houses. Few observations were collected on A. caballus (A. juppi) due to its scarcity in the main areas studied, but it might also transmit infection to man. C. pipiens was found to be almost exclusively ornithophilic and on this account could only be involved in the wild cycles, or in infection among domestic fowls.

Because of these uncertainties further field studies were made on the feeding habits of Highveld mosquito species. It was hoped that such studies would lead to the acquisition of further evidence as to the identity of likely link vectors which transfer virus from the feral cycle to man thus causing human infection. The extent to which each species was ornithophilic and anthropophilic, its vertical distribution during feeding and whether endophagy occurred were

aspects investigated. Such studies also provided additional information on prevalence. The investigations reported were made during the summer months of 1964 to 1972, most observations being collected in 1971 and 1972. The study areas, noted in Chapter 1, were Olifantsvlei near the southern outskirts of Johannesburg, two farms at Lake Chrissie in the eastern Transvaal and several suburbs in north-eastern Johannesburg.

As frequent reference to the initial paper on mosquito bionomics (Jupp and McIntosh, 1967) will be necessary, it will be referred to simply as 'the previous study'.

METHODS

Mosquito collecting

In the case of most of the collecting methods used only a brief outline is necessary as a full description is given in the previous study.

(1) Fowl-baited traps. A Bellamy and Reeves (1952) larg-can baited with a single adult fowl secured in a nylon stocking, a fowl-pen containing eight - ten unrestrained adult fowls and a net-trap containing 14 pullets with their legs taped together were set overnight. The proportion of engorged to unengorged mosquitoes in each catch was recorded. The catch in the net-trap was removed using a mechanical aspirator.

(2) Catches with human bait. A man-baited net-trap was used and hand collections off human bait in the open were also undertaken. In the former an individual sat within the trap collecting with test tubes those mosquitoes which alighted on his bare legs, while mosquitoes which merely entered the trap were removed at the completion of the catching period. In this trap the mosquitoes caught alighting were recorded as fed and the others as unfed. Hand collections off human bait were conducted as in the previous study except that each catcher operated individually collecting mosquitoes which alighted on his bare legs. At Lake Chrissie six catchers were employed and one or two at Olifantsvlei. In both methods collecting started half-an-hour after sunset and lasted for 1 or 1½ hours.

(3) Pigeon-baited suction trap. This trap was not used in the previous study. Operation of a suction trap powered by a 12-volt car battery on a cage containing pigeons was first used to sample mosquitoes attracted to pigeons exposed as sentinels (McIntosh, unpublished). The same technique was used in the present study at Lake Chrissie. The cage, housing two pigeons, was made of 5 cm wire-mesh and was fastened to poles so that the bottom was about 1½ m above the ground. The suction motor unit was inserted through an opening in the floor of the cage so as to apply suction close to the pigeons' perch. Mosquitoes were drawn downwards into a collecting cage. Catches were almost exclusively non-engorged individuals showing that the draught prevents mosquitoes from feeding.

(4) Solid carbon dioxide-baited lard-can traps. These were used for the vertical distribution study.

(5) Room collections. The door and window of a room at Olifantsvlei were left open and the light switched on from sundown until 2130 hours when the occupant returned and collected resting mosquitoes with a mechanical aspirator.

(6) House collections. These were conducted in several houses of three residential suburbs in north-eastern Johannesburg. Resting mosquitoes were collected early in the morning either in test tubes by hand or with a mechanical aspirator. After killing the mosquitoes, the proportion of fed to unfed was recorded and in the case of most of the engorged specimens a precipitin test was carried out to determine the vertebrate host from which the blood-meal originated.

Analysis of results

The results were presented in a manner similar to that of the previous study, using Williams' modification of the geometric mean (Mw), such means expressed as percentages and the 'mean percentage fed'. The latter is the arithmetic mean of the percentages engorged from all catches of a series. In the hand collections off human-bait in the present study, however, relative mosquito attraction was expressed as the number of mosquitoes biting per man-hour. Such figures were also converted to a percentage basis.

Mosquito identifications

The nomenclature used for anopheline mosquitoes follows that of Gillies and De Meillon (1968), while that used for culicines is after Stone et al. (1959) and Stone (1967) except in the case of their Culex (Culex) pipiens quinquefasciatus Say. The two members of the Culex pipiens complex occurring in South Africa are referred to as C. (Culex) fatigans Wiedemann and C. (Culex) pipiens Linnaeus as it is believed that they are two distinct species in this country.

During the course of the present study taxonomic revisions (McIntosh, 1971, 1973) have resulted in some nomenclative changes affecting two Highveld culicines. Among Highveld populations of what have previously been regarded purely as Aedes (Neomelaniconion) lineatopennis (Ludlow) and as Aedes (Ochlerotatus) caballus (Theobald), two new species, Aedes (Neomelaniconion) unidentatus McIntosh and Aedes (Ochlerotatus) juppi, McIntosh respectively, have been identified. The identification of these new species has shown that A. unidentatus is the dominant Neomelaniconion species on the Highveld at the localities sampled in the present study, although A. lineatopennis does also occur. A. caballus (sensu stricto) does not occur and it appears unlikely that it occurs at all in the Highveld. The existence of the two Neomelaniconion species was known before the Lake Chrissie collections were made and before most of the collections containing aedine mosquitoes were undertaken at the other localities. A. lineatopennis was not detected at Olifantsvlei but a small proportion of this species was detected together with A. unidentatus in samples taken at Lake Chrissie. However, only A. unidentatus is listed in the collections from this locality because for practical reasons it was not possible to separate the two species. It was assumed retrospectively that all the Ochlerotatus material collected belonged to A. juppi.

Some other specimens collected at Lake Chrissie were identified as Culex (Culex) terzii Edwards but these might have been C. (C.) vansomereni Edwards from which it is not readily distinguishable in the female. However, C. vansomereni has not been encountered previously in the Transvaal.

C. pipiens complex

The members of this complex include the most widely distributed

mosquitoes in the world and it is usually regarded as a single polytypic species (Mattingly, 1967). However, the two South African forms appear to meet the requirements for two discrete species. The basis for this view is that although they occur sympatrically in South Africa the writer has not been able to detect any hybridization, even after examining material of both forms reared from larvae which were collected together at the same breeding site. Larvae of the two forms have been frequently found together.

The two forms can be clearly differentiated morphologically on the basis of the usual male genitalia characters, and under results clear differences in their feeding habits will be presented. The South African form of C. fatigans is probably the same or very close to the mosquito referred to in other countries as either C. pipiens fatigans or C. pipiens quinquefasciatus, which is fairly uniform in appearence and biological characteristics (Barr, 1967). South African C. pipiens, on the other hand, appears to differ from the C. pipiens pipiens found elsewhere. According to Barr (1967), C. pipiens pipiens is a very variable form, both morphologically and biologically and the writer has found the local form to be more eurygamous than the forms of the mosquito occurring in Europe, North America and Egypt which apparently mate in relatively small cages. C. pipiens pipiens in other countries may also be more strongly anthropophilic than the local form.

At the time of the previous study it was thought that only C. pipiens occurred in the rural habitats of Olifantsvlei and Lake Chrissie. More recently larvae of C. fatigans have been found on two occasions at Olifantsvlei. However, as these were the only C. fatigans identified by examination of males reared out from either adult females or larvae collected on many occasions at this locality, it would seem that C. fatigans is very rare at Olifantsvlei.

In studying females belonging to the complex collected from the north-eastern suburbs of Johannesburg it was necessary to distinguish the two species since both commonly occur in this area. This was done on the basis of a difference in the wing venation.

Precipitin test

The method used for the preparation of antisera and the technique employed for the test itself were those described by Anderson (1967).

RESULTS

Collections with human bait

(a) Hand collections. The results of these collections are given in Table 3. At Olifantsvlei the most avid feeders on man were Culex (Culex) theileri Theobald, A. unidentatus, C. pipiens and Aedes (Aedimorphus) dentatus (Theobald) in that order. These species together comprised 93% of the total catch per man hour. However, the unexpectedly high attraction for man shown by C. pipiens is believed to be misleading and is probably the result of the exceedingly high autumn densities reached by this species at the time when two of the five collections were made. In 4½ man-hours over the two evenings concerned 216 of the total 232 C. pipiens were collected off human bait and the catch in dry-ice baited lard-can controls set overnight on one of the two evenings averaged 3573 C. pipiens per trap. This observation serves to underline that at very high densities a mosquito's normal host preference range becomes modified. In this case a usually poorly anthropophilic mosquito (Jupp and McIntosh, 1967) bit man readily. At Lake Chrissie three of the same species as at Olifantsvlei showed the most preference for man but in a different order, namely, A. dentatus, C. theileri and A. unidentatus. These made up nearly the whole catch per man-hour and the density of A. dentatus was notably high. Culex univittatus was almost absent in the catch from each locality indicating the poor anthropophilism of this species which was prevalent at both localities at the time of these collections. C. pipiens was not taken at Lake Chrissie. Reference to Table 3 shows that several anophelines occurred in small numbers in the collections but only two species, An. (Cellia) schwetzi Evans and An. (Anopheles) tenebrosus Donitz were at all prevalent. These were taken biting at Olifantsvlei. Of interest was the collection of C. terzii biting man at Lake Chrissie.

(b) Net-trap. The results for the man-baited net-trap used at Olifantsvlei and Lake Chrissie are given in Table 4; those species collected only in small numbers have been omitted. This trap permitted determination of the proportion of each species alighting on man, presumably to feed, in relation to the total number attracted

TABLE 3

Relative attraction of mosquitoes for man according to human-bait catches at Olifantsvlei and Lake Chrissie

	Olifantsvlei			Lake Chrissie		
	Total mosqs. collected (8.25) [a]	No. per man hour	No. per man hour as %	Total mosqs. collected (30.5)	No. per man hour	No. per man hour as %
An. coustani	13	2	1	20	<1	<1
An. marshalii	0	-	-	28	<1	<1
An. schwetzi	43	5	3	0	-	-
An. squamosus	2	<1	<1	6	<1	<1
An. tenebrosus	27	3	2	0	-	-
A. centatus	108	13	8	1282	42	76
A. juppi	8	1	<1	2	<1	<1
A. mixtus	17	2	1	7	<1	<1
A. uniuentatus	252	31	20	86	3	6
Coq. fuscopennata	0	-	-	3	<1	<1
C. annulioris	0	-	-	11	<1	<1
C. pipiens	232 [b]	28	18	0	-	-
C. terzii	0	-	-	14	<1	<1
C. theileri	513	74	47	296	10	18
C. univittatus	1	<1	<1	1	<1	<1

a - Number of man-hours

b - This high figure is misleading - for explanation refer text.

TABLE 4

Mosquitoes collected in a man-baited net-trap
at Olifantsvlei and Lake Chrissie

	Olifantsvlei (7) [a]			Lake Chrissie (3)		
	Total mosqs. collected	No	Mean % fed	Total mosqs. collected	No	Mean % fed
An. tenebrosus	15	4	93	-	-	-
A. dentatus	18	5	97	126	22	73
A. juppi	21	3	54	-	-	-
A. mixtus	59	9	70	1	<1	
A. unidentatus	51	8	90	1	<1	
C. pipiens	243	45	3	1	<1	
C. theileri	478	39	52	29	7	72
C. univittatus	147	8	2	4	1	0

a - Number of collections

to him. Experience with the two methods of human-baited collecting showed, however, that on an equal effort basis, considerably fewer mosquitoes were collected alighting on bait inside the trap compared with the numbers collected alighting on bait in the open. The net apparently excludes some of the mosquitoes. This may account partly for the much smaller number of C. pipiens alighting on man in the net-trap at Olifantsvlei as compared with numbers caught off human bait in the open at this locality. However, it is believed that the main reason for this difference is that the net-trap series of collections did not take place on any evenings during the autumn when densities of C. pipiens had reached such high levels. It can be seen that An. tenebrosus, the four aedine species which include Aedes (Aedimorphus) mixtus Edwards, and C. theileri fed readily while C. pipiens and C. univittatus were only slightly anthropophilic. The number of collections at Lake Chrissie was insufficient to sample adequately A. unidentatus which was shown by the hand collections to be quite prevalent there.

Collections with avian bait

(a) Net-traps baited with fowls. The percentages fed for a series of these collections carried out at Olifantsvlei and at Lake Chrissie respectively are shown in Table 5. The higher feeding rates shown by the mosquitoes at Lake Chrissie are thought to have two possible causes, (a) the omission to tape together the legs of the bait pullets at every trapping of the series at Olifantsvlei and (b) the high density of the strongly ornithophilic C. pipiens entering the trap at this locality tending to displace the other species during feeding by monopolizing the bait. As demonstrated in the previous study C. pipiens is relatively rare at Lake Chrissie. The anophelines which entered the traps in small numbers are omitted from the Table. However, only a few females of those anopheline species collected were engorged, indicating that An. (Anopheles) coustani Laveran, An. (Cellia) marshalli (Theobald), An. schwetzi, An. (Cellia) squamosus Evans and An. tenebrosus do not prefer birds. Culex (Culex) annulioris Theobald, C. pipiens and C. univittatus showed high feeding rates and C. tarsalis a low one; that for C. theileri was moderate at Lake Chrissie.

However, taking the results from both localities into account

TABLE 5

Mosquitoes collected in a net-trap baited with
fowls at Olifantsvlei and Lake Chrissie

	Olifantsvlei (5) [a]			Lake Chrissie (6)		
	Total mosqs. collected	Mw	Mean [b] % fed	Total mosqs. collected	Mw	Mean % fed
A. dentatus	53	11	9	963	90	16
A. mixtus	38	5	(20)	11	1	(40)
A. unidentatus	60	11	13	46	5	28
Coq. microannulata	-	-	-	19	3	(6)
C. annulioris	-	-	-	41	3	18
C. pipiens	2821	500	21	81	11	57
C. terzii	-	-	-	41	3	2
C. theileri	732	114	6	280	21	13
C. univittatus	988	134	20	313	43	46

a - Number of nights trap set.

b - Arithmetic mean of percentage feeding in each collection, figures in parenthesis indicate means calculated from percentages which are based on inadequate numbers of mosquitoes.

C. theileri, A. dentatus, and A. unidentatus showed appreciable rates of feeding. Noteworthy is the unusually high catch of A. dentatus in the series of collections at Lake Chrissie and the mean percentage feeding for this species which in the present study is higher than that obtained in net-traps at Olifantsvlei. In respect of A. unidentatus, if the two feeding rates (13% and 28%) obtained here are compared with that previously obtained at Olifantsvlei (33%) it is evident that this mosquito shows a significant degree of ornithophilism.

(b) Fowl-pens. In Table 6, which shows the results from a series of 11 fowl-pen trappings at Lake Chrissie, figures are given for the relative attraction for fowls of the three main Culex species, the only species which entered the trap in any number. The feeding rates are also shown which give a similar picture of preferential feeding as determined with the net-traps, except that the rate for C. theileri is considerably higher (45%). This rate indicates that this mosquito will feed readily upon fowls on the ground and may be compared with the rate of 14% determined for fowl-pen traps at Olifantsvlei in the previous study. The difference between the two localities, also evident in the net-trap described above but more marked in the fowl-pen collections, may indicate that the population of C. theileri at Lake Chrissie is more ornithophilic than that at Olifantsvlei, or possibly that at Olifantsvlei the large number of C. pipiens displaces C. theileri during feeding.

(c) Fowl-baited lard-cans. Such traps were used at Lake Chrissie and at Rietfontein, a north-eastern suburb of Johannesburg. At the first locality this trap was used mainly to investigate the ornithophilic tendency of the Coquillettidia species which do not enter the net-trap readily and at the second to find if C. fatigans was ornithophilic. The latter species is common in the suburbs of Johannesburg in addition to C. pipiens. Confinement of the bait chicken in a nylon stocking gave high feeding rates for all species of mosquito entering the trap which precludes a comparison of such rates. However, the results given in Table 7 confirm the bird-feeding habit of C. annulioris, C. pipiens, C. univittatus and C. theileri shown in the other traps and also demonstrate ornithophilism in C. fatigans, Coq. (Coquillettidia) microannulata (Theobald) and Coq. (Coquillettidia) fuscopennata (Theobald). Small numbers of Culex (Lutzia) tigripes Grandpré & Charmoy, not included in Table 7, also

TABLE 6

Mosquitoes collected in a fowl-pen trap over eleven nights at Lake Chrissie

	Total mosqs. collected	Mw	Mw as %	Mean % fed
C. pipiens	22	1	3	45
C. theileri	1048	29	73	45
C. univittatus	452	10	24	76

entered the traps at both localities.

(d) Suction traps. It was hoped that this type of trap would catch representatives of all species of mosquito, including the biting species which feed upon pigeons and that the results obtained would complement those from the fowl-baited net-traps. Table 8 gives the results for a series of such traps set together with empty control traps at Lake Chrissie. Only C. univittatus, Coq. microannulata and probably Coq. fuscopennata entered the traps readily thus confirming the ornithophilism of these species demonstrated by fowl-baited lard-cans. A large number of Coq. microannulata was collected. Unfortunately the trap appears selective in that it collected so few C. theileri and A. dentatus, both species common at the same time in human-baited catches. The explanation may lie in the height of this trap above ground-level as compared with the net-trap where the bait is on the ground.

Vertical distribution study

A series of 29 collections was made at Olifantsvlei to compare mosquito densities within 1 m of ground level and 7.6 m above in the

TABLE 7

Mosquitoes collected in a fowl-baited lard-can trap at Lake Chrissie and at Rietfontein.

	Lake Chrissie (12) [a]			Rietfontein (7)		
	Total mosqs. collected	Mn	Mean % fed	Total mosqs. collected	Mn	Mean % fed
C. annulioris	71	2	100	2	<1	
C. fatigans	-	-	-	78	10	85
C. pipiens	154	7	91	129	16	92
C. theileri	420	8	86	0	-	
C. univittatus	2014	55	94	119	15	89
Coq. fuscopennata	18	1	100	0	-	
Coq. microannulata	646	18	92	0	-	-

a - Number nights trap set.

TABLE 8

Mosquitoes collected in suction traps attached to sentinel pigeon cages and in unbaited controls at Lake Chrissie

	Suction trap + pigeons (18) [a]			Control (18)	
	Total mosqs. collected	Mw	Mw as %	Total mosqs. collected	Mw
A. centetus	3	<1	<1	1	<1
A. juppi	0	-	-	1	<1
A. mixtus	0	-	-	1	<1
A. unidentatus	1	<1	<1	1	<1
Coq. fuscopennata	13	<1	<1	0	-
Coq. microannulata	1135	38	65	72	1
C. annulioris	6	<1	<1	0	-
C. pipiens	8	<1	<1	0	-
C. theileri	7	<1	<1	13	<1
C. univittatus	787	20	34	9	<1

a - Number of traps set.

canopy of a tree. The trap was the lard-can baited with solid carbon dioxide and it was assumed that mosquitoes collected represented those seeking a blood-meal. This project was a continuation of the exploratory trapping reported in the previous study. The numbers with their geometric means of the different species collected are shown in Table 9. It is clear that C. pipiens and C. univittatus are much more active in the canopy than at ground level, whereas C. theileri is far more active on the ground. As expected the four sedine species were rare but with each a larger number was collected at ground level. In the case of A. dentatus and A. unidentatus the catches were sufficiently large to indicate that this ground-haunting habit was real.

Room collections

The terms used to classify house-frequenting mosquito behaviour are those of Senior-White (1954). Endophily defines the habit shown by the mosquito that remains within a man-made shelter throughout the whole or a definite part of its gonotrophic cycle and exophily that of the one where the greater part of the gonotrophic cycle is spent out of doors. In both these cases food may be sought within or without a man-made structure. Endophagy is the habit of obtaining the blood-meal within a man-made structure, be this a human dwelling or an animal dwelling, whereas exophagy is the habit of seeking the blood-meal out-of-doors.

Those mosquitoes which entered a lighted empty room at Olifantsvlei on four successive nights were collected resting on the walls. The numbers of the different species taken are given in Table 10 and as a comparison the numbers collected in the human-baited and fowl-baited net-traps for the same period. In interpreting the table it should be remembered that the fowl-baited net-trap was set overnight, while the other two catches occurred during the first half of the evening only. The results indicate that all the species usually found at Olifantsvlei enter a lighted room and it appears likely that each species will do this readily, probably a phototaxic response. These findings may be compared with those from similar collections made in the same room during the previous study in which the light was turned off except while collecting was in progress and in which a man was present as bait. In those collections only three species were prevalent, An. tenebrosus, A. unidentatus and C. theileri,

TABLE 9

Numbers of mosquitoes, with their geometric means, collected in lard-can traps with dry ice bait at ground level and in a tree canopy over 29 nights at Olifantsvlei

	Ground level		7½ metres	
	No.	Mw	No.	Mw
A. dentatus	165	2	3	<1
A. juppi	13	<1	3	<1
A. mixtus	8	<1	1	<1
A. unidentatus	84	<1	8	<1
C. pipiens	4382	82	12882	271
C. theileri	999	23	191	5
C. univittatus	702	15	1560	37

TABLE 10

Numbers of mosquitoes collected in a lighted room on four nights at Ulifantsvlei. As controls the results for man-baited and fowl-baited net-traps set outside are shown.

	Total mosquitoes collected		
	Room	Net + man	Net + fowl
An. coustani	1	0	.
An. schwetzi	0	3	.3
An. squamosus	1	0	0
An. tenebrosus	4	14	4
A. centatus	24	16	42
A. juppi	11	4	2
A. mixtus	17	41	37
A. unidentatus	28	38	47
Cog. fuscopennata	3	1	3
C. pipiens	75	43	1952
C. theileri	101	64	422
C. univittatus	91	21	597

indicating that these were probably endophagic species entering the room to feed. In the present series nearly all the mosquitoes were neither engorged nor gravid. Probably this catch was composed of hungry individuals of both endophagic and exophagic species, but phototaxis would serve to enhance the juxtaposition of these mosquitoes and the human host indoors. In the case of an endophagic species and to a lesser extent in an exophagic species this would be expected to lead to feeding. Evidence for the identity of endophagic species is given by the house collections dealt with below.

Collections in houses

The object of these collections was to determine the endophagic man-biting species in suburban Johannesburg. Table II shows the number of mosquitoes of different species taken resting in 29 morning house collections. From the proportion of mosquitoes engorged and the number of human blood-meals detected by the precipitin test, it is evident that C. fatigans was the most highly endophagic man-feeder. The strongly anthropophilic tendency shown by this species is particularly noteworthy. The next most endophagic mosquito was C. theileri which probably fed mainly on man, although too few of the engorged individuals of this species were precipitin-tested to confirm this. The collections also indicate that A. juppi and C. univittatus show a degree of endophagism and C. univittatus, according to the precipitin test results, bites man indoors at least to a small extent. The small number of aedine species appearing in the catches may be a reflection of the rarity of these species at the localities of collection rather than an indication of their unwillingness to enter houses. Of interest were the few individuals of Culiseta (Allotheobaldia) longiareolata (Macquart) in the catches. These collections were not designed to investigate endophilism and exophilism. However, it was noticed that C. fatigans may remain inside houses for one or more days after the blood-meal which indicates endophilic behaviour.

DISCUSSION

Most collections were carried out by using baited catches to study host preferences. Preferences were assessed mainly from

TABLE 11

Numbers of mosquitoes with percentage engorged collected resting inside houses in three Johannesburg suburbs. In some cases the origin of blood-meals as determined by the precipitin test is included.

	Mosquitoes collected and proportion engorged (29) [a]			Blood-meal origin from precipitin tests		
	Total No.	No. fed	% fed [b]	No. mosqs. tested	Human	Other host
An. tenebrosus	1	-	-			
A. centatus	1	-	-			
A. juppi	15	3	20			
A. unicentatus	6	1	25			
C. annulioris	1	-	-			
C. fatigans	205	184	90	124	118	6
C. pipiens	26	1	4			
C. pipiens/fatigans	22 [c]	9	41			
C. theileri	100	20	20	6	1	5
C. univittatus	33	5	15	5	3	2
Cu. longiareolata	5	-	-			

a - Number of collections.

b - Percentage of total collected.

c - In the case of these mosquitoes the 2 species were not separated.

engorgement rates or, in the case of man-baited catches, alighting rates. Several different trapping methods were included in the study in an attempt to eliminate false conclusions resulting from trap bias, since experience has shown that nearly all methods influence the number of mosquitoes attracted to and feeding on baits. As mentioned earlier, larger numbers of mosquitoes are taken biting man in the open than in a net-trap. However, the net-trap will provide a better indication of the extent of anthropophilism in species occurring at low density. This is possible because such a collecting method permits a determination of the proportion of the mosquitoes alighting to feed, out of the total attracted, for example, A. juppi and A. mixtus in Tables 3 and 4. The restriction of man-baited catches to a short period after sunset meant that biting activity through the remainder of the night until the morning was missed. It is unlikely, however, that any anthropophilic species were overlooked.

According to the work of Dow et al. (1957), it seems reasonable to assume that mosquitoes feeding on fowls will also feed on wild birds. Of all the fowl-baited traps, the net-trap proved to be the most useful since all Highveld mosquitoes except Coquillettidia species enter it readily. Feeding rates seem to increase in the net-trap if the legs of the fowls are tied together to restrict movement. The fowl-pen trap proved suitable for sampling only Culex species and the fowl-baited lard-can for only Culex and Coquillettidia species. The value of collections made by suction traps attached to pigeon cages is reduced by the mosquitoes being unable to feed on the bait. Furthermore, the control suction traps indicate that a significant number of mosquitoes are trapped which are not attracted by the bait but by the trap itself, perhaps the noise of the motor. Aedine species do not enter the lard-can trap readily so that in the vertical distribution study limited numbers of these species were collected. An alternative approach for assessing the extent to which they are arboreal would seem to be conducting a human-baited catch at both levels.

Feeding habits of seven species

The results obtained for the feeding habits of seven species will be discussed in turn. These account for the mosquitoes most frequently prevalent in the Highveld.

(1) C. univittatus. This strongly ornithophilic species feeds nocturnally both on the ground and in the canopy of trees, but to a greater extent in the canopy. Such nocturnal arboreal feeding behaviour ensures the juxtaposition in time and habitat between the mosquito and birds which roost in trees. Since this important mosquito was shown to be poorly anthropophilic in the previous study, attempts were made to define more clearly the extent to which it feeds on man. The present results show that C. univittatus at Lake Chrissie and Olifantsvlei was only slightly anthropophilic. However, they also indicated that the species can be endophagic on man to a small extent.

(2) C. theileri. This mosquito was shown to be a highly anthropophilic as well as a moderately ornithophilic species, findings which confirm the results of the previous study. However, in addition, the present investigations indicate that C. theileri is significantly endophagic on man. They also show that it is more of a ground than a canopy feeder which would diminish its feeding on species of nocturnally-roosting birds.

(3) C. pipiens. The results indicate that the feeding habits of C. pipiens are very similar to those of C. univittatus except that it does not appear to be at all endophagic.

(4) C. fatigans. Although this domestic mosquito is absent or rare in the rural study areas of Olifantsvlei and Lake Chrissie, it is probably common in all urban or suburban habitats throughout the Highveld. Certainly it is common in Johannesburg's suburbs. It is ornithophilic, highly anthropophilic and enters houses readily to bite man. The house collections also showed that it is endophilic.

(5) and (6) A. unidentatus and A. dentatus. A. unidentatus followed by A. dentatus were the most prevalent aedine species at Olifantsvlei and the order was reversed at Lake Chrissie. The latter was not the case in the previous study and was due to the unusually high densities of A. dentatus encountered at this locality during the present observations. These grass-dwelling aedine species are highly seasonal, only appearing after rainfall, so that population densities vary tremendously. Both species are highly anthropophilic, and A. unidentatus is probably endophagic. A. unidentatus feeds quite readily on fowls and A. dentatus moderately so, an indication

that both would probably feed on wild birds. However, both feed largely at ground level which would preclude their feeding on many arboreal birds.

(7) A. juppi. Few observations were made on this species owing to its scarcity at the time of collections. Hence, little advance was achieved in understanding its feeding habits. A. juppi is probably not so prevalent in the Highveld as was at one time believed and it is thought that it is less common in this region than A. subdentatus and A. dentatus. Like the other Aedes this mosquito appears to feed at ground level, it is anthropophilic and can show endophagy in feeding on man.

Other species

The five anopheline species collected all bite man but appear only slightly ornithophilic. The collections at Lake Chrissie, where two species of Coquillettidia were collected, indicate that Coq. microannulata can reach a high density while Coq. fuscopennata is rather rare. Both species were shown to be bird-feeders but because it is rare Coq. fuscopennata barely featured in the human-baited collections. However, this is an important anthropophilic species in Uganda (Haddow, 1961).

CHAPTER 3.

LABORATORY STUDIES ON VECTOR CAPABILITY

The six commonest species of Highveld mosquito were studied in the laboratory to assess the vectorial capability of each with WN and SIN viruses. This entailed determining the degree of susceptibility and the level of transmission efficiency and to this end experiments were carried out to ascertain the infection rate, infection threshold and transmission rate for each of the species. It was hoped that the information gained could be correlated with the ecological characteristics-prevalence and feeding habits and the level of infectivity in wild mosquito populations. Well founded conclusions could then be reached on the qualification for vectorship of each species, either as a vector in the feral cycles of WN and SIN viruses or as a link vector between these cycles and man.

In order to undertake the vector experiments, a supply of mosquitoes belonging to each of the four Culex and two Aedes species concerned was required, necessitating the colonization or rearing of these different species, an aspect dealt with in Section A of this chapter. The vector capability experiments referred to above are reported in Section B, while in Section C an experiment is described in which the infected salivary gland is assessed as an indicator of a mosquito's ability to transmit virus. It was hoped that this possible alternative method for demonstrating the potential to transmit might facilitate experiments embracing the aedine mosquitoes. Finally, in Section D the results of experiments on C. univittatus to investigate factors influencing the transmission rate with WN virus are given. They were undertaken in view of the results obtained in the infection and transmission tests with this species (Section B.)

SECTION A

MOSQUITO REARING AND COLONIZATION.

At different times each of the four commonest species of Culex from the Highveld was colonized in the insectary to provide a constant supply of mosquitoes for the vector capability experiments. Colonies of all four species were not, however, in existence at all times over the several years that vector experiments were carried out so that it was sometimes necessary to rear a fresh batch of insects from wild-caught females. In the case of A. unicentatus and A. dentatus all experimental work was done with mosquitoes reared in this way.

During the period of the vector experiments three different colonies of the stenogamous C. fatigans were established from mosquitoes collected in Johannesburg and were used for these experiments. Colonization was easily accomplished in 35 cm cages using techniques well established for this domestic mosquito. On the other hand, the related species C. pipiens needed special treatment including the use of a large 'walk-in' cage before mating would occur on account of its eurygamy. In this connection it is interesting to note that colonies have been established in smaller cages in Europe, North America and Egypt. With these colonies it seems that the minimum space required for mating is a cube of between 34 and 40 cms.

A description of the methods used for the colonization or rearing of each of the five mosquito species other than C. fatigans follows, together with notes on the history of the colonies successfully established.

C. univittatus

The initial colonization of this Highveld species has already been described (Jupp and Brown, 1967) including details of the methods employed. This successful colonization was basically due to a special artificial lighting arrangement giving simulated dawn, day and dusk periods and the initial use of a 'walk-in' cage which caused mating to occur. The original sub-colony was maintained through 48 generations by which time the emerging adults became weak and many died so that the colony had to be discontinued. A second colony

was then started by placing adults reared from field mosquitoes collected near Johannesburg directly into six 35 cm^3 cages. These were continuously exposed to the same lighting regime used for the original colony and a viable colony was rapidly established without the use of the 'walk-in' cage. The speed with which these mosquitoes became conditioned to mate in the small-sized cages provided is evident from the viability indices for rafts deposited in successive generations. These were 20% (F_1), 55% (F_2), 73% (F_3) and 83% (F_4). The lighting used was essential for the establishment of this colony. It is thought that an improvement in the culture method for the larval stage may have contributed to the faster establishment of the second colony. For this, dried green kikuyu grass (Pennisetum clandestinum) was added to tap water in white enamel trays with internal measurements 86 x 24 x 5 cm, instead of dried brown grass in polythene bowls, and fewer newly hatched larvae, about 200, were seeded into each dish. The laying tubes described previously (Jupp and Brown, 1967) were used for oviposition with two modifications, an infusion was substituted for tap water and sugar-water pledgets were not provided. The infusion, prepared by soaking dried green kikuyu grass in water for five days and filtering this mixture through organdie, stimulated oviposition. Oviposition, which occurred in the dark, was inhibited if the mosquitoes imbibed sugar water, and also if beakers of infusion were placed in the cages instead of confining the mosquitoes in laying tubes.

C. theileri

The earlier attempts to colonize this mosquito were unsuccessful because it did not mate, but mating did occur when the special artificial lighting regime was used. The methods used to establish and maintain the colony were almost the same as those found successful for the second colony of C. univittatus, a walk-in cage not being used. Gravid females from Lake Chrissie were used to start the colony. The percentage viability indices for the F_1 - F_{16} rafts were 26, 60, 36, 38, 74, 47, 54, 61, 85, 41, 69, 73, 48, 92, 56 and 67 respectively. Although this demonstrates a considerable fluctuation in viability, indices were consistently high enough in the later generations for the colony to be useful for experimental purposes. All stages of the mosquito were robust, with very little mortality,

and the colony was discontinued in the 16th generation.

The F_3 adults were observed during the dusk period. Swarming by males in the manner observed for the C. univittatus colony (Jupp and Brown, 1967) did not occur. There was only hovering near the side of the cage, mainly by males, which increased as the light dimmed. Males, however, copulated with females that were resting on the sides of the cage; sometimes a male disengaged while the female was still stationary, while at other times the pair flew in copula to the bottom of the cage before disengaging. The frequency of mating increased as the light intensity decreased.

A variety of animals have been used as the source of the blood-meal but infant mice or chickens were usually employed. The mosquitoes were starved for 24 hours before a blood-meal, which was offered overnight.

Hatching of rafts took place one to two days after oviposition. The duration of the larval stages, at a water temperature of 24°C - 25°C, varied from seven to 23 days and averaged 14 days. The pupal period usually lasted two days.

C. pipiens

Previously I attempted to colonize this species in 35 cm^3 cages exposed to lighting which represented a 12 hour day - 12 hour night and subsequently in a larger cage, 75 x 50 cm. in cross-section and 150 cm. high, exposed to illumination simulating a 12 hour day, one hour dusk of constant intensity, and 11 hour night. However, the mosquitoes did not mate because of the eurygamous nature of the species until the method described for C. univittatus (Jupp and Brown, 1967) was used with a walk-in cage and with lighting incorporating a gradual increase in intensity in the morning and a decrease in the evening. A research student, Mr. Motara, successfully colonized C. pipiens with this method and with two adjacent walk-in cages in my insectary (unpublished). He also used the same refinements in technique as already described for the second C. univittatus colony. His colony, started from C. pipiens collected near Johannesburg, was treated in a manner very similar to that used for the original colony of C. univittatus except that laying tubes were found unnecessary for oviposition since early in the colony's history rafts were readily

deposited in trays of tap water placed on the floor inside the walk-in cage. By the eighth generation a high raft viability index of about 80% was attained which indicated a similarly high frequency of matings.

At the 11th generation this colony of C. pipiens was given to the author who transferred the adults to 35 cm^3 cages. These cages were still exposed to the special illumination, and oviposition occurred either in a small black plastic dish of grass infusion placed inside each cage or in laying tubes. The percentage viability indices for the F_{12} - F_{21} rafts were 3, 2, 9, 22, 9, 8, 8, 30, 14 + 33 respectively and the colony is still being maintained. As can be seen, the viability fell dramatically when the species was transferred to small cages and although it improved somewhat in the last three generations, it did not follow the rapid rate of increase shown by the second colony of C. univittatus and is also less than that recorded for C. theileri. However, all stages of the mosquito are robust, so it is expected that the viability index of the rafts in future generations will improve.

In order to guard against accidental contamination of this colony with C. fatigans, in each generation larvae coming from each egg raft were seeded in a different rearing tray and subsequently the identity of several fourth stage larvae was checked. In some cases the male genitalia of adults reared from individual dishes were also checked.

A. unidentatus

Adult mosquitoes were reared from eggs deposited by wild-caught females but the species was not colonized in the laboratory.

Gravid females were collected near Johannesburg or at Lake Chrissie. Eggs were obtained by confining these mosquitoes individually in laying tubes containing a layer of moist cotton-wool about 1 cm. thick at the bottom and a strip of filter paper down the side. These eggs were stored in the insectary at a temperature of 25°C - 26°C and a relative humidity of 75% - 80% for up to two months. They were then flooded with deoxygenated (boiled) water after which a proportion of the eggs usually hatched in each laying tube. The

tubes were then allowed to dry and after an interval were reflooded when further eggs hatched. This procedure of drying and flooding was repeated a third time when further but fewer eggs hatched - in the case of one mosquito the third flooding occurred four months after the original oviposition and a few eggs still hatched. It was observed that, in several tubes, some of the eggs hatched only after the second or third flooding.

Larvae were reared using the method developed for C. univittatus - dried kikuyu grass in tap water with the addition of food powder. Larval mortality was minimal and the adults which emerged appeared to be robust.

These adults were housed in a 35 $cm.^3$ cage which was exposed to the special illumination in the hope that mating would take place. A hamster was exposed to these mosquitoes as a source of blood and six days later gravid mosquitoes were confined individually in laying tubes to obtain F_1 eggs. Most mosquitoes laid eggs and then died. Mortality after oviposition was usually the case and those mosquitoes which still survived did not usually live long.

The F_1 eggs obtained from 46 different females were dried for three months in insectary conditions and were then flooded. No hatchings occurred but it is just possible that the withdrawal of humidification for 36 hours because of a power failure during the three month period killed the embryos inside those eggs which were viable.

A. dentatus

The methods described for A. unidentatus were also used for A. dentatus to obtain eggs and larvae from wild-caught females originating from Lake Chrissie. The resulting adults were again placed in 35 $cm.^3$ cages but were found to be somewhat more robust than those of A. unidentatus. The mosquitoes failed to feed readily when offered a chick but fed upon a hamster. F_1 eggs were deposited by a total of 88 mosquitoes and mortality was less after oviposition than with A. unidentatus. After being held for three months under insectary conditions except for the 36 hour interval when humidification ceased, these F_1 eggs were flooded and those from one female hatched.

SECTION B

INFECTION AND TRANSMISSION TESTS

Six species of mosquito were tested in the laboratory to determine their susceptibility to infection ('infection test') with WN and SIN viruses and their ability to transmit them ('transmission test'). These mosquitoes were the four commonest Highveld *Culex* species, *C. univittatus*, *C. theileri*, *C. fatigans* and *C. pipiens* and two of the three commonest Highveld *Aedes*, namely *A. unidentatus* and *A. dentatus*. Infection thresholds were determined for each species from the results of the infection tests, in the course of which it was discovered that the susceptibility of *C. pipiens* to WN virus differed greatly depending upon the infecting method used. When mosquitoes of this species fed through a membrane on a blood mixture containing WN virus they were only slightly susceptible, whereas if they engorged on viraemic chicks they were much more readily infected. Because of this, the susceptibility of each *Culex* species to each virus was tested by both methods. The susceptibility of the two *Aedes* species, however, was tested only by feeding on viraemic animals. Transmission was evaluated by determining a transmission rate except in the two equine species where due to the weakness shown by these mosquitoes and their reluctance to take a second blood-meal the transmission tests done were more qualitative.

MATERIALS AND METHODS.

Mosquitoes. The origin of the six species is described in Section A and is also indicated in the tables of results in the present section. In the case of the *Culex* colonies, the particular colony employed and the generation number of the mosquitoes at the time of each experiment is shown. This generation number is exact for all colonies except for the C.83 colony of *C. fatigans* and the first colony of *C. univittatus* where the number of generations was estimated. The age of the mosquitoes when given the infective feed varied from one to 30 days but was usually four to 13 days. All the experiments were undertaken in an insectary where the mosquitoes were maintained at 24°C to 26°C at a relative humidity of 75% to 85%.

Virus. The viruses used were the H442 strain of WN virus (Kokernot and McIntosh, 1959) and the AP66 strain of SIN virus (Weinbren et al. 1956) at their third or fourth intracerebral mouse-passage level.

Infective meal; membrane feeding. In this method, which is referred to as the 'membrane method', a series of blood-virus mixtures were prepared as infective meals from stocks of lyophilized virus of known potency. To prepare such blood-meals, virus dilutions were made in 0.75% ovine plasma albumen, so as to provide four 10-fold dilutions estimated to cover virus dosages over the range 5.0 - 2.0 logs of virus when added to defibrinated rabbit blood in the proportion of one part of virus dilution to three parts of blood. The actual amount of virus present in the blood-virus mixture containing the highest concentration of virus was determined by titration in mice immediately before the mixtures were given to the mosquitoes. In a few experiments only one dose of the highest viral concentration was used while in the experiment described for C. pipiens and WN virus each different blood-virus mixture used was prepared, titrated and offered to the mosquitoes concerned on different evenings. Titrations were carried out in infant (SIN) or adult or infant (WN) mice inoculated intracerebrally with an inoculum of 0.02 ml. For WN virus, adult mice were used more often than infants and when infant mice were used the titres thus determined have been reduced by 1 log so as to permit comparison with those determined in adults. This was done because comparative titrations of infected blood in mice of each age-group had shown that infants are slightly more susceptible to WN virus than adults and on average give titres 1 log higher. In some experiments titrations were also done on the mixtures containing the lowest concentration of virus and, furthermore, as a control on the viability of the virus at the high temperature of the insectary, additional titrations were sometimes carried out on the mixtures containing the strongest concentration of virus at the completion of the infective feed.

The mosquitoes were starved for 24 hours before being given their infective feeds. For each graded dose of virus, two to four Perspex canisters each containing 25 or 30 mosquitoes, were used. A canister was 7.5 cm. long and 5 cm. in diameter : the top was closed by mosquito netting and the bottom either by freshly-harvested chicken skin or

by Badruche membrane. Each canister was placed in a shallow plastic dish, on the floor of which had been cemented a 7 mm high Perspex ring of the same diameter as the canister. The ring was filled with the blood-virus mixture so as to allow adequate contact between skin or membrane and the mixture. The dishes were held at 30°C - 32°C in a water bath during feeding so that the temperature of the suspensions was 29°C - 31°C. Feeding took place between 1800 and 2100 hours, from 1800 - 1900 hours in dim light and from 1900 - 2100 hours in darkness. At the end of the feed all the mosquitoes were transferred into 20 cm^3 cages.

In one experiment only - the first experiment with C. fatigans and SIN virus - the mosquitoes were given the infective feed on a cotton-wad ('cotton-wad method'). These wads were saturated in the four blood-virus mixtures and placed on top of 20 cm^3 cages each of which contained 150 mosquitoes.

Infective meal: viraemic chicks or hamsters. For the Culex species the second infecting method of allowing the mosquitoes to feed on viraemic birds was used and with the Aedes species viraemic hamsters ('viraemia method'). To obtain different levels of virus circulating in avian blood the following procedure was carried out. For high titres of both viruses domestic chicks were inoculated intramuscularly on the day of hatching, or 2-day-olds were still suitable in the case of SIN virus, and 24 hours later they were exposed to the mosquitoes. For low titres adult pigeons (Columba livea) or 2-day-old domestic chicks were inoculated and used 24 hours or three to four days later, respectively. Adult Syrian hamsters (Mesocricetus auratus) were inoculated intramuscularly with either virus and exposed to the aedine mosquitoes three days later. A range of titres was obtained in this way because the level at which virus circulated in the blood varied from hamster to hamster.

For the mosquito feed, chicks with their legs tied together or pigeons restrained inside nylon stockings were placed within 35 cm^3 cages containing the mosquitoes. Hamsters were anaesthetized and placed on top of 20 cm^3 cages containing the mosquitoes. Culex species were starved for 24 hours prior to being given the infective feed, while aedine species were starved only for the preceding six

hours. Feeding was allowed to go on for one to three hours in the dark in the insectary with the Culex species, but with the two Aedes species, the mosquitoes were transferred, for the feed, to another room with the same temperature but with uncontrolled relative humidity. These mosquitoes were returned to the insectary directly afterwards. Immediately before the exposure of a particular viraemic animal, a blood-sample was collected to determine the concentration of virus present. Details concerning the titration of virus described above under membrane-feeding also apply to the experiments done by the viraemia method.

Post-infection procedure. On the day after the infective feed (Culex species) or immediately afterwards (Aedes species), the engorged mosquitoes from each blood-virus mixture or animal were placed in separate cages and held in insectary conditions. Subsequently in those experiments where a transmission test was to follow, the mosquitoes were allowed to oviposit. With Culex species this took place either in beakers of water placed inside the cages or by confining the mosquitoes individually in laying tubes. The aedine mosquitoes, however, were not always placed in laying tubes before attempted transmission because of the mortality this sometimes caused. Details concerning the laying tubes used for the two genera are described in Section A.

Transmission. The transmission rate in the present study means the percentage of infected mosquitoes which successfully transmit virus. However, the definition for this rate made by Chamberlain et al. (1954[1]) is 'the percentage of specimens transmitting infection by bite to susceptible animals after ingesting a meal having a high virus titre and after a suitable extrinsic incubation period'. According to this, therefore, the rate is based on groups of mosquitoes which may include an unknown number of non-infected individuals and clearly the rate will vary depending upon the quantity of virus ingested. The same basis has been used by others (Collins et al. 1965; Sudia et al. 1971). It would seem that a more valid basis for this rate would be to include only known infected mosquitoes.

Determination of the transmission rate was made in transmission tests carried out with some of the groups of mosquitoes after their

infective meal. The mosquitoes used were those groups of C. univittatus, C. theileri and C. fatigans which had fed on the blood-virus mixture containing the highest dose of WN or SIN virus, or occasionally the second highest dose. In addition to this, such a test was also done with a group of C. pipiens which had been infected by feeding them on a chick with circulating WN virus. On the 14th to the 20th day after attempted infection each mosquito was allowed to engorge on a different susceptible 2-week-old chick ('transmission chick'). These mosquitoes, which had been deprived of sugar water during the preceding 24 hours, were placed singly in 20 cm^3 cages. A chick, restrained within a nylon stocking, was suspended into each cage through an access sleeve at the top. Feeding took place from 1600 - 2100 hours or from 1600 - 0830 hours. In each experiment, 20 to 50 chicks were used and were given numbers corresponding to mosquito cages. Chicks on which mosquitoes had engorged were kept separate from one another for the first week after the transmission feed to exclude the possibility of cross-infection. Successful transmission was assessed by testing the sera of these chicks three weeks after the attempted transmission for antibodies against WN or SIN virus with the haemagglutination-inhibition test. For each transmission attempt undertaken with the Aedes species one anaesthetized hamster was exposed to a number of mosquitoes 10 - 20 days after the infective feed. Again, as with the transmission chicks, to assess whether transmission had occurred hamster sera were tested with the haemagglutination-inhibition test three weeks after the transmission feed.

Determination of infection rates. The infection rate, i.e., the proportion of mosquitoes feeding which became infected, was determined for mosquitoes which fed on each infective meal, either blood-virus mixture or viraemic animal, by testing mosquitoes individually for virus by the inoculation of a group of 12 infant mice. The mosquitoes were killed for testing from the 14th - 24th day (Culex species) or the 10th - 26th day (Aedes species) after attempted infection, those which fed a second time in the transmission tests being tested usually four days (Culex species) or two days (Aedes species) after the latter feed to allow prior digestion of the blood-meal. The mosquitoes were judged to have been infected if all 12 inoculated mice died within the expected death time of the respective virus.

Estimation of infection threshold. The infection threshold was taken to be the dose of virus in logs which infected 10% of the mosquitoes (log ED_{10}). For each experiment this threshold was estimated or calculated from the infection rates obtained for the four or more different viral titres (blood-virus mixtures or animal viraemias). The probit method as recommended by Dougherty (1964) was used for the calculation and could be applied in those cases where there were at least three infection rates with values between 1% and 99% or where a value occurred above and another below the 10% value.

RESULTS

In three of the experiments in which the membrane method was used the strongest viral dose was titrated on completion of the infective feed and it was found that the titre only decreased by 0.3 - 0.8 logs during the feeding period.

The mosquitoes engorged through the thinner skins which came from younger chickens much more readily than through those from older chickens - skin from chicks five to seven weeks old gave the best results. At a time when most of the experiments had been completed a change was made to the Badrucha membrane as it was much more convenient to use, but, for some species, the proportion of mosquitoes successfully engorging through it may have been lower than with chicken skin.

West Nile Virus and six mosquito species.

(1) C. univittatus (Table 12). The results of single experiments with each of the two infecting methods are shown. The 10% infection thresholds calculated from the infection rates in each experiment were very low (<1.0 logs), although the infection rates obtained with the second mosquito colony (C.132) using the viraemia method in Experiment 2 indicate a higher susceptibility. However, in both cases it is evident that the actual end point for the log ED_{10} value is below that which can be determined in the test mice. The transmission rates for mosquitoes infected at both 4.7 and 3.7 logs were high (100% and 89%) with an overall 12 out of 13 (92%) infected mosquitoes transmitting the virus.

TABLE 12. Results of quantitative infection and transmission tests with C. univittatus and West Nile virus including comparison of 2 infecting methods.

EXPT. No.	Infecting Method	Mosq. colony generation	Titre of infective feed in logs	No. days after infective feed when mosqs. tested	MOSQUITO INFECTION: Infection rate [a]	MOSQUITO INFECTION: Infection threshold log ED_{10} [b]	TRANSMISSION: No. days after infective feed	TRANSMISSION: Transmission rate [c]
1	Membrane	1st col. F_4	4.7	18 - 22	14/15 (93%)		18	4/4
		"	3.7		15/16 (94%)	<1.0	"	8/9 (89%)
		"	2.7		20/25 (80%)			
		"	1.7		6/15 (40%)			
2	Viraemia	C.132; F_5	4.1 - 4.6	15 or 16	37/37			
		" F_{12}	2.6		24/24	<1.0		
		" F_6	1.0[d]		21/25 (84%)			
		" "	0.2[d]		12/29 (41%)			

a - Numerator = No. mosquitoes infected; denominator = No. mosquitoes tested.

b - This is calculated from infection rates and is the titre of virus to infect 10% of mosquitoes.

c - Numerator = No. mosquitoes transmitting virus; denominator = No. of infected mosquitoes feeding.

d - These titres have been corrected - actual titrations were done in infant mice and were 1 log higher.

(2) C. theileri (Table 13). In experiment 1, in which graded doses of virus were given by the membrane method, mosquitoes were readily infected and the 10% infection threshold was as low as 1.5 logs. None of the 18 mosquitoes infected with a virus concentration of 4.5 logs transmitted the virus. However, in experiment 2, where the same infective dose was used, eight out of 32 (25%) mosquitoes successfully transmitted, bringing the overall transmission rate for both experiments to 16%, i.e., eight out of 50 mosquitoes transmitting the virus. In experiment 3 the log ED_{10} value determined with the viraemia method was also low (<2.4 logs).

(3) C. fatigans (Table 14). In experiments 1 and 2 (membrane method) infection occurred only with the stronger concentrations of virus so that the infection threshold was about 4.0 logs for both colonies C.83 and C.113. Successful transmission occurred in both experiments although transmission rates were very low (22% and 10%) with an overall three out of 19 (16%) infected mosquitoes transmitting the virus. The infection threshold determined by the viraemia method in experiment 3 was about 1.5 logs lower than in the other experiments, as the mosquitoes proved to be much more susceptible to the virus after feeding on the viraemic birds.

(4) C. pipiens (Table 15). When the membrane method was used (experiment 1) the mosquitoes were almost completely refractory to infection, with the 10% infection threshold about 5.2 logs which corresponded to the titre of the mixture containing the highest concentration of virus. However, in experiment 2, in which the viraemia method was employed, the value calculated for log ED_{10} (1.0 log) was more than 4 logs lower. In a transmission test subsequently carried out in experiment 2 with those mosquitoes which had been infected at 5.2 logs four out of 16 (25%) infected mosquitoes transmitted virus.

Some of the infection tests carried out on C. pipiens, which are shown in Table 15, were also designed to try to explain the discrepancy between the infection thresholds determined by the two infecting methods. These comparative infection tests were as follows:-
(a) At the same time as the batch of mosquitoes concerned fed on the mixture with a viral titre of 5.1 logs (experiment 1), a batch of C. univittatus, also from a laboratory colony, fed on it. Although

TABLE 13. Results of quantitative infection and transmission tests with C. theileri and West Nile virus including comparison of 2 infecting methods.

				MOSQUITO INFECTION			TRANSMISSION	
EXPT. NO.	Infecting Method	Mosq. colony generation	Titre of infective feed in logs	No. days after infective feed when mosqs. tested	Infection rate	Infection threshold log ED_{10}	No. days after infective feed	Transmission rate
1	Membrane	F_5	4.5	21 or 22	25/25		18 or 20	0/18
		"	3.5		23/25 (92%)			
		"	2.5		13/25 (52%)	1.5		
		"	1.5		3/21 (14%)	($\pm$0.1)[a]		
2	"	F_7	4.4[b]	18	34/34	-	14 or 15	8/32 (25%)
3	Viraemia	p	4.5[b]	14	18/18	< 2.4		
		"	2.4		27/27			

a - Standard error.

b - These titres have been corrected - actual titrations were done in infant mice and were 1 log higher.

p - The progeny reared from wild-caught females.

TABLE 14. Results of quantitative infection and transmission tests with C. fatigans and West Nile virus including comparison of 2 infecting methods.

			MOSQUITO INFECTION				TRANSMISSION	
EXPT. NO.	Infecting Method	Mosq. colony generation	Titre of infective feed in logs	No. days after infective feed when mosqs. tested	Infection rate	Infection threshold log ED_{10}	No. days after infective feed	Transmission rate
1	Membrane	C. 83; F_9	5.5	22 or 24	14/22 (64%)		20	2/9 (22%)
		"	4.5		5/23 (22%)	ca 4.6		
		"	3.5		0/10			
		"	2.5		0/9			
2	"	C.113; F_1	4.7	18 or 23	16/17 (94%)		19	1/10 (10%)
		"	3.7		0/3	ca 4.0		
		"	2.7		0/5			
		"	1.7		0/23			
3	Viraemia	C.139; F_9	4.5	15 or 16	25/25			
		" "	3.8		20/25 (80%)	1.4		
		C. 83; F_{72}	2.2		13/27 (48%)	(± 0.3)		
		" "	1.2		1/25 (4%)			

TABLE 15. Results of quantitative infection and transmission tests with C. pipiens and West Nile virus including comparison of 2 infecting methods.

				MOSQUITO INFECTION			TRANSMISSION	
EXPT. NO.	Infecting method	Mosq. colony generation	Titre of infective feed in logs	No. days after infective feed when mosqs. tested	Infection rate	Infection threshold log ED_{10}	No. days after infective feed	Transmission rate
1	Membrane	♂	5.2[a]	14 - 18	3/26 (12%)			
		F_9	5.1[a]		0/18	ca 5.2		
		♀	3.7[a]		0/23			
		♀	3.3[b]		0/29			
		F_{11}	2.7[c]		1/25			
2	Viraemia	♀	5.5	14 - 18	29/29			
		♀	5.2		25/26 (96%)	1.0	14	4/16 (25%)
		F_{11}	4.5[c]		24/25 (96%)	(± 0.2)		
		♀	3.3[b]		25/28 (89%)			
		F_{11}	2.2		6/25 (24%)			
		F_{11}	1.2		0/16			

a - These titres have been corrected - actual titrations were done in infant mice and were 1 log higher.

b. + c - These two pairs of infection tests were each conducted with mosquitoes drawn from the same broods.

the C. pipiens were refractory, 20 out of 25 (80%) of the C. univittatus mosquitoes became infected.

(b) Mosquitoes drawn from the same broods of C. pipiens were given an infective feed by the two different methods on the same evening to eliminate the possibility of previous differences being due to the use of different sibling species of the C. pipiens complex, which might have been employed unwittingly in the experiments. This was done both in the case of progeny reared from field mosquitoes and for F_{11} mosquitoes from the laboratory colony as shown in Table 15. In each of these tests, those mosquitoes which fed through the membrane were refractory to infection, whereas a large proportion of individuals in the other group became infected.

Mosquitoes which had engorged by both methods were also dissected and the blood-meal was found to fill the midgut in every insect. Leakage to the dorsal diverticula and ventral diverticulum occurred only when a very large meal was taken. This was found to apply to C. fatigans also.

(5) A. unidentatus and A. dentatus (Table 16). From the infection rates determined it is evident that the 10% infection threshold is high in both these species, between 3.5 and 4.5 logs for A. unidentatus and >4.5 logs for A. dentatus. One transmission feed was accomplished with a single infected A. dentatus which had previously been infected at 4.5 logs, but this mosquito failed to transmit the virus to a hamster.

Sindbis Virus and six mosquito species.

(1) C. univittatus (Table 17). Three experiments (Nos. 1 - 3) were undertaken using the membrane method and one only (No. 4) with the viraemia method. A value is not given for log LD_{10} in experiment 1 because the infection rates obtained for the 4.6 and 3.6 log doses are based on inadequate numbers of mosquitoes and that for the 2.6 log dose remained undetermined. These deficiencies were due to limited numbers of mosquitoes feeding and complete failure to feed respectively. But in experiment 2, a log LD_{10} value of 1.6 logs was calculated, a low value although not as low as that previously determined for UN virus. In experiment 4 an exact value for log LD_{10} could not be calculated from the percentage infection rates obtained

TABLE 16. Results of quantitative infection tests and transmission attempts with two Aedes species and West Nile virus using viraemic hamsters for infective feeds.

		MOSQUITO INFECTION				TRANSMISSION ATTEMPTS		
	Mosqs.	Titre of infective feed in $\log_e$[a]	No. days after infective feed when mosqs. tested	Infection rate	Infection threshold log ED_{10}	No. days after infective feed	No. of infected mosqs. that fed	Results
A. unidentatus	P	4.5	13 - 17	5/9 (56%)				
	"	3.5	"	0/12	3.5 - 4.5			
	F	2.7	12 - 26	0/24				
A. dentatus	F	4.5	10 - 18	1/28 (4%)	>4.5	10	1	Negative

a - Titres have been corrected - actual titrations were done in infant mice and were 1 log higher.

F - Field-collected mosquitoes taken out of traps.

TABLE 17. Results of quantitative infection and transmission tests with C. univittatus and Sindbis virus including comparison of 2 infecting methods.

				MOSQUITO INFECTION			TRANSMISSION	
EXPT. NO.	Infecting Method	Mosq. colony generation	Titre of infective feed in logs	No. days after infective feed when mosqs. tested	Infection rate	Infection threshold log ED_{10}	No. days after infective feed	Transmission rate
1	Membrane	1st col. F_6 -F_7	5.6	18 - 23	10/12 (83%)		19	2/3
		" "	4.6		3/6 (50%)			
		" "	3.6		3/5 (60%)			
		" "	2.6		No test [a]			
2	"	" F_{10}	3.9	18 - 22	7/12 (58%)		18	0/2
		" "	2.9		2/3 (67%)			
		" "	1.9		1/16 (6%)	1.6 (± 0.3)		
		" "	0.9		1/19 (5%)			
3	"	" F_{14}-F_{15}	5.1	22	23/32 (72%)	-	18	12/21 (57%)
4	Viraemia	C.132: F_{13}	6.3	14 or 15	23/25 (92%)			
		" F_{16}	5.5		19/20 (95%)			
		" "	3.7		22/25 (88%)	ca 2.9		
		" F_{15}	3.2		13/26 (50%)			
		" F_{16}	2.5		0/18			

a - The mosquitoes failed to feed on the infective meal at this dose level.

for the different viral titres because they did not cover a wide enough range. The threshold was greater than 2.5 logs and about 2.9 logs which means that it was 1.0 - 1.6 logs higher than the value calculated in the membrane experiment (1.6 ± 0.3 logs). This difference in susceptibility is due to the refractoriness of the mosquitoes which fed on the bird with the lowest virus titre, i.e., 2.5 logs. However, the infection rates obtained for the two intermediate viral titres in the same experiment, i.e., 3.2 and 3.7 logs indicate that this second colony of C. univittatus is probably as susceptible as the first colony tested in the other experiments and suggest that the degree of susceptibility of the mosquito at 2.5 logs in this experiment needs confirmation.

Because of some deaths of transmission chicks and the relatively small number of mosquitoes upon which a transmission rate could be calculated in experiments 1 and 2, a third experiment was performed. In it (No. 3) only a single dose (5.1 logs) was used and in the subsequent transmission test 12 out of 21 (57%) infected mosquitoes successfully transmitted virus. The infection rate in this experiment is in fair agreement with the values obtained in experiment 1 with the two higher concentrations of virus. By combining the results of all three transmission tests, a transmission rate of 54% is obtained, i.e., 14 out of 26 infected mosquitoes transmitting the virus.

(2) C. theileri (Table 18). The log ED_{10}'s calculated in experiment 1 (membrane method) and estimated in experiment 3 (viraemia method) from the infection rates are fairly close and show that the mosquito was infected readily. This value of 2.0 - 2.9 logs was slightly higher than that determined for WN virus. In the only experiment (No. 1) in which transmissions were attempted two out of 23 (9%) mosquitoes successfully transmitted. This is not a significantly lower rate ($2\alpha > 0.2$ in a 2 x 2 table test using tables of the hypergeometric distribution) than the rate of eight out of 32 (25%) obtained in experiment 2 with WN virus (Table 13).

(3) C. fatigans (Table 19). In experiment 1, cotton-wad feeding was used as the infecting method and, because of this, the level of mosquito infection obtained in this experiment may not be strictly comparable with that obtained in the other three experiments. It has been shown by Jupp et al. (1966) that mosquitoes may take up

TABLE 18 Results of quantitative infection and transmission tests with C. theileri and Sindbis virus including comparison of 2 infecting methods.

				MOSQUITO INFECTION			TRANSMISSION	
EXPT. NO.	Infecting Method	Mosq. colony generation	Titre of infective feed in logs	No. days after infective feed when mosqs. tested	Infection rate	Infection threshold log ED_{10}	No. days after infective feed	Transmission rate
1	Membrane	F_3	5.5	15	23/23	-	14	2/23 (9%)
2	"	F_{11}	4.8	18	18/26 (69%)			
		"	3.8		14/19 (74%)	2.0	-	-
		"	2.8		9/26 (35%)	(± 0.2)		
		"	1.8		1/27 (4%)			
3	Viraemia	p	3.7	14	13/25 (52%)			
		p	3.2		14/26 (54%)	ca 2.9	-	-
		"	2.5		0/14			

TABLE 19. Results of quantitative infection and transmission tests with C. fatigans and Simbis virus including comparison of 3 infecting methods.

			MOSQUITO INFECTION				TRANSMISSION	
EXPT. NO.	Infecting method	Mosq. colony generation	Titre of infective feed in logs	No. days after infective feed when mosqs. tested	Infection rate	Infection threshold log ED_{10}	No. days after infective feed	Transmission rate
1	Cotton-pad	C.83; F_1	4.8	24	4/33 (12%)	ca 4.8	20	2/4 (50%)
			3.8		0/25			
			2.8		0/25			
			1.8		0/25			
2	Membrane	C.83; F_{10}	5.1	24	0/13	>5.1	20	0/0
			4.1		0/25			
			3.1		0/11			
			2.1		0/12			
3	"	C.113; F_1	5.3	19 or 23	4/25 (16%)		19	0/2
			4.3		1/25 (4%)	ca 5.0		
			3.3		0/25			
			2.3		0/25			
4	Viraemia	C.139; F_{11}	6.3	15 or 17	5/26 (19%)	ca 5.0		
		C.83; F_{73}	2.3		0/25			

additional virus through the tarsi when they are infected by feeding upon blood-soaked cotton-wads. This may explain the slightly lower value for log ED_{10} obtained in this experiment. The high titre of virus in the avian feed in experiment 4 (6.3 logs) infected a small proportion of the mosquitoes but the concentrations of virus in the infective meals in experiments 1 and 3 were barely sufficient to infect and in experiment 2 the mosquitoes were not infected by 5.1 logs of virus. There was a close agreement between the log ED_{10} values obtained in the four experiments, which were higher than those determined for WN virus, and neither infecting method influenced this infection threshold. In the transmission tests virus was transmitted in experiment 1 but not in experiments 2 or 3 and, if the results of all three tests are combined, a transmission rate of 33% is obtained, i.e., two out of six infected mosquitoes transmitting the virus.

(4) C. pipiens (Table 20). In experiment 1 (membrane method) the mosquitoes were refractory to infection after feeding on a mixture containing a concentration of 5.0 logs of virus, whereas in experiment 2 (viraemia method) in which most of the infecting titres were high the value for log ED_{10} was very high (6.4 logs). Therefore the infection thresholds determined with each method appear to agree closely, although the viral dose employed with the membrane method was not sufficiently high to eliminate the possibility of infection occurring at high levels of virus, 6.3 logs and more, as used in experiment 2. It was decided not to undertake a transmission test with this species and SIN virus before its degree of susceptibility had been established. The refractoriness of the mosquito which emerged then made such a test unnecessary.

(5) A. unidentatus and A. dentatus (Table 21). The infection rates obtained indicate that the 10% infection threshold is low in A. unidentatus ($<$ 2.6 logs) and rather high in A. dentatus (ca 3.5 logs). Three individuals of A. unidentatus infected with 3.8 logs of virus failed to transmit to a hamster. Similarly with A. dentatus, in two separate transmission attempts, two and one mosquitoes respectively infected at 6.6 logs failed to transmit the virus to hamsters.

TABLE 20. Results of quantitative infection tests with C. pipiens and Sindbis virus according to 2 infecting methods.

EXPT. NO.	Infecting Method	Mosq. colony generation	Titre of infective feed in logs	MOSQUITO INFECTION: No. days after infective feed when mosqs. tested	Infection rate	Infection threshold log ED_{10}
1	Membrane	F	5.0	17 - 19	0/26	>5.0
2	Viraemia	F	>7.5	15 or 17	38/54 (70%)	
		F	6.5		8/26 (31%)	6.4
		"	6.3		1/24 (4%)	(± 0.1)
		F_{12}	2.3		0/25	

TABLE 21. Results of quantitative infection tests and transmission attempts with two Aedes species and Sindbis virus using viraemic hamsters or chicks for infective feeds.

		MOSQUITO INFECTION				TRANSMISSION ATTEMPTS		
	Mosqs.	Titre of infective feed in logs	No. days after infective feed when mosqs. tested	Infection rate	Infection threshold log LD_{10}	No. days after infective feed	No. of infected mosqs. that fed	Result
A. unidentatus	P	3.8	7 - 12	14/25 (57%)		10	3	Negative
	F	3.5	15	5/8 (63%)	<2.6			
	P	2.6	10 - 12	2/8 (25%)				
	"	2.1	"	1/2				
A. dentatus	P	5.6[a]	18 - 22	10/10		13	2	Negative
					ca 3.5	20	1	"
	F	3.5	12 - 16	3/34 (9%)				

a - A viraemic chick was used for the infective feed in this experiment only.

DISCUSSION

The results obtained for each species will be discussed in turn and this will be followed by a comparison of the susceptibilities determined by the two different infecting methods. It is interesting to note that all four Culex species were shown to have a greater vectorial capacity with WN than with SIN virus. In the experiments with the Culex species no mosquitoes were used from laboratory colonies which had passed through more than 16 generations except in three infection tests with C. fatigans (two tests in experiment 3 with WN virus and one test in experiment 4 with SIN virus). In these tests mosquitoes with a much longer history of colonization were used because, at the time, they were the only ones available. However, it is not thought that this influenced the infection rates obtained.

(1) C. univittatus. This species is an efficient vector of WN and SIN viruses. Not only was a high proportion of mosquitoes infected after feeding on low concentrations of virus, but a high proportion of infected mosquitoes transmitted the viruses. Using WN virus, the results of further experiments described in Section D of this chapter confirm these findings.

The quantitative methods used have also revealed a difference in vectorial capacity with the two viruses. With WN virus, not only is the 10% infection threshold up to 1 log of virus lower but the transmission rate, based on overall rates, i.e., 12/13 (92%) for WN virus as against 14/26 (54%) for SIN virus, is significantly higher ($P < 0.05$). Hence, when the vectorial capacity of this mosquito is assessed by both critical criteria of susceptibility and transmitting ability, it is superior in both cases with WN virus.

It has already been pointed out (Chapter 1, Page 1[illegible]) that the Egyptian mosquito referred to as C. univittatus by Hurlbut is probably another species but since morphological study has shown that it is clearly closely related to C. univittatus it might be expected to possess similar viral susceptibility. In previous experiments undertaken with this Egyptian C. univittatus, Hurlbut (1953, 1956) successfully transmitted both WN and SIN viruses between mice and also tested the mosquito's susceptibility to WN virus. He infected mice of different ages so that they circulated virus at different levels,

and by feeding mosquitoes on such mice determined infection rates for a range of virus titres. From his results he concluded that a titre of approximately 2.5 logs of WN virus would infect 50% of the mosquitoes. If the probit method, as applied in the present study, is applied to Hurlbut's figures, a value of 1.0 log for the Log ED_{10} is obtained which is close to the value of < 1.0 log which was determined for South African C. univittatus. Hurlbut (1956) also tested the susceptibility of C. (Culex) antennatus (Bekker) to WN virus and his results indicate a value for log ED_{10} of < 2.4. However, his mosquitoes of all species were held for 14 days at $28^{o}C$ - $32^{o}C$ so that the temperature, higher than that of the writer's insectary, may have promoted viral infection making comparisons invalid.

(2) C. theileri. It is evident that this mosquito is readily infected with both viruses, even at low concentrations, but possesses a rather poor ability to transmit WN virus and a very limited ability to transmit SIN virus. Although the differences in the infection threshold and transmission rates obtained between the two viruses are not statistically significant, the trends indicate a possible greater vectorial capacity with WN virus. Comparison of the log ED_{10} values with those for C. univittatus show that C. theileri is almost as easily infected with either virus.

(3) C. fatigans. The 10% infection threshold determined with WN virus for mosquitoes infected by feeding on viraemic birds (1.4 logs) shows that this species is highly susceptible to the virus but the low overall transmission rate of 16% would preclude the mosquito from being an efficient vector. On the other hand, although the transmission rate determined for SIN virus is based on six mosquitoes only, this species is also unlikely to be an important vector of this virus because of the high viraemia levels necessary to infect it.

Workers in India (Varma, 1960) and in Algeria (Vermeil et al., 1960), using the same species of mosquito, have transmitted WN virus between chicks, but in both cases batches of infected mosquitoes were fed on the same susceptible chick so that transmission rates were not determined. In South Africa, Donaldson (1966) using a laboratory colony of C. fatigans which originated, as did the writer's, from mosquitoes collected in Johannesburg, did not determine transmission

rates. In her experiments with both viruses, batches of mosquitoes, fed on viraemic chickens or on wild birds from which infection rates were obtained, transmitted the virus to infant mice. Infection rates were 13% and 50% for mosquitoes fed on sparrows circulating WN virus at 2.0 - 2.6 logs which are in accord with the present results. However, the infection rates of 38% and 13% for mosquitoes fed on fowls circulating SIN virus in the blood at 2.0 - 2.5 and 3.4 logs respectively indicate that Donaldson's colony of C. fatigans was considerably more susceptible to this virus than the present colonies used by the writer, even taking into account only the susceptibility demonstrated in experiment 4 (Table 19) because this was obtained in the same manner by infective feeds on viraemic birds. It is possible that Donaldson's colony had undergone a genetic change as a result of lengthy colonization and this resulted in an enhanced vectorial capacity with SIN virus. This colony had been colonized for five years before Donaldson's experiments (pers. commun.).

(4) C. pipiens. In the case of WN virus, the 10% infection threshold determined by feeding on viraemic birds shows that the species is highly susceptible to this virus. This, together with the moderate transmission rate obtained, suggests that this mosquito could have some significance as a vector. But as it is refractory to infection with SIN virus within the normal range of avian titres (as will be explained more fully in Chapter 5), it is unlikely to be a vector of this virus.

In Egypt, Hurlbut (1956) tested the susceptibility of C. pipiens to WN virus by feeding the mosquito on mice previously infected at different ages - the same way he tested C. univittatus. From his mosquito infection rates it is evident that the value for log ED_{10} would be < 2.2, which shows a susceptibility similar to that of South African C. pipiens. Furthermore, the present results are in accord with those of Hurlbut (1953) in the case of SIN virus. He demonstrated transmission by mosquitoes which had been infected by feeding them on viraemic mice with circulating virus titres of 7.5 - 8.0 logs. South African C. pipiens also became infected when the titres were high, much higher than would usually occur in the natural avian hosts of C. pipiens.

(5) A. unidentatus and A. dentatus. The proportion (56%) of A. unidentatus which were infected with 4.5 logs of WN virus indicates a limited vector potential because such a titre can be reached in the blood of wild birds on which this mosquito might feed. The low susceptibility (4%) of A. dentatus at the same viral dose indicates that it has little or no significance as a potential vector.

A. unidentatus was fairly readily infected with SIN virus but the single transmission attempt carried out suggests that the transmission rate of mosquitoes infected at 3.8 logs of virus is low and the vectorial capacity of the species poor. However, the rate of transmission might possibly be higher at 4.0 - 5.0 logs. The rather high 10% infection threshold of A. dentatus with SIN virus, together with the fa[illegible]hree mosquitoes previously infected at very high titre t[illegible]mit, suggest that this mosquito need not be considered as a vector.

Comparison of infecting methods. With SIN virus, the values for the 10% infection threshold determined by each of the two infecting methods agreed closely for all four species of Culex. On the other hand, with WN virus, while there was good agreement between the log ED_{10} values determined by each method for C. univittatus and C. theileri, there was a clear, statistically significant difference in the case of both C. pipiens and C. fatigans. It is noteworthy that it was with one virus only and with these two mosquito species which are closely related that this difference occurred. This lower susceptibility of C. pipiens and C. fatigans to infection with WN virus when fed on blood-virus mixtures through a membrane indicates that this method should be used with caution if information on the susceptibility of natural populations of mosquitoes is required. It should not be used in the first instance to test the susceptibility of a mosquito to a particular virus. If, however, the susceptibility determined for a mosquito by feeding it on blood-virus mixtures through a membrane is similar to that obtained by feeding it on viraemic animals, the membrane method could justifiably be used in further experiments with the species and virus concerned.

With respect to other methods of artificially infecting mosquitoes with virus, in a previous study with WN virus (Jupp et al. 1966) it was concluded that it was inadvisable to use intra-thoracic inoculation

of virus and feeding upon cotton-wads soaked in blood-virus mixture. However, in the light of the present findings, it seems that the result obtained in the first part of that study may have been misleading and that the comparative experiment with A. aegypti would be worthwhile repeating so as to record infection rates resulting, not only from the use of inoculation and membrane-feeding, but also from feeding on viraemic animals. In the experiment previously carried out, A. aegypti was completely refractory to infection by the membrane method, whereas when suspensions of virus in BPA were inoculated into it a log ED_{10} of about 2.9 was obtained. It seems likely that the mosquitoes used in that experiment would have been infected after feeding on viraemic animals with similar titres of virus in their blood, in view of the demonstration by three different groups of workers that A. aegypti can transmit WN virus between mice or between hamsters by natural feeding (Goldwasser and Davies, 1953; Davies and Yosha-Purer, 1954; Vermeil et al. 1960). Hence the refractoriness of A. aegypti to infection with WN virus by the membrane method may perhaps be similar in nature to the refractoriness shown by C. pipiens and C. fatigans with this method.

It seems significant that the degree of susceptibility of C. fatigans to WN virus by cotton-wad feeding shown in the second part of the same previous study (Jupp et al. 1966) is about equal to that demonstrated with the membrane method in the present study. This suggests that the refractoriness of C. fatigans to infection with WN virus, when infant mouse brain virus in defibrinated rabbit blood is used as the infective feed, is due to the nature of this blood-virus suspension in this particular mosquito species and that the use of the membrane is unimportant. Several other workers have used a membrane and a blood-virus mixture in infection and/or transmission tests. A. aegypti has usually been the mosquito investigated, however, and the method does not appear to have been applied by others to any species of Culex. There is one other comparative study known to the writer (Nye and Bertram, 1960) in which the susceptibility of A. aegypti to Semliki Forest virus was tested by feeding this mosquito on the one hand on viraemic mice and on the other hand on a suspension of mouse brain virus and blood through a mouse skin. These workers did not find a difference in susceptibility with the two methods. The real reason for the differences shown in the present study could probably be found by undertaking further controlled experiments with the two species of mosquito concerned.

SECTION C.

ASSESSMENT OF THE SALIVARY GLAND METHOD FOR SHOWING TRANSMISSION.

One of the main difficulties frequently encountered in an experiment on the mosquito transmission of a virus is the reluctance of the infected mosquito to feed a second time, that is, to take blood from the susceptible animal to which transmission is being attempted. As was seen in Sections A and B of the present chapter, this difficulty occurs in the aedine species which often die or weaken after ovipositing so that they are unable to take the second blood-meal. If a mosquito is still gravid when offered the second blood-meal it is unlikely to feed. A possible solution to this problem might be the demonstration of the ability of a mosquito to transmit virus by a method which does not depend upon blood-feeding. For a mosquito to be able to transmit it must have developed a salivary gland infection. If, after an adequate incubation period, the salivary glands of a mosquito which has previously taken an infective meal are dissected out and found to be infected, the presence of virus in these glands is thought to indicate the probable ability of the species to transmit by bite (Chamberlain, 1968). An experiment was therefore undertaken to compare this 'salivary gland method' with the conventional one to assess its reliability as a possible alternative method for determining the transmission rate.

METHODS

The salivary glands were dissected out and tested for virus in the course of one of the series of infection and transmission tests reported in Section B of the present chapter. This was experiment 1 of Table 18 in which a batch of *C. theileri* mosquitoes infected with SIN virus by feeding them on a blood mixture containing 5.5 logs of virus, were then allowed to feed on susceptible 2-week-old chicks 14 days later. On the day following this transmission feed, those mosquitoes which had engorged successfully were frozen on solid carbon-dioxide and, 12 days later, were removed from storage and allowed to thaw at room temperature. The salivary glands were quickly dissected out and ground up in a solution of bovine plasma

albumen ready for inoculation into infant mice. The procedure adopted for this is described separately. The salivary gland suspension and one prepared from the decapitated body of each mosquito were tested separately for virus by inoculating them intracerebrally into infant mice, operations carried out immediately after the salivary glands had been dissected out.

Mosquito dissection. A few mosquitoes at a time were allowed to reach room temperature, the balance being kept frozen until required. The salivary glands were dissected out from each mosquito by the method of Shute and Maryon (1960), with slight modifications. The head was severed from the body with a safety razor blade and dissected with two 'minutin' needles. The two glands were isolated in a small drop of normal saline solution and then rinsed and ground up with the needles in a second drop of saline. This suspension was transferred to a tube containing 0.3 ml of bovine plasma albumen with a glass micro-pipette and was then ready for inoculation into mice.

RESULTS AND DISCUSSION

The results of the transmission attempts made by individual mosquitoes and for the infection tests done on these mosquitoes are given in Table 22. Although the decapitated bodies of all 23 mosquitoes tested were found to be infected, only two individuals, Nos. 16 and 23, transmitted virus successfully to chicks. As anticipated, the salivary glands belonging to each of these mosquitoes were found to be infected, but the glands from a further seven individuals were also positive for virus. Thus it would appear, at any rate in the case of C. theileri and SIN virus, that although the salivary glands are infected, transmission will not necessarily follow when the mosquito bites the susceptible host. Perhaps in the infected glands of the seven unsuccessful transmitters a sufficiently high titre of virus had not been reached so as to enable these mosquitoes to infect the chicks. The results obtained by Miles et al. (1973) in Aedes australis with Wheteroa virus lend some support to such an explanation. They found that those individuals which transmitted the virus had a titre of 4.5 $\log_{10}$ pfu of virus or more in their glands, whereas the glands from those mosquitoes which failed to transmit had one of

TABLE 22. *C. theileri* and Sindbis virus; results of transmission attempts and of tests for infection in the salivary glands and in the remainder of the mosquito.

Mosquito No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Mosquito minus head	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salivary glands	+	+	-	-	-	+	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-	+
Transmission	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+

\+ : presence of infection

\- : absence " "

less than 1.7 $\log_{10}$ pfu. Furthermore, Thomas (1963), working with C. tarsalis and western equine encephalomyelitis (WEE) virus did not always obtain transmission by mosquitoes with infected salivary glands, although the interpretation of his results are difficult because he allowed groups of up to four mosquitoes to feed on each chick in his transmission attempts. More recently McLean et al. (1974) tested the salivary glands from infected A. aegypti individuals which had each bitten a mouse. In the case of California encephalitis virus, most of the mosquitoes with infected glands failed to transmit but when the mosquitoes had glands infected with dengue-2 virus all the individuals transmitted this virus successfully.

Clearly then the salivary gland method is not a suitable method to measure the transmission ability of C. theileri with SIN virus and it is likely that it would be misleading to employ it whatever the mosquito species or virus.

SECTION D

FACTORS INFLUENCING THE TRANSMISSION RATE IN CULEX UNIVITTATUS WITH WEST NILE VIRUS

In Section B of this chapter, C. univittatus, the maintenance vector of the two viruses, was among those species of mosquito whose vector capability was evaluated by experiments carried out in the insectary at 25°C. With each virus it was shown that the species is easily infected and transmission occurs readily, although the latter is more efficient with WN virus. However, instead of using a constant optimal temperature, such experiments could be conducted with a temperature regime which approximates more closely to summer conditions on the Highveld. This should lead to a better understanding of the natural transmission cycles. But before such improved experiments could be designed, some fundamental studies were necessary on the extrinsic incubation period and other factors which influence transmission.

The extrinsic incubation period is the interval between ingestion of virus-infected blood by an arthropod and the time when transmission of virus first occurs (Whitman, 1951). It is only with relatively few different viruses and species of mosquito that the extrinsic incubation period has been studied. Most work of this kind has been done with the viruses of yellow fever and the equine encephalitides of the New World. The period of extrinsic incubation can vary according to virus or mosquito species. Chamberlain et al. (1954[2]) showed in Aedes aegypti (Linnaeus) and Aedes triseriatus (Say) that optimal transmission with Western equine encephalitis (WEE) virus occurred after a much shorter incubation than with Eastern equine encephalitis (EEE) virus. It has also been demonstrated (Chamberlain et al. 1959) that a considerably longer incubation period for St. Louis encephalitis virus (SLE) is required in Culex quinquefasciatus Say than in Culex pipiens Linnaeus. Experiments reported by Hurlbut (1956) indicate that C. univittatus from Egypt may have an extrinsic incubation period as short as five days with WN virus.

The purpose of the present study was to determine the effect of three factors that possibly influence transmission of WN virus by C. univittatus, viz., length of the extrinsic incubation period,

environmental temperature and concentration of virus in the blood-meal.

MATERIALS AND METHODS

As these were essentially the same as those of Section B, only a short description is necessary in which any differences will be noted. For clarity the series of experiments will be referred to by the letters A to G both in the text and in the tables of results.

In experiment G, the mosquitoes belonged to the 48-49th generation from the first colony of C. univittatus while, in all the others, they were F_5 or F_6 females from the second colony (C.132). The membrane-feeding method was employed in Experiment G so as to infect the mosquitoes with two selected doses of virus. Thus there were two blood-virus mixtures prepared so that one contained twice the virus concentration of the other. The actual dose of virus in each mixture was determined by titration in infant mice immediately before the feeding of the mosquitoes commenced. In all the other experiments, infective meals were obtained from viraemic 1-day-old chicks, and immediately before these were exposed to the mosquitoes a blood-sample was collected which was titrated in adult mice to determine the virus titre. The day after the infective feed the engorged mosquitoes from each chicken or blood-virus mixture were placed in separate cages and held either at insectary conditions (26°C and 75% - 85% R.H.) or, in experiment F, at a mean temperature of 18°C and 75% - 80% R.H. Subsequently the mosquitoes were encouraged to oviposit. Transmissions were attempted at various intervals after the infective feed, as shown in the tables, by feeding each 'infected' mosquito on a different susceptible 7-day-old mouse (a few mosquitoes only in Experiment A), on a 2-4-day-old chick (Experiment B-F) or on a 11-12-day-old chick (Experiment G). The feedings took place under insectary conditions and in Experiment F the mosquitoes which had previously been held at 18°C were transferred to the insectary an hour before the feed. Successful transmissions were established by testing dead or dying chicks or mice for viraemia, or, three weeks later, by testing the sera of surviving chicks for WN virus antibodies with the haemagglutination-inhibition test. In all experiments except Experiment E a transmission test was done and

a transmission rate determined. For the determination of infection rates mosquitoes were killed usually 14 - 18 hours after the transmission feed.

RESULTS

The influence of time-lapse after infective feed. The infection rates and transmission rates for mosquitoes at increasing intervals after the infective feed are given in Table 23. It is evident that a 100% transmission rate can be obtained as early as the seventh to eighth day. A prolonged period of up to 49 days old not reduce the transmission rate and mosquitoes tested after 81 days were all infected, although by this time they were too weak to feed in a transmission test.

The influence of reduced temperature. Table 24 shows the results for two experiments in which batches of mosquitoes were held at 26°C (Experiment B) and 18°C (Experiment F), until transmission tests were conducted on the 17th day after the infective feed. Experiment B is one of the experiments from the series given in Table 23. The reduction in temperature did not reduce the infection rate as in both experiments it was 100%. However, in Experiment F only 13 out of 27 infected mosquitoes transmitted virus (48%), whereas in Experiment B 35 out of 36 (97%) did so. These two transmission rates are significantly different ($P < 0.001$).

The influence of viral dosage in infective feed. In Experiment G, transmission tests were carried out using two groups of mosquitoes which had previously fed on blood-mixtures containing 5.0 and 2.6 logs of virus respectively, according to titrations done in infant mice. These two doses are representative of the range of viraemia titres that occurred most frequently in wild birds, the hosts of C. univittatus, on the first two days after inoculation with virus (McIntosh et al. 1969). The findings made in Experiment G are shown in Table 25. In the high dose group of mosquitoes it was determined that all 38 mosquitoes were infected and that 34 (89%) of these successfully transmitted virus. On the other hand, of the 35 mosquitoes which fed on the lower viral dose, 15 (43%) were infected and only five of them (33%) transmitted virus. There is a significant difference ($P < 0.001$) between these two transmission rates.

TABLE 23

C. univittatus and West Nile virus; infection and transmission rates at various intervals after the infective feed for mosquitoes held at 26°C.

Expt.	Titre of infective feed in logs	Mosquito Infection		Transmission Tests	
		No. days after infective feed when mosquitoes tested	Infection rate	No. days after infective feed [a]	Transmission rate
A	4.1 - 4.6 [b]	8 - 9	24/24	7 - 8	15/15
B		16	37/37	17	35/36 (97%)
C		42	36/38 (95%)	41	33/37 (89%)
D		50	25/25	49	21/21
E		81	19/19	-	-

a - No. days after infective feed when transmission occurred.

b - As determined by titration in adult mice.

TABLE 24.

C. univittatus and West Nile virus; comparative infection and transmission rates of mosquitoes held at two different temperatures after the infective feed.

Expt.	Temperature °C.	Titre of infective feed in logs [a]	Mosquito infection		Transmission tests	
			No. days after infective feed when mosqs. tested.	Infection rate	No. days after infective feed	Transmission rate
B	26	4.1 - 4.6	18	37/37	17	35/36 (97%)
F	18		"	30/30	"	13/27 (48%)

a - As determined by titration in adult mice.

TABLE 25

C. univittatus and West Nile virus; comparative transmission rates of mosquitoes infected with two different viral doses and held at 26°C.

Expt.	Titre of infective feed in logs [a]	Mosquito Infection		Transmission Tests	
		No. days after infective feed when mosquitoes tested	Infection rate	No. days after infective feed	Transmission Rate
G	5.0	15	38/38	13	34/38 (89%)
	2.6	16	15/35 (43%)	14	5/15 (33%)

a - As determined by titration in infant mice, titres in adult mice are about 1 log lower.

DISCUSSION

The results of various studies (Davis 1932, Bates and Roca-Garcia 1946, Chamberlain et al. 1954[2], 1959) show that at 25°C - 26°C, the same temperature as used in most of the present experiments, the extrinsic incubation period varies widely, from three to 28 days, depending on the species of mosquito and strain of virus used. The present results show that with C. univittatus infected with WN virus the extrinsic incubation period is less than seven to eight days. However, it would not seem important to determine the exact incubation period since wild populations of this mosquito would rarely re-feed before the seventh day after the previous meal. This is clear because even at a constant optimal insectary temperature of 26°C, C. univittatus requires a minimum of four to five days after the first blood-meal to complete its first gonotrophic cycle before it can re-feed. This cycle would take longer, about seven days, at the fluctuating temperature experienced in the field during summer on the Highveld.

There is evidence from other laboratory studies carried out at 25°C - 26°C that transmitting ability decreases markedly after lengthy periods (Chamberlain et al. 1954[2], Chamberlain and Sudia, 19[illegible] 1957, McIntosh and Jupp 1970). However, after 41-49 days C. univittatus still transmitted WN virus at peak rates.

With C. univittatus and WN virus, a reduction in temperature for the post-infection period from 26°C to 18°C halved the transmission rate (97% to 48%), although the infection rates of both groups of mosquitoes were 100%. This result is similar to one obtained by Chamberlain and Sudia (1955) which formed part of a detailed study of A. triseriatus and EEE virus. After the same post-infection period of 17 days they obtained transmission rates of 53% and 85% with mosquitoes held at 21°C and 26°C respectively. Similarly their mosquitoes were all found to be infected. In both their and my experiments a gut infection presumably occurred in all those mosquitoes held at the lower temperature, while a salivary gland infection ensued in only some of them. For these the extrinsic incubation period was probably lengthened. It would be interesting to undertake further determinations of the transmission rate in the low temperature group at longer intervals after the infective feed

to see whether the rate would increase with time.

The finding that C. univittatus, infected after ingesting a lower concentration of WN virus, has a lower transmission rate (89% reduced to 33%) is noteworthy. This relationship between viral dosage in the infecting blood-meal and the subsequent transmitting ability does not seem to have received much attention. Chamberlain et al. (1959) observed that C. quinquefasciatus and C. pipiens transmitted SLE virus more efficiently if the mosquitoes had ingested blood with a high viral titre. Also, Thomas (1963) showed, with Culex tarsalis Coquillet infected with EEE virus, that the proportion of mosquitoes with infected salivary glands was higher in insects which had ingested blood containing a high viral titre. He concluded that the ingestion of small amounts of virus may not lead to infection of the salivary glands so that transmission cannot occur. It would be worthwhile to find out if the transmission rate of mosquitoes infected with a low virus dose increased with time. However, the result obtained shows that, if the transmission rates of different mosquito species are compared, a similar infecting dose should be used in each case.

CHAPTER 4

WINTER BIOLOGY

Previous epidemiological investigations at Lake Chrissie and at Olifantsvlei (McIntosh *et al.* 1967; ARU unpublished) have indicated that each summer on the Highveld, from December through March, birds and *C. univittatus* are regularly infected with both viruses. But it is not known how the viruses survive the unfavourable winter to re-appear in the following summer. If the mechanism for such virus survival lies in the mosquito maintenance vector, apart from trans-ovarian transmission, there are two possible ways in which *C. univittatus* could be responsible. The first is by its participation in retarded transmission cycles between mosquitoes and wild birds during the winter months and the second by overwintering adult mosquitoes carrying virus through this unfavourable season. The first step towards assessing the likelihood of either being correct is to gain an understanding of the winter biology of Highveld *Culex* species, a subject which has not received the attention of other investigators in South Africa. In Section A of this chapter, field studies are described designed to investigate the winter biology of the commoner species of Highveld *Culex* and to find out how they survive the Highveld winter. Aspects studied during this season were mosquito prevalence, life history stages, whether development of immatures continues and whether blood-feeding occurs. In addition, the proportion of parous females which occurred in mosquito collections from autumn until spring was determined during one year (1972). The reason for this was that if overwintering, perhaps hibernating, females are to carry virus through this season they would need to take an infective pre-winter blood-meal and re-feed after overwintering. If such female mosquitoes occurred overwintering populations should include a significant proportion of parous individuals. The ovarian trachestion technique was chosen for determining the parous rates after its applicability had been tested on the four commonest Highveld *Culex* species. The results of this evaluation are presented in Section B.

SECTION A.

FIELD STUDIES.

Field investigations carried out at localities around Johannesburg are recorded. Studies were undertaken from May through October in 1967, 1970 and 1972 and from June until September in 1969. The month of May was regarded as autumn, June - July - August as winter, and September - October as spring. Although an examination of temperature records shows that in some years the monthly mean derived from maximum and minimum temperatures has been slightly higher for August than for May, the mean minimum temperature for August is invariably lower. Except for one series of observations on larvae occurring at Rivonia, a suburb to the north of Johannesburg, field observations were restricted to Olifantsvlei. In addition, field experiments were undertaken at Olifantsvlei and at Rietfontein. Adult females and larvae of the three Culex species commonest at Olifantsvlei, C. pipiens, C. theileri and C. univittatus were studied. Observations were also made on the larvae of C. fatigans and Culex (Eumelanomyia) rubinotus Theobald and once only on those of An. tenebrosus. I did not read temperatures in 1967 but recordings made by the meteorological staff at Jan Smuts Airport, situated to the east of Johannesburg, are included in Table 27. In 1970 daily temperatures were recorded with a thermograph at Olifantsvlei and with maximum and minimum thermometers at Rietfontein in 1972.

COLLECTION OF ADULT FEMALES IN BAIT-TRAPS

Three series of collections of adult female mosquitoes were made at Olifantsvlei with different types of bait-traps:-

(1) 1967: six lard-can type traps based on the design of Bellamy and Reeves (1952) but each trap was smaller, 55 cm. long and square in cross-section (17.5 cm^2). The bait was a single domestic pigeon (Columba livia) restrained within a nylon stocking. This trap is referred to as the 'small lard-can'.

(2) 1970: three standard Bellamy and Reeves lard-cans each baited with 2.3 kg of solid carbon dioxide which was broken up and enclosed

in a cardboard box.

(3) 1972: three pigeon-baited suction traps (see methods in Chapter 2) each containing two pigeons.

Pigeons were chosen as avian bait because they withstand winter temperatures well. In each series, traps were set once weekly at one site to run overnight from 1700 - 0800 hours. Lard-can traps were suspended from trees and the wire pigeon cages with the suction traps by means of poles about 1½ metres above ground level. After each collection the traps were returned to the laboratory where the mosquitoes were killed, pooled and identified. Mosquitoes which entered the pigeon-baited suction traps were not able to feed on the bait but in the case of the small lard-cans the totals of fed and unfed mosquitoes were recorded. For the 1972 series, part or all of each identified catch was stored on dry-ice for the later removal of the ovaries to determine the parous rate.

The small lard-can baited with a pigeon was selected for the first series of trappings because at that time it was considered the best method for collecting C. univittatus. In 1970 a change was made to the larger standard-size-lard-can baited with solid carbon dioxide as it was believed that all three species of Culex at Olifantsvlei, including C. theileri, would enter this trap equally as readily and thus observations could be made on the activity of all three. C. theileri had entered the small lard-cans in the 1967 series less readily than individuals of the other two species. The numbers of C. univittatus and C. pipiens taken in the 1970 series proved to be very low however, and probably larger catches of these strongly ornithophilic species would have been taken if avian bait had been used. The pigeon-baited suction trap was chosen for the 1972 collections because it had been decided to concentrate on C. univittatus in that series and the preceding summer a comparison of several different traps had shown that the suction-trap with pigeon-bait collected the largest numbers of C. univittatus.

The 1967 series (Table 26). The number of mosquitoes collected in the small lard-cans from the middle of May through October are shown, together with records of temperature. Temperatures were lowest during the three winter months and, not shown in the table, dropped to below

TABLE 26

Number of mosquitoes and number fed collected weekly in six small lard-can traps baited with pigeons at Olifantsvlei (1967). Temperatures in °C recorded at Jan Smuts airport are also given: bracketed figures are monthly means while unbracketed are daily recordings.

Date	Max. temp.	Min. Temp.	Max.+ Min. 2	C. univittatus		C.pipiens		C.theileri	
				No. mosqs.	No. fed.	No. mosqs.	No. fed.	No. mosqs.	No. fed.
May 16				10	4	107	16	0	0
" 23	(18.0)	(7.1)	(12.6)	19	1	125	11	2	0
" 30				158	32	352	43	7	6
June 6	8.7	-0.5	4.1	0	0	0	0	0	0
" 13	13.5	-2.5	5.5	0	0	0	0	0	0
" 20	17.7	3.7	10.7	2	0	6	2	0	0
" 27	17.2 (14.7)	7.6 (3.4)	12.4 (9.1)	20	3	10	1	0	0
July 4	15.7	6.4	11.1	10	1	3	1	1	1
" 12	8.4	1.2	4.8	0	0	0	0	0	0
" 18	15.6	1.7	8.7	0	0	0	0	0	0
" 24	18.9	4.9	11.9	0	0	0	0	0	0
" 31	19.6 (14.7)	6.9 (2.2)	13.3 (8.5)	0	0	0	0	0	0
Aug. 7	17.7	0.6	9.2	1	0	1	0	0	0
" 14	19.1	9.0	14.1	15	4	2	1	0	0
" 21	16.1	0.7	8.4	0	0	0	0	0	0
" 28	20.1 (17.7)	7.6 (5.1)	13.9 (11.4)	34	7	1	0	1	1
Sept. 6				109	35	0	0	0	0
" 13	(23.0)	(9.1)	(16.1)	45	9	2	0	0	0
" 19				50	9	3	0	0	0
" 26				20	1	1	0	0	0
Oct. 3				80	14	2	0	0	0
" 12	(24.1)	(10.7)	(17.4)	139	27	0	0	2	2
" 17				306	172	0	0	1	0
" 24				594	134	2	0	3	3

freezing point on five nights in June, seven nights in July and three in August. As C. theileri is not so strongly ornithophilic as the other two species, the small numbers collected may not reflect its true density, relative to that of C. pipiens and C. univittatus. However, the single specimens of C. theileri collected on two occasions during the winter months prove that there were active females of this species present during that season. Small numbers of C. pipiens and C. univittatus were present in some of the weekly collections during each of the winter months and usually some blood-feeding occurred. The absence of all species from the traps on the 6th and 13th June and between the 12th and 31st July was probably mainly because temperatures were very low on those dates (Table 26). On the first four of the six nights concerned, the minimum temperature fell to between -2.5°C and 1.7°C. At such temperatures mosquitoes could not be expected to be active. Throughout the winter C. univittatus was somewhat more prevalent than C. pipiens and showed much higher densities during the spring in September and October. C. pipiens, however, had the higher density in the autumn in May.

The 1970 series (Table 27). In 1970, small numbers of C. theileri were taken intermittently in the carbon dioxide-baited lard-cans in June, July and August and a few C. pipiens in early June. Apart from these, no other catches were recorded during these three winter months. In the 1970 winter, mean monthly minimum temperatures at Olifantsvlei were very low (Table 27) and daily temperatures dropped to below freezing point on at least 10 nights in June, 21 in July and seven nights in August.

The 1972 series (Table 28). As with the pigeon-baited small lard-cans, the numbers of C. theileri collected in the pigeon-baited suction traps probably do not represent the true density of this mosquito relative to that of the other two species. The proportion of parous mosquitoes in each catch was found by examining the ovarian tracheation and, at the same time, the size of the fat body was observed. The catches indicated that C. univittatus, C. pipiens and C. theileri were all scarce during the 1972 winter. Very small numbers of C. pipiens and C. theileri were collected during June and then none until September 1st. C. univittatus entered the traps

TABLE 27

Number of mosquitoes collected weekly in three solid CO_2-baited lard-can traps at Olifantsvlei (1970). Temperatures in °C recorded at Olifantsvlei are also given: bracketed figures are monthly means, while unbracketed are daily recordings.

Date	Max. Temp.	Min. Temp.	Max.+Min. 2	Number of Mosquitoes		
				C. univittatus	C. pipiens	C. theileri
May 26	26.2 (21.8)	2.0 (3.4)	14.1 (12.6)	2	88	100
June 3	19.0	-	-	0	3	0
" 11	21.5	-1.0	10.3	0	2	4
" 17	-	-	-	0	0	1
" 24	17.0 (18.4)	-1.5 (-0.3)	7.8 (9.0)	0	0	0
July 1	18.0	-2.5	7.8	0	0	0
" 8	20.0	-3.0	8.5	0	0	0
" 15	14.5	-3.0	5.8	0	0	0
" 29	21.0 (17.7)	-0.5 (-0.7)	10.3 (8.5)	0	0	1
Aug. 5	24.0	4.0	14.0	0	0	1
" 12	19.5	2.5	11.0	0	0	0
" 20	21.5	2.0	11.8	0	0	0
" 26	20.0 (21.5)	11.0 (1.7)	16.5 (11.6)	0	0	3
Sept. 2	25.5	4.0	14.8	0	0	5
" 9	27.0	10.5	18.8	0	0	15
" 23	29.0	5.5	17.3	0	0	5
" 30	16.5 (24.5)	7.0 (7.0)	11.8 (15.8)	0	0	14
Oct. 7	27.0	10.5	18.8	1	0	11
" 14	28.0	8.0	18.0	0	0	22
" 28	- (24.4)	- (8.1)	- (16.3)	7	0	93

TABLE 28

Numbers of mosquitoes with proportion parous collected weekly in three pigeon-baited suction traps at Olifantsvlei (1972). Temperatures given were recorded at Rietfontein.

Date	Max. temp.	Min. temp.	Max. + Min. 2	C. univittatus		C. pipiens		C. theileri	
				No. mosq.	Parous rate.	No. mosq.	Parous rate.	No. mosq.	Parous rate
May 10	-	-	-	6	0/6	287	2/20 * (10%)	35	8/34 (24%)
" 17	21.4	8.1	14.8	8	2/8 (25%)	586	0/20	31	9/28 (32%)
" 24	19.6	4.9	12.3	4	1/4 (25%)	262	2/25 (8%)	7	3/7 (43%)
" 30	22.1 (20.2)+	5.3 (4.8)+	13.7 (12.5)+	14	2/14 (14%)	500	1/24 (5%)	32	9/29 (31%)
June 7	15.6	2.7	9.2	0		2	0/2	0	
" 14	19.0	-1.0	9.0	0		1	0/1	1	0/1
" 21	17.5	-1.5	8.0	0		1	0/1	0	
" 28	14.4 (17.8)	-2.7 (0.1)	5.9 (9.0)	0		0		0	
July 5	18.3	0.0	9.2	0		0		0	
" 12	18.8	-3.2	7.8	0		0		0	
" 19	18.8	4.2	11.5	0		0		0	
" 26	20.1 (19.1)	-0.2 (0.6)	10.0 (9.9)	0		0		0	
Aug. 2	8.8	-4.2	2.3	0		0		0	
" 9	21.6	-0.5	10.6	0		0		0	
" 16	21.3	4.2	12.8	1	1/1	0		0	
" 23	26.4 (23.6)	4.3 (2.9)	15.4 (13.3)	0		0		0	
Sept. 1	22.1	10.7	16.4	11	8/8	4	0/4	2	0/2
" 6	19.4	0.0	9.7	0		1	0/1	0	
" 15	27.6	8.6	18.1	0		0		2	0/2
" 20	28.0	7.0	17.5	11	10/11 (91%)	1	1/1	0	
" 27	25.4 (26.1)	10.8 (6.7)	18.1 (16.4)	46	29/42 (69%)	0		0	
Oct. 5	18.9	11.4	15.2	22	8/19 (42%)	2	0/2	0	
" 12	27.8	10.2	19.0	71	27/40 (68%)	19	7/15 (47%)	5	1/5 (20%)
" 26	23.3 (27.0)	10.2 (11.3)	16.8 (19.2)	258	19/46 (41%)	176	15/44 (34%)	27	5/24 (21%)

* Numerator = number parous, denominator = total number dissected.

\+ Bracketed figures for May are mean temperatures for period from 15th through 31st only, while other bracketed figures given are monthly means.

only once in the winter, on August 16th. Temperatures dropped to below freezing point on 12 nights in June, eight in July and nine in August, i.e. on about half the nights on which traps were set. By the beginning of winter, at the end of May, most individuals of all three species had developed a moderate-sized fat body which was still present at the end of October. In the autumn (May) some of each of the three species were parous, the highest rate occurring in C. theileri followed by C. univittatus and then by C. pipiens. Of the mosquitoes appearing in the winter months, C. pipiens and C. theileri were all nulliparous and continued so into the early spring (September), while one parous C. univittatus was collected on 16th August and eight further parous females of this species on September 1st. The density of C. univittatus increased in the spring more than that of the other two species. A high parous rate was maintained by C. univittatus, while the rates for C. pipiens and C. theileri increased with the increasing spring populations.

OBSERVATIONS ON WINTER LARVAE

(1) C. fatigans larvae at Rivonia. On 29th June, 1967, many C. fatigans larvae, estimated as 2nd, 3rd and 4th instars, were discovered in a concrete ornamental 'wishing well' situated in the grounds of an hotel in Rivonia, Johannesburg. The well contained 60 cm of water covering dead leaves. Four pupae were found. These and 13 larvae were brought to the laboratory where the pupae were maintained at ambient temperature and in natural daylight outside, while the larvae were kept inside at 23°C (water temperature) but also in natural daylight. One pupa died after three days. From the other three, adults emerged after about nine days but died from drowning directly after emergence. The larvae continued their development at rates similar to those of comparable larvae in a laboratory colony with the production of healthy adults. The identity of the mosquito was determined by examination of larvae and confirmed subsequently by examining the adult male genitalia.

In order to find when adult emergence would occur from the well, it was enclosed with copper mesh to prevent the escape of any adults which subsequently emerged. After enclosure it was not possible to

make a proper inspection for the presence of pupae, although adults could be seen readily.

The well was examined fortnightly and by 26th August the water level had dropped to 20 cm. when the first adults - 12 including one female - were seen, together with larvae and pupae. It would appear as if emergence had commenced on about this date, as it is known that the males of C. fatigans emerge first. The date when the last larva pupated was unknown as the experiment was terminated on 26th August.

(2) Larval collections at Olifantevlei. A search for larvae was conducted on several occasions at Olifantevlei during the winter and a total of seven positive collections resulted, the details of which are shown in Table 29. Instars of these larvae were estimated and mosquito identifications were made from the larvae themselves and confirmed by examination of adult mosquitoes subsequently reared out from such larval samples which were brought back to the laboratory and maintained at [illegible]°C (water temperature) and in natural daylight. There they continued their development to adulthood at the rate of laboratory-reared larvae. The identity of C. pipiens was confirmed by an examination of male genitalia. In the case of two of the collections, Nos. 4 and 7, further observations were made on the development of those larvae still remaining in the field. From the site where collection No. 4 was obtained in June, 1969, a large sample of C. theileri larvae was taken back to the laboratory at Rietfontein and placed in two buckets within an 'over-wintering cage'. This experimental exposure of these larvae is described in the next sub-section. Collection No. 7 of C. pipiens larvae was taken from a 22 litre plastic container filled with an infusion made from dried grass and cow dung. This was one of eleven such containers distributed at Olifantevlei as artificial oviposition sites in the autumn of 1970, but the only one in which winter larvae were subsequently found. On the 30th June it contained 4th instar larvae, a sample of which was brought inside the heated laboratory. This sample underwent pupation after two days whereas the remaining larvae left behind in the field did not start pupation until 14 days later. On 28th July the first adults emerged so that the developmental period from 4th instar larvae to adulthood was at least 28 days. Adults continued to emerge until about 23rd August when the last pupa became an imago.

TABLE 29

Winter larval collections at Olifantsvlei.

Collection No.	Date collected	Developmental stage	Species and No. larvae/pupae	Habitat
1.	12. 6.67	2nd instar larvae	C. pipiens : 6 C. theileri : 1 C. univittatus: 1	Small ground pool, muddy water.
2.	20. 6.69	2nd + 3rd instar * larvae	C. rubinotus : 6 An.tenebrosus: 3	Furrow with aquatic plants, emergent vegetation and reed margins.
3.	"	Pupa	C. pipiens : 1	Earthenware pot.
4.**	"	2nd, 3rd + 4th instar Larvae and Pupae	C. theileri : many (sample taken)	Pond with emergent vegetation.
5.	11. 7.69	2nd instar larvae	C. rubinotus : 6	Furrow as in No. 2
6.	"	2nd + 3rd instar larvae	C. rubir t s : 3-6	Furrow similar to No. 2.
7.**	30. 6.70	4th instar larvae	C. pipiens : >20 (sample taken)	Plastic container, 22 litres.

* larval instars were estimated. ** the development of these larvae was observed further in the field - see text.

EXPERIMENTAL EXPOSURE OF LARVAE AND ADULTS

(1) C. pipiens and C. univittatus (1967) (Tables 30 and 31) One way of discovering the developmental stage in which the mosquitoes overwintered was the exposure of larvae and adults in the field from autumn until spring to see which stage survived. Whether development would occur in the immature stages and whether a further generation would be produced from gravid adults were also questions needing an answer. Special 'over-wintering' cages were set up inside which were provided environments thought suitable for one or the other possible overwintering (perhaps hibernating) stages.

The nylon mesh cages were 0.8 m. high, 1.7 m. long and 0.5 m. wide. The bottom of each cage was removed. Access was provided at the top through a cotton sleeve. Cages were sited in the field so that about half the bottom of a cage was over thick grass which reached to the top so that nearly half the volume was filled by grass, while the contents of the other half varied according to whether a cage was used for exposure of adults or larvae. Cages for adults had in this area a pile of large stones and a 13½ litre bucket sunk into the ground, while cages for larvae had two sunken buckets. Each bucket was quarter-filled with mud and then topped up with water to which green grass was added to form an infusion. The top and one of the long sides of each cage were sheltered with board.

Adults and larvae of C. pipiens were exposed in two cages (A and B) at Olifantsvlei while larvae and adults of C. univittatus were placed in two cages (C and D) at Rietfontein, all situated in the open. Larvae of both species and most of the C. univittatus adults had been reared in the laboratory from egg rafts deposited by gravid females brought in from the field in April. Rearing conditions had been 21°C (air temperature) and a 12-hour artificial 'day'. Blooded C. pipiens adults had been collected in traps and transferred to cage A for exposure on the same day while the 73 engorged C. univittatus adults exposed in cage D along with laboratory-reared adults had been collected three days previously (see Tables 30 and 31). The age of the reared C. univittatus adults on exposure was 1 - 24 days.

After exposure at the beginning of May, cages A and B were observed weekly and cages C and D more frequently, although the presence or

TABLE 30

Experimental exposure of *C. pipiens* adults and larvae (1967)

Date	Cage A	Cage B
May 9	352 Field-collected blooded adult females exposed.	1st batch of laboratory-reared larvae, 2nd instar, exposed.
May 16	Adults	Larvae from 1st batch seen. 2nd batch of laboratory-reared larvae, 3rd & 4th instar, exposed.
May 22	Adults + rafts	larvae
May 29	2 adults	larvae + pupae
June 5	Larvae	larvae, pupae + 1 adult male.
June 12	Larvae	larvae + pupae.
June 19 to July 24	Larvae	larvae
July 31	Larvae	nothing visible.
Aug. 7 to 21	Nothing visible due to dark coloured water	Nothing visible
Aug. 29	Cages removed due to grass fire and replaced.	
Sept. 6	4th instar larvae + pupae	Buckets returned to laboratory No mosquitoes found.
Sept. 7	56 adults emerged	No mosquitoes found.

TABLE 31

Experimental exposure of C. univittatus larvae and adults (1967).

Date	Cage C	Cage D
May 12	Laboratory-reared 3rd and 4th instar larvae exposed	Laboratory-reared unblonded and unfertilized male and female adults, and 73 field-collected blooded adult females exposed
May 15 - 17	Larvae + pupae	Adults + rafts
May 19	Larvae, pupae + drowned adults	Adults + rafts
May 22 - 26	Larvae, pupae + drowned adults	Adults
June 1	Pupae	Adults
June 14	Nothing visible	Adults from 1st batch seen. 2nd batch of laboratory-reared adults exposed.
June 22	Nothing visible	Adults
June 28 to July 12	Nothing visible	Adult females
July 23	Cages + buckets finally inspected : no adults or larvae found.	

absence of the different stages could not be seen with any certainty through the mesh of the cages. The results are summarized in Tables 30 and 31 which give the developmental stages seen at different times until the cages and buckets were thoroughly investigated at the end of the experiment.

Referring to Table 30, it should be mentioned that, unfortunately, cages A and B had to be removed on 29th August because of a nearby grass fire, but were replaced over the buckets within an hour. This occurred before the final inspection of the cages so it was not known if any adults were present at the end of the exposure period. In cage A egg rafts were laid by the gravid C. pipiens adults on or before 22nd May and these gave rise to the next generation of adults on 7th September. In cage B, some development was shown by the larvae, pupae being seen between 29th May and 12th June, but no larvae or pupae were found in the buckets at the end of the experiment although some larvae survived until 24th July.

Table 31 shows that no C. univittatus larvae or adults were found on 23rd July when cages C and D were finally inspected. This inspection was carried out a month earlier than planned because the ground on which the cages were sited had to be ploughed. In cage C, pupae were seen between 15th May and 1st June and these appeared to give rise to adults which drowned after emergence. The two batches of adults exposed in cage D each survived for at least a month after exposure. Rafts laid by the 73 gravid adults did not appear to produce larvae.

(2) C. theileri (1969) (Table 32). In the winter of 1969 one experiment was carried out with C. theileri, in a way similar to that used in 1967 but with the larval stage only. A sample of winter larvae, estimated as 2nd, 3rd and 4th instars, collected at Olifantsvlei (collection No. 4 in Table 29) was placed in two buckets within an overwintering cage at Kistfontein. This cage was similar to that already described except it was larger, measuring 1.8 m. high, 1.8 m. long and 1.2 m. wide, which permitted a person to enter, thus facilitating observations. The larvae were exposed on 21st June and examined periodically. The observations made are given in Table 32. The first of the 4th instars exposed on 21st June pupated on about the 9th and 10th July giving rise to adults from about 21st

TABLE 32

Experimental Exposure of *C. theileri* larvae (1969)

Date	larvae	pupae	pupal castes	adults	dead emerging adults	drowned adults
June 21	+*					
" 30	+					
July 9	+	+(1)				
" 10	+	+(4)				
" 12	+	+(8)			+(1)	
" 15	+	+				
" 17	+	+			+(1)	
" 21	+	+	+(9)	+(2)		
" 31	+	+	+(>8)	+(1)		+(9)
August 11	+	+		+(1)	+(1)	+(3)
" 20	+	+	+			+(3)
" 25	+(6)	+(25)	+(58)	+(5)		+(7)
Sept. 3		+(8)	+(40)	+(16)		+(6)

* - field collected 2nd, 3rd + 4th instar larvae placed inside overwintering cage.

"+" - indicates the presence of the developmental stage concerned; figures in parenthesis are the number of individuals counted.

July onwards. Hence, about one month elapsed from 4th instar larvae to adult emergence, during which time the pupal per. d lasted 11 to 12 days. The 2nd instar larvae initially exposed, on the other hand, account for the large batch of pupae observed on 25th August giving rise to adults on 3rd September. Hence, the period from 2nd instar larvae until adulthood was as long as 74 days. The death of a few adults during emergence in the winter months may have been due to falls in temperature which occurred on those occasions.

DISCUSSION

Quiescence and diapause. Quiescence and/or diapause are the biological mechanisms which enable Culex mosquitoes to survive the winter. Quiescence in insects refers to the temporary suspension of activity under the direct action of an unfavourable environmental influence, with removal of the influence permitting a prompt return to normal activity (Bellamy and Reeves, 1963, after Lees, 1955). Diapause, on the other hand, is a physiological state of suspended activity or arrested development that facilitates survival through a period of unfavourable conditions, but is usually initiated before onset of these conditions. Once entered into, diapause must run its course or can be broken only with some difficulty (Bellamy and Reeves after Lees, op cit.). Culex (C) tarsalis Coquillet in Kern County, central California, overwinters by nulliparous females going into diapause. These are mosquitoes which emerge in the late autumn, fail to take blood and develop a distended fat body. Their diapause is induced by the shortened day length at this time and is terminated by an accumulated heat budget in the insect as temperatures rise in spring, thus allowing the first blood-meal to be taken (Bellamy and Reeves, 1963, Burdick and Kardos, 1963 and Nelson, 1964). Larvae are normally absent in Kern County during winter. A similar diapause has been recorded for C. (C) pipiens pipiens L. in England (Tate and Vincent, 1936), and C. tritaeniorhynchus in Japan (Kawai, 1969). Gonotrophic dissociation, the formation of a fat body instead of eggs after a blood-meal, has been shown to occur in the laboratory in C. pipiens pipiens, but not in C. (C. pipiens quinquefasciatus Say, in response to low temperature and short photoperiod (Eldridge, 1968).

Under natural winter conditions, this phenomenon has been shown to occur sometimes in C. tritaeniorhynchus in Japan (Kawai, 1969) and has also been demonstrated by a field experiment in C. pipiens pipiens in Illinois (Hayes, 1973). Mosquitoes in this condition are nulliparous in spite of having taken a blood-meal.

The Imperial Valley, Southern California, has a milder winter than Kern County with mean minimum temperatures 5°C to 7°C and mean maximum temperatures 18°C to 21°C. (Nelson, M., 1971). He showed that throughout the winter females of C. tarsalis were attracted to CO_2-bait, although numbers were reduced, and adult males, larvae of all instars and pupae were always present. His conclusions were that C. tarsalis in Imperial Valley has a short weakly expressed diapause because of the relatively high autumn and winter temperatures. Both adults and larvae seem to show a degree of quiescence in winter in this region so that they resume blood-feeding and continue their normal development respectively at any time after a rise in temperature. The adult is usually the more important stage for carrying a Culex species through the unfavourable season but Chapman (1959) reported that C. erythrothorax in Nevada overwintered principally as larvae, and those he saw appeared to be quiescent.

Catches of adult females. The overwintering biology of Highveld Culex species seems closest to that exhibited by C. tarsalis in Southern California in that small numbers of female mosquitoes take blood during the winter, it being assumed that the adult females which entered the bait-traps were seeking blood. The 1967 collections showed a low density of adults of C. pipiens through most of the autumn and winter, with a noticeable absence from the 4th to 31st of July, which was the coldest month of that year. They also showed that blood-seeking adults of C. theileri are present in the winter, although their density relative to the other two species was probably higher than indicated by the pigeon bait. The largest catches were of C. univittatus which did, however, as did C. pipiens, disappear from the traps from 4th to 31st July. From the end of August until 24th October the catches of C. univittatus increased greatly in contrast to the other two species. This increase might indicate the emergence of a spring generation of adults from over-wintering larvae. The 1970 catches agree with the 1967 series in

demonstrating the presence of a small number of C. theileri during the winter. Bait-traps were not set in a locality where C. fatigans normally occurs so that no information was obtained about adult stages of this species in the winter.

The catches made in the winter of 1972 were very small and the nulliparous females of C. pipiens and C. theileri collected in June probably emerged in May in the autumn. Nulliparous females of these two species collected at the beginning of September were probably mosquitoes just emerged, or those which had emerged earlier either during a warm spell in the winter or before the winter had started and which had been quiescent until temperatures rose again in September. Parous C. univittatus was found in the collections of 1972 at the tail-end of winter and was not nearly so prevalent as in the 1967 winter. The numbers of all three species increased in the spring with an accompanying increase in parous rates over such rates for the winter, or, in the case of C. univittatus, a high parous rate was maintained. These spring samples are thought to have been due to new broods of mosquitoes which were active in biting and ovipositing.

Winter larvae. The observations made on naturally occurring winter larvae and the results of the experiments in which both larvae and adults were exposed in the field from autumn onwards show that the larval stage plays a role in the overwintering biology of Highveld Culex species.

The study of winter larvae of C. fatigans at Rivonia in the winter of 1967 showed that development from 4th instar larvae to adults lasted about 60 days and that these mosquitoes had remained in the larval and pupal stages throughout the winter months of June, July and August and, in the case of some larvae, for longer. Similarly, the exposure of gravid C. pipiens in 'overwintering' cage A in 1967 (Table 30), showed that the developmental period from egg to adult of the subsequent generation lasted a minimum of 108 days from mid-May, against one of about 14 days under optimum laboratory conditions. The emergence of the C. fatigans at the end of August and of the C. pipiens adults at the beginning of September occurred at a time when temperatures started to rise at the end of winter (see Table 26).

These results, therefore, show that C. fatigans and C. pipiens can overwinter as larvae throughout the three winter months, developing slowly until the adult stage is reached at about the end of August. The collection of 2nd instar C. pipiens larvae from a ground pool in June during the same winter is further evidence of this. It is concluded that the larvae studied overwintered by quiescence as opposed to diapause. This view is held because, when both the C. fatigans and C. pipiens larvae collected in the field were brought into the laboratory where the temperature was higher but the daily photo-period the same, development to adulthood continued at rates similar to those of laboratory-reared larvae.

The results obtained in overwintering cage B with C. pipiens, again in 1967, (Table 30), suggest that if larvae succeed in pupating while temperatures are still low, the pupae die or fail to produce adults which stay alive till spring. The few C. fatigans pupae found on 29th June at Rivonia produced adults which died on emergence, probably because of the low temperature. Possibly, if young larvae had been exposed in cage B at the beginning of June, these would, as in cage A, have eventually given rise to adults at the beginning of September. Alternatively, perhaps the laboratory-reared larvae failed to survive the winter because they first became acclimatized to the high laboratory temperature and as a result were unable to survive the lower temperatures subsequently experienced in the field.

Little definite information can be gleaned from the 1967 experimental exposure of C. univittatus larvae and adults. Pupae arising from the larvae exposed from the middle of May onwards gave rise to adults which drowned. Possibly, as has already been suggested in the case of C. pipiens laboratory-reared larvae, the C. univittatus larvae in cage C (Table 31) would have overwintered if first instars had been exposed at the beginning of June. It is likely that if a larger number of field-collected gravid adults had been exposed in cage D, as with C. pipiens in cage A, C. univittatus larvae would have been found at the beginning of September. However, the collection of one C. univittatus and one C. theileri larva in June of the same year from a ground pool and the accompanying laboratory observations suggest that quiescent overwintering larvae may also be of importance in these two species.

The results of the larval collecting during the winters of 1969 and 1970 at Olifantsvlei and the extended observations made on larvae of two of these collections confirm the 1967 study by indicating that C. pipiens can overwinter as quiescent larvae. Furthermore, they indicate that C. theileri can bridge the winter in this way and suggest that C. rubinotus also possesses an overwintering quiescent larval stage. Emergence began as early as the end of July from immature stages of C. theileri studied in 1969 and from those of C. pipiens studied in 1970. This was probably due to the higher temperatures of the winter months of these years compared with those of the same months of 1967. Such emergence during mild periods in the winter could account for the small numbers of nulliparous C. theileri which entered the bait-traps at this time in 1970.

Overwintering of the mosquito: conclusion. Did the bait-trap catches of feeding females made in the winter represent the entire mosquito population of each species present at this time or was part of the population in diapause overlooked? It seems unlikely that diapause occurs on the Highveld similar to that of C. tarsalis in Central California because searches for diapausing females were fruitless at Olifantsvlei and also because, on the Highveld, day length in the autumn and winter is not shortened to the same extent as in California. However, further searches for diapausing females in shelters of man-made origin (e.g. bridges and culverts), potential hibernation sites which have been neglected on the Highveld, must be undertaken before the possibility of diapause is entirely discounted. There was no evidence of gonotrophic dissociation in Highveld Culex species.

Winter temperatures at Olifantsvlei are similar to, although a little lower, than those in southern California and it would appear that Highveld Culex species have an overwintering biology similar to that of C. tarsalis in the Imperial Valley. In this type of overwintering the immature stages and adult females would seem to show alternating periods of quiescence and activity according to fluctuations in winter temperatures, the growth of larvae and blood-feeding by adults being resumed in warmer periods. The parous individuals of C. univittatus collected in the winter of 1972 might indicate that adults of this species have a winter biology different from

those of C. pipiens and C. theileri, overwintering in the parous condition. However, this would seem unlikely as all three species belong to the same subgenus and show a similar summer biology. Only tentative conclusions can be drawn from the parous rates because of the small size of the samples.

Overwintering of the viruses: conclusion. As was pointed out at the beginning of this chapter, virus survival through the winter, if it is dependent upon the arthropod host, C. univittatus, could take place in two possible ways, apart from transovarian transmission. It could occur through retarded transmission cycles between the mosquito and species of wild birds during winter or through mosquitoes, infected in the previous autumn, remaining alive throughout the winter and passing on their infection to susceptible vertebrate hosts at their next blood-meal in the following spring.

The first alternative is highly unlikely because the density of biting C. univittatus drops to very low levels during the winter and because, moreover, the monthly infection rate of natural summer populations of C. univittatus is not usually more than 2.0 (McIntosh et al. 1967). As concerns the second alternative, the 1972 study of adult mosquitoes failed to show clearly whether or not C. univittatus females which have taken blood in the autumn could overwinter and re-feed in the following spring. If, as might be expected, C. univittatus has the same overwintering biology as C. pipiens and C. theileri, the results obtained for the other two species would suggest that C. univittatus is more likely to occur in a nulliparous condition at the beginning of winter, which would prevent it from being a vehicle for carrying virus through the unfavourable season. It is unlikely, therefore, that the arthropod maintenance vector could be responsible for the overwintering of either virus on the Highveld.

Taylor et al. (1956) obtained some evidence to support the overwintering of WN virus in Egypt by retarded transmission in C. pipiens. This evidence consisted of one unconfirmed isolation of virus from C. pipiens in winter, a few serological conversions to antibody status against WN virus at this time in children and the persistence of active biting C. pipiens in houses through the winter. However, Hurlbut & Weitz (1955) found that the Egyptian form of C. pipiens, although

anthropophilic, was only mildly ornithophilic, which would limit the likelihood of this mosquito acquiring infection from avian hosts. In France, Mouchet et al. (1970) found that the probable maintenance vector of WN virus in the Rhone Delta, C. modestus, overwintered in the form of hibernating or diapausing adult females and suggested that the virus might overwinter in them

SECTION 6

EVALUATION OF THE OVARIAN TRACHEATION TECHNIQUE FOR DISTINGUISHING NULLIPAROUS FROM PAROUS FEMALES IN CULEX SPECIES

In terms of both simplicity and reliability, it seems that the best method for distinguishing nulliparous from parous females in mosquitoes generally, is by the ovarian tracheation. This was developed by Detinova on Anopheles in 1945 and reviewed by her in 1962. The principle is that, in the ovaries of a nulliparous female, the tracheoles are coiled in tight knots or skeins at the ends of the trachese, and that once eggs have been developed and deposited, the trachese are irreversibly stretched causing the skeins to unwind and d ppear. The technique has been us successfully on a number of ulicines and is probably applicable to mosquitoes generally, although Corbet (1959) said that the degree of tracheolar coiling in nulliparous females varies from one species to another. For Culex species, it has been used extensively on C. (Culex) tarsalis Coquillet in California (Kardos and Bellamy, 1951, Burdick and Kardos, 1963, Nelson, 1966) and lately has been employed by Davies et al. (1971) in Brazil on nine species, although most dissections were confined to C. (Melanoconion) portesi Senevet and Abonnenc and C. (Melanoconion) taeniopus Dyer and Knab.

Before the method could be utilized for parity determinations on collections of Culex species made at Olifantsvlei in the 1972 winter study (Section A), its application was evaluated on the three species concerned, namely C. univittatus, C. pipiens and C. theileri and also on C. fatigans.

METHODS

The mosquitoes re from laboratory colonies of C. fatigans and C. univittatus, while for C. pipiens and C. theileri progeny reared from females collected in the field were used. Nulliparous and parous individuals were prepared, killed, and stored on solid carbon di xide, either dry in small screw-capped bottles or, following a suggestion by Davies (1969), in lots of up to 25 in such bottles

containing 3.5 ml of physiological saline and a single drop of household liquid detergent ('Sunlight Liquid'). The specimens were allowed to thaw, preperatory to dissection, and were placed in a solution consisting of nine parts distilled water and one part liquid detergent for about ten minutes. This wetted the specimen and softened the tissues, which may have become dry and brittle between death and freezing and thus facilitated subsequent dissection. The specimen was then washed in distilled water and the abdomen cut off. This was transferred to a clean microscope slide on which both ovaries, after removal under a dissecting microscope, were placed in a drop of water and allowed to dry. Usually only a pair of ovaries were mounted on one slide. Ovaries were examined under a compound microscope immediately after drying or up to several months later. Slides of parous and nulliparous ovaries were made for each of the four species. These were then given to a colleague who prepared the slide labels to avoid any personal bias when I tested my ability to recognize the two types of ovary.

RESULTS AND DISCUSSION

As can be seen from Table 33, all specimens were diagnosed correctly except three parous *C. univittatus* and one parous and one nulliparous *C. theileri*. The incorrectly diagnosed specimens of *C. univittatus* were intermediate. In their ovaries only a small number of tracheolar skeins had become unravelled because the mosquitoes had only taken small blood-meals and had consequently laid small rafts. The same applied to the single parous *C. theileri*, while the nulliparous specimen of this species was mis-diagnosed because the ovary had been damaged and stretched causing some skeins to be unravelled.

It is clear, therefore, that the ovarian tracheation technique can be applied reliably to material belonging to the four *Culex* species tested. Since these species do not, as far as is known, exhibit autogeny, 'intermediates' should be interpreted as parous unless the ovary in question has been damaged. Ovaries in an intermediate condition are not encountered so often in wild-collected, as in the laboratory-reared mosquitoes used here. If an ovary is unduly stretched or damaged it may be wrongly interpreted which

TABLE 33.

The results of an assessment of ovarian tracheole classification in 4 Culex species.

	No. slides prepared		No. slides correctly diagnosed	
	Parous	Nulliparous	Parous	Nulliparous
C. fatigans	29	39	29	39
C. pipiens	9	11	9	11
C. univittatus	27	25	24	25
C. theileri	32	44	31	43

underlines the importance of having both ovaries available for examination. Failure to remove pieces of fat body from an ovary before drying it may also prevent a proper diagnosis. The method is restricted to those mosquitoes with abdomens 'empty' or containing a fresh blood-meal. In order to age all categories of mosquito the dilatation technique as evaluated by Nelson (1966) would have to be employed.

Specimens which had been stored dry on solid carbon dioxide for up to five months were still suitable for dissection and there did not seem to be any advantage in storage under liquid. In one instance, where wet storage had been used, a tube of specimens was refrozen after thawing because of failure to dissect all the specimens in the time available. It was found subsequently that the remaining mosquitoes were too soft for dissection.

CHAPTER 5

GENERAL CONCLUSIONS.

MOSQUITOES AS VECTORS

Qualification for vectorship, either as a vector in the feral cycles of WN and SIN viruses or as a link vector between these cycles and men, varies among the seven main species which have been included in the study. Each species is assessed as a vector on its prevalence and feeding habits (Jupp and McIntosh, 1967; and Chapter 2), the results of laboratory vector capability experiments (Chapter 3, Sections B and D) and the level of infectivity in wild populations (McIntosh *et al.* 1967; and ARU unpublished work). Each species will be discussed in turn below. In order to facilitate an evaluation of each one and the comperison between species, the vector capability determined experimentally for six of the seven mosquitoes is summarized in Table 34.

C. univittatus. The results obtained in the laboratory vector experiments are in accord with the epidemiological observations made over several years at Olifantsvlei and Lake Chrissie (McIntosh *et al.* 1967). The epidemiological studies showed that while infection rates of wild-caught *C. univittatus* were nearly the same for both viruses during each summer season, the number of infections recorded in sentinel fowls exposed at both study localities was consistently higher for WN virus. This suggested that the mosquito transmitted WN virus to the sentinel birds more efficiently than SIN virus. The previous field studies had also indicated that *C. univittatus* was the only vector of importance infecting the sentinels, so the possibility of a different vector for each virus and providing an alternative explanation for the difference in sentinel infection rates did not arise. The findings made in another field study (ARU unpublished) at Bethulie situated in a more arid area on the edge of the Karoo in the Orange Free State, were similar in that, over three years, there were two and a half times more WN virus than SIN virus infections in sentinel pigeons. However, the infection rate with SIN virus in wild-caught *C. univittatus* was twice that with WN virus. This differs

TABLE 34

Summary of the vector capability determined experimentally for six mosquito species with West Nile and Sindbis viruses.

		Infection threshold log ED_{10}	Transmission Rate [a]
C. univittatus	WN	<1.0	12/13 (92%)
	SIN	1.6	14/26 (54%)
C. theileri	WN	1.5	8/50 (16%)
	SIN	2.0 - 2.9	2/23 (9%)
C. pipiens	WN	1.0	4/16 (25%)
	SIN	6.4	-
C. fatigans	WN	1.4	3/19 (16%)
	SIN	ca 5.0	0/2
A. unicentatus	WN	3.4 - 4.5	-
	SIN	<2.6	0/3 [b]
A. centatus	WN	>4.5	0/1
	SIN	ca 3.5	0/3 [b]

a - the overall rate determined for each species.

b - These mosquitoes did not all feed on separate animals.

from the Highveld findings but does emphasize the superior ability of the mosquito to transmit WN virus.

Taking into consideration the levels of viraemia shown to occur in birds (McIntosh et al. 1969) and the infection thresholds required to infect C. univittatus it is clear that most birds common on the Highveld are a potential source of infection of either virus for this mosquito. In the avian studies, nearly all species which were inoculated with virus circulated both viruses at levels well above the demonstrated infection thresholds. Usually the levels of SIN virus were higher than those of WN virus which may perhaps serve to compensate for the slightly higher 10% threshold demonstrated for SIN virus and result in the approximately similar infection rate between the two viruses found in wild-caught C. univittatus on the Highveld.

Thus, in several respects, the degree of vector potential shown by C. univittatus with SIN and WN viruses under laboratory conditions supports the earlier conclusion (derived from field observations on the infectivity, prevalence and host preferences of this mosquito) that it is the maintenance vector of both viruses on the Highveld in feral cycles between the mosquito and wild birds. The results of the present studies on feeding habits support this conclusion.

The reduced ability of C. univittatus to transmit at a decreased temperature, as demonstrated in the laboratory with WN virus, is probably significant. It suggests that the temperature fluctuations which Highveld populations of this mosquito experience are likely to influence transmission in the feral cycle of WN virus and probably of SIN virus. Under normal summer conditions the average minimum daily temperature ranges from 13°C to 15°C, although on some nights it may descend below this, occasionally as low as 9°C. On the other hand, the maximum temperature in the daytime averages 26°C to 30°C, although it may reach 36°C. It is possible that these high day temperatures may nullify, or partially nullify, the effects of the low night temperatures on mosquito transmitting ability, a point that should be investigated. Over and above this, the occurrence of unseasonal low temperatures in the summer or autumn would be expected to reduce transmission. In order to undertake laboratory investigations along these lines, a temperature regime could be set up based

on the daily summer temperature pattern. Mosquitoes would be held under such a regime during transmission tests.

The findings that the species can, to a small extent, be endophagic on man coupled with its high vector capability suggests that C. univittatus is probably an important transmitter of both viruses to man. The increase in host range at high densities shown by C. pipiens in the study on feeding habits suggests that C. univittatus might also become more anthropophilic at unusually high densities and this would greatly enhance its ability to infect man. The event observed in C. pipiens may well have the same cause as shifts in preference from birds to mammals recorded in species of Culex in North America: C. nigripalpus (Edmund & Taylor, 1968) and C. tarsalis (Hayes et al. 1973). Reeves (1971) suggested in the case of C. tarsalis that the avian host showed an intolerance to attack by mosquitoes at high density.

C. theileri. From the experimental results reported in Chapter 3, it is evident that C. theileri is almost as easily infected as C. univittatus. This raises an interesting point with regard to the infection rates found in wild populations of these two species on the Highveld, because C. univittatus has frequently been found infected but C. theileri only rarely (McIntosh et al. 1967). The answer probably lies in differences in the feeding habits of the two species. C. theileri is only moderately ornithophilic whereas C. univittatus is highly so, but it is possible that there are other important reasons. These are differences between the two mosquito species in their host preferences among various avian species and the habit of C. theileri of feeding largely at ground level would deny to it certain avian hosts which are fed upon by C. univittatus in the canopy of trees. This apparent frequent failure to take up virus from wild birds coupled with a rather poor transmission ability, would be expected to limit severely the ability of the mosquito to transfer infection from birds to man. It seems, therefore, that C. theileri could play only a negligible role as a transmitter of the viruses to man.

C. pipiens. Its ecological attributes, very similar to those of C. univittatus, should make C. pipiens ideally suited as a maintenance vector in the feral virus cycles, but its laboratory vector capability alters the picture. With SIN virus, the experimental results eliminate it from consideration as a vector, since the levels of viraemia shown to occur in various Highveld avian species (McIntosh et al. 1969) never reached as high as the 10% infection threshold of 6.4 logs determined for this mosquito. The only way in which a few mosquitoes belonging to natural populations of C. pipiens might become infected with SIN virus is by their feeding on viraemic nestling chicks of wild birds which probably can have a viraemia with a titre in excess of 7.0 logs as with experimentally inoculated day-old domestic chicks. On the other hand, with WN virus the vector potential of this mosquito seems slightly superior to that of C. theileri. It may be important here that the C. pipiens individuals used in the transmission test were infected at 5.2 logs of virus, whereas the C. theileri individuals used were infected at only 4.4 - 4.5 logs. The transmission rate for C. theileri may have equalled that for C. pipiens if the infecting dose had been the same. However, the infectivity of feral populations of C. pipiens shown in field studies on the Highveld (McIntosh et al. 1967) seem incompatible with both the mosquito's prevalence and feeding habits and its vector capability, because, after testing 106524 C. pipiens over four summers, no isolations of WN virus and only three of SIN virus were made. Why C. pipiens does not become infected in the feral cycle of WN virus is not clear. As suggested for C. theileri, the answer may lie, at least partially, in preferential feeding by the mosquito on different species of birds resulting in the selection of birds which are relatively resistant to the virus. However, no field observations have been made in support of this.

C. fatigans. As extensive mosquito collecting in South Africa has shown that C. fatigans is usually rare in rural environments its participation in feral cycles of the viruses would be limited accordingly. However, it is prevalent in urban and peri-urban environments both on the Highveld and in South Africa generally, so that such a distribution together with its feeding habits would make it a likely candidate for link vectorship. However, the mosquito does not possess a high vector capability. Its 10% infection threshold is

about 5.0 logs with SIN virus and as most avian species on the Highveld rarely have a viraemia at a higher level than this (McIntosh et al. 1969), the likelihood of large numbers of this mosquito becoming infected seems remote. Its vector potential would, furthermore, be impeded by its apparently low ability to transmit this virus. C. fatigans is highly susceptible to infection with WN virus, so that wild populations of the mosquito would become infected if they fed on viraemic birds. Feral populations of C. fatigans on the Highveld have not yet, however, been tested for infectivity. The transmission rate with WN virus is rather poor (an overall rate of 16%) but this, coupled with its high susceptibility, would permit the species to play a secondary role in carrying infection between birds in the wild transmission cycle as well as in transferring virus from wild birds to man. In view of the domestic distribution of this mosquito it would seem that the latter might be of some importance.

A. unidentatus and A. dentatus. These two species possess feeding habits which on the whole are suited to a link vector. However, their low susceptibility to WN virus in the vector capability experiments exclude A. dentatus and probably A. unidentatus from playing important roles as vectors of this virus. In the case of SIN virus, the low log ED_{10} value obtained for A. unidentatus indicates that this species might play a minor vectorial role, but to substantiate this the transmitting ability of more than three infected mosquitoes requires determination. The rather high value for log ED_{10} determined for A. dentatus and SIN virus, together with the failure of three infected individuals of this species to transmit, is probably sufficient evidence to exclude it as a vector of any significance.

A. unidentatus with SIN virus, therefore, appears to be the only instance where some vector potential seems possible with either of these species. However, the intermittent nature of the appearance of Aedine mosquitoes on the Highveld would not favour transmission. A. limnetopenni was previously collected from the Highveld (McIntosh et al. 1967) and found not to be infected with either virus. In retrospect, these mosquitoes were almost certainly all A. unidentatus (see Chapter 2 - Methods) and although the number of isolations of SIN virus per 1000 C. univittatus was low in the same study, the absence of infection in 4650 A. unidentatus tends to suggest that vectorship

with SIN virus by this species would be negligible.

A. juppi. Laboratory vector studies with A. juppi have so far been precluded because of the intermittent appearance of this species, which makes mosquitoes difficult to secure for experiments. In view of its feeding habits, however, it may have some potential as a link vector.

Other species. The species which occur less frequently on the Highveld generally, but which were collected sometimes in considerable numbers, were not subjected to experiments to determine their vector capability. Hence an evaluation of their potential as vectors is based purely on ecological characteristics. As the five Anopheles species collected do not appear to be ornithophilic, they would not participate in the feral virus transmission cycles. Aedes mixtus is apparently rather rare, but the indications are that it might bite wild birds which means that it might act as a minor vector in virus transmission. The four other species collected as adults in the field, Cog. microannulata, Cog. fuscopennata, C. terzii and C. annulioris were all shown to be ornithophilic and must be regarded as potential minor vectors in the feral cycles. Cog. microannulata can occur in large numbers in areas where suitable water with vegetation is available for its larvae, such as Lake Chrissie with its numerous pans. Furthermore, one isolation of WN virus has been made from field collections of this species in Natal (ARU unpublished) so that it is possible that it might have more vectorial importance than the other three species in those areas where its characteristic larval habitat occurs.

The sum of the evidence indicates that C. univittatus acts not only as a maintenance vector in the feral cycle of each virus and in cycles between domestic fowls, but is also the most important vector for transmitting both viruses to man. Because it transmits WN virus more readily than SIN virus man is more likely to be infected with WN virus. The poor arthropophilism of C. univittatus is probably an important reason for the low level of infection which occurs in man on the Highveld. From a study of the other more prevalent mosquito species it seems that none can act as a link vector of any

great significance, but C. theileri, C. fatigans and possibly A. univentatus could be responsible for occasional human infections with one or both viruses. C. theileri can transmit both viruses, WN virus more efficiently, but is not likely to be able to cause such human infection because feral populations of this mosquito usually fail to take up virus from avian hosts. C. fatigans is able to transmit WN virus as efficiently as C. theileri in the laboratory and could transfer this virus from wild birds to man, particularly as it is more highly ornithophilic. It would seem worthwhile to test samples from wild populations of this mosquito for natural infectivity, particularly if they occurred at a site where human cases of WN infection arose. The only other species which might act as a link vector is A. unidentatus which, according to the incomplete evidence available, might be able to cause infections with SIN virus.

MOSQUITOES AND OVERWINTERING OF VIRUS

Five alternative hypotheses have been advanced to explain the endemic maintenance of viruses through the unfavourable seasons, either winter in temperate climates or prolonged dry or rainy seasons in sub-tropical and tropical regions, when continuous virus transmission is disrupted. Reeves (1961) has reviewed these hypotheses which are based on virus persistence during this period through one or more of the following: (1) a long-lived infected primary arthropod vector, (2) a long-lived primary vector capable of transovarian transmission of virus to its progeny, (3) alternative arthropod or metazoal vectors, (4) chronic latent infection in vertebrate hosts, or (5) annual re-introduction by infected migratory or dispersing hosts or vectors.

It was concluded from the observations described in Chapter 4 that the mosquito vector is not likely to be the means by which WN and SIN viruses overwinter on the Highveld, either as a long-lived infected vector or as a vector which can continue to participate in retarded transmission cycles in the winter months. It is also thought that transovarian transmission of virus by C. univittatus is unlikely as there is no conclusive evidence that any of the mosquito-borne viruses develop transovarially in Culex mosquitoes, in fact until recently this could be said for mosquitoes generally (Burgdorfer

et al. 1967). However, the recent laboratory experiments and field observations of Watts et al. (1973) strongly suggest that La Crosse virus is transmitted transovarially by Aedes triseriatus in the northern United States, constituting the overwintering mechanism for this virus and in view of this perhaps a laboratory study should be undertaken before transovarian transmission is finally discounted in C. univittatus. Mosquito-borne viruses seem to be limited specifically to mosquitoes so that it is not considered likely that they would be transmitted by other arthropods or by Metazoa. In keeping with the fifth hypothesis, it could be suggested that WN and SIN viruses might be re-introduced annually to the Highveld by migratory birds. This does not seem probable because on the Highveld most migratory birds from other parts of Africa arrive between the end of September and the end of October when the population density of C. univittatus is still low and, moreover, when wild populations of the maintenance vector are not usually yet infected. In a three year field study at Olifantsvlei (McIntosh et al. 1967) infections were not recorded until December, except in one November (1962) when a single isolation of each virus was made. This leaves the fourth hypothesis, i.e., chronic latent infection in vertebrate hosts, which I favour as the most likely way in which WN and SIN viruses survive the Highveld winter. The hosts would be various wild avian species harbouring a latent infection which would lead to the re-appearance of virus in their bloodstream at a time when the C. univittatus populations had reached a density sufficient for mosquito transmission to be resumed. Reeves (1958) obtained some evidence in support of this possibility when he recovered Western equine encephalomyelitis virus from the tissues of eight species of wild bird, one to ten months after they had been inoculated with the virus.

CHOICE OF METHODS FOR LABORATORY TRANSMISSION EXPERIMENTS

There were three aspects of the laboratory studies on vector capability dealt with in Chapter 3 which are pertinent to the execution of experiments designed to investigate vector potential in mosquitoes with arboviruses generally. These were the use of blood-

virus mixtures versus viraemic animals for the infective feed, infected salivary glands as an indicator of a mosquito's ability to transmit virus and the relationship between the concentration of virus in the infective feed and the transmission rate.

In accord with experiments along similar lines undertaken by other workers, it was found in the experiment performed that the salivary gland method was not suitable as a substitute for the conventional method for demonstrating transmission by a mosquito. It was concluded that feeding on blood-virus mixtures should not be used, at any rate in the first instance, as the infecting method for testing the susceptibility of a mosquito, as this might give misleading results. Lastly, the concentration of virus in the infective feed would appear to be most significant in experiments designed to assess the vector potential of natural mosquito populations, as a very large dose of virus, larger than those found in the blood of the mosquito's natural host, could give unrealistically high values for the transmission rate. This could also be critical in experiments comparing the transmission rates of different species of mosquito.

ADDENDUM

Epidemics of either of the two viruses have not been recorded in South Africa before 1974. As mentioned in Chapter 1, sporadic summer infections usually occur, mainly in the Highveld region, and are more common with WN virus. However, this year (1974) an extensive epidemic caused by WN virus occurred, not on the Highveld but in the Karoo region of the Cape Province (fig. 1). Dr. B.M. McIntosh of the Arbovirus Unit carried out field investigations at Upington during the first half of April near the end of the epidemic. It is interesting to compare his observations with the observations and experimental results given in this thesis since the findings made in the epidemic tend to bear out its main conclusions.

Mosquito densities were much higher than usual this year in the Karoo because the unprecedented summer rains in this normally arid area created an abundance of aquatic sites for mosquito breeding. It is estimated that the epidemic occurred over a vast area stretching

from Laingsberg (situated 177 km. east of Cape Town) to Upington, a distance of 515 km. (fig. 1). According to clinical data about 20000 human infections occurred in the Upington area alone and a study of blood specimens taken from a sample of the population showed that WN virus was responsible (isolations of the virus were made and also serological conversions to WN antibody were demonstrated). The only species of mosquito prevalent at the time were C. univittatus and C. theileri, with C. pipiens in smaller numbers. No virus isolations were made from C. pipiens but the infection rates per 1000 mosquitoes were 39 for C. univittatus and 0.8 for C. theileri. Mosquitoes biting man were mainly C. theileri and to a small extent C. univittatus, but it is thought possible that from March to the beginning of April C. univittatus was probably much more abundant and that a significant number may well have deviated from birds to feed on other hosts, including man, because the normal avian hosts would have become intolerant to the unusually high attack rate (see also page 112). It was concluded by both Dr. McIntosh and myself that the field data, in conjunction with the results of the laboratory vector studies, indicated that the majority of human infections were transmitted by C. univittatus. The basis for this conclusion was the high infection rate in the wild populations of this mosquito, the high laboratory transmission rate (Table 12 page 53) and the high population density of the species. It was concluded that C. theileri could not have been responsible for more than a few human cases because of its low infection rate in feral populations and rather poor laboratory transmission rate (Table 13 page 55). Furthermore, it was considered that C. univittatus could have passed infection to man, not only by transferring virus from wild birds to man but also, to a more limited extent, from man to man. This view is based on the level of viraemia which can be reached in the human bloodstream, 1.8 logs of virus (Goldblum et al. 1957), taken in conjunction with the results of the laboratory studies. At least 40% of C. univittatus which feed on blood with such a titre could become infected (Table 12 page 53) and about 25% would transmit, allowing for a decrease in transmissibility at the lower infecting dose in proportion to that shown in the relevant laboratory experiment (Table 25 page 81). This means that 10% of infected mosquitoes would be able to accomplish man to man transmission, a conservative

estimate. No other species of mosquito, other than the three mentioned, were collected at Upington except in very small numbers, so that aedine species were probably not implicated in the epidemic, although it is possible that they were more prevalent earlier on and may have played a part then.

It does seem, therefore, that this unusual event in the Karoo bears out the main conclusions which have been drawn in this thesis from the studies described in Chapter 2 and Chapter 3, Sections B and D, on the identity of the mosquito species responsible for transmitting disease to man. These conclusions, presented earlier in this chapter, were reached before the epidemic occurred.

SUMMARY OF FINDINGS.

1. From a study of the feeding habits of mosquitoes in the field and of vector capability in the laboratory, together with a previous knowledge of infectivity levels in feral Highveld populations, it is concluded that Culex univittatus is maintaining both West Nile (WN) and Sindbis (SIN) viruses among wild birds on the Highveld.

2. This species, it is concluded, is also the main vector transmitting both viruses to man. As it is a more efficient vector of WN virus, human disease caused by this virus is more likely to occur. Furthermore, results from the vector experiments show that it is theoretically possible for it to transmit WN virus from man to man because a low concentration of this virus will infect a proportion of C. univittatus and some of these will successfully transmit virus. The low level of anthropophilism of C. univittatus is probably an important reason for the usual low incidence of human infection with either virus on the Highveld.

3. It appears that none of the other common Highveld mosquito species can act as vectors of any significance but Culex theileri and Culex fatigans could act in a minor capacity by infecting man with WN virus acquired from birds. C. theileri and Aedes unidentatus might possibly fulfill the same rôle with SIN virus.

4. Field studies on the winter biology of mosquitoes indicate that low-level populations of both immature and adult Culex pipiens, C. theileri and C. univittatus are present during the winter. They have periods of activity during warm periods and periods of quiescence when temperatures fall. Diapause does not seem to occur. The same is thought to apply to C. fatigans, although evidence was only obtained for the occurrence of the immature stages.

5. From the field observations it is concluded that C. univittatus is unlikely to serve as the overwintering mechanism for WN and SIN viruses on the Highveld, either as an infected quiescent overwintering adult or by transmission of virus during warm spells during the winter.

6. Laboratory transmission experiments using different techniques showed the following:-

(a) A blood-virus mixture used as an infecting meal can sometimes

give a misleading assessment of vector susceptibility.

(b) The demonstration of virus in the salivary glands of adult mosquitoes cannot be taken as an indication that a mosquito will transmit virus.

(c) A decrease in the concentration of virus in the infective feed can cause a significant lowering of the subsequent transmission rate.

7. Laboratory colonies of C. fatigans, C. univittatus, C. theileri and C. pipiens were established for the vector capability experiments, the last two species not having been colonized before from Highveld mosquitoes. The use of the ovarian tracheation technique for parity determination was assessed on these four species and found applicable.

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