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THE EFFECT OF TEMPERATURE ON THE VECTOR COMPETENCE OF *CULEX UNIVITTATUS* THEOBALD (DIPTERA:CULICIDAE) FOR WEST NILE AND SINDBIS VIRUSES

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A Dissertation Submitted to the faculty of Medicine
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ABSTRACT

The mosquito Culex univittatus is the primary vector of West Nile (WN) and Sindbis (SIN) viruses on the inland plateau in South Africa. The influence of different temperatures on the vector competence of this mosquito for both viruses and on the longevity of uninfected mosquitoes was investigated.The replication and distribution of each virus within the mosquito was studied by the fluorescent antibody test and additionally,the salivary glands from infected and uninfected insects were compared histologically.

Orally infected mosquitoes were held at 14°C,18°C,26°C and 30°C and also at fluctuating outside summer ambient temperature for the vector competence studies. At regular intervals,a sample of the insects were fed individually by a capillary method to demonstrate virus transmission. The capillary fluid with salivary secretions was titrated in Vero cells as was an extract of the whole mosquito. This enabled three graphs to be constructed showing variations in the titre of the mosquito,the titre of virus transmitted and the transmission rate against time. Infection rates and titres of virus in individual mosquitoes for both these viruses were unaffected by temperature. However,both WN and SIN viruses multiplied slower at lower temperatures,and both the extrinsic incubation period and the period before maximum transmission rates were attained,increased as incubation temperature decreased. Incubation temperatures as high as 30°C,however,appeared to adversely affect the vector capability of Cx.univitt for SIN virus by reducing the transmission rate. T vector competence of WN-infected Cx.univittatus at outside summer temperatures was similar to that obtained at constant 26°C. This was not the case with SIN virus,as the

result at outside temperatures differed greatly from that obtained at any of the constant temperatures. It showed that Cx.univittatus,under natural temperature conditions,is not such an efficient vector of SIN virus as originally proposed from past studies conducted at constant temperatures.

Both viruses reduced the average mean life expectancy of orally infected Cx.univittatus when held at 26°C,but SIN virus appeared to be pathogenic by disrupting salivary gland cells,which considerably reduced the life span of the mosquito at 14°C.

Fluorescent antibody studies confirmed that WN and SIN viruses,first multiplied in the posterior midgut region of the mosquito,and thereafter spread to other organs. SIN virus also spread to other organs such as the ventral diverticulum and abdominal ganglia.

According to the longevity and vector competence studies,transmission of WN and SIN viruses by Cx.univittatus was most favoured at temperatures of 30°C and 26°C respectively,and epidemics of these viruses in South Africa would most likely be expected when mean summer temperatures approximate these respective temperatures.

Declaration

I declare that this dissertation is my own,unaided work.It is being submitted for the degree of Master of Science in the University of the Witwatersrand,Johannesburg.It has not been submitted before for any degree or examination in any other University.

A.S. CORNER

2 day of November, 19 87.

PREFACE

This study was undertaken as an MSc under the supervision of Dr.P.G.Jupp and Dr.R.Swanepoel,and I would like to express my gratitude for their guidance and support.

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ABBREVIATIONS USED IN THE TEXT.

Ae.....Aedes
CE.....California encephalitis
CHIK....Chikungunya
CPD_{50}...50% cytopathic dose
Cx.....Culex
DEN.....Dengue
E.....Eosin
EEE.....Eastern equine encephalitis
EI.....Extrinsic incubation
FA.....Fluorescent antibody
H.....Haematoxylin
HAF.....Hyperimmune ascitic fluid
HI.....Haemagglutination inhibition
IR.....Infection rate
JBE.....Japanese B encephalitis
PBS.....Phosphate buffered saline
PI.....Post infection
RH.....Relative humidity
RVF.....Rift Valley fever
SF.....Semliki Forest
SIN.....Sindbis
SLE.....St.Louis encephalitis
TAH.....Tahyna
TOT.....Transovarial transmission
TR.....Transmission rate
WEE.....Western equine encephalitis
WHA.....Whataroa
WN.....West Nile
WR.....Resistant to infection with Western equine encephalitis
WS.....Susceptible to infection with Western equine encephalitis
YF.....Yellow fever

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CHAPTER 1.

INTRODUCTION.

An aspect of prime importance in the epidemiology of arthropod-borne viruses(arboviruses) is an understanding of the ecological conditions and circumstances which favour the occurrence of human epidemics due to a particular virus. Several field studies have enhanced our understanding as to the reasons for the development of epidemics of certain arboviruses. However,the evidence provided by some field observations tends to be too circumstantial so that additional evidence is needed from parallel laboratory studies. One such environmental factor recorded in the field which needs assessment in the laboratory is environmental temperature and the role it has on influencing the competence of several arthropod vectors to transmit associated viruses. Field observations conducted on West Nile(WN) and Sindbis(SIN) viruses in South Africa (McIntosh et al,1976;Jupp et al,1986) have suggested that ambient temperature might be important in favouring epidemics of these two viruses. The present study further investigates the effect of environmental temperature on the longevity and vector competence of the vector Culex univittatus Theobald for both WN and SIN viruses respectively by laboratory experimentation. These experimental observations are then correlated with field observations to strengthen our understanding of the importance of ambient temperature in the epidemiology of WN and SIN viruses.

Temperature is not the only environmental factor influencing transmission of mosquito-borne viruses and the other factors are also discussed in this chapter. A detailed review of the epidemiology of WN and SIN

viruses in South Africa is also given as this is necessary for an interpretation of the results obtained from the temperature vector competence studies.

1.1.Factors influencing transmission of arboviruses.

Several factors influence the occurrence of arboviruses and Hardy et al(1975) divided these factors into two groups,namely,extrinsic and intrinsic factors. Extrinsic factors influence the exposure of mosquitoes to viraemic and clinically susceptible hosts(Hardy et al,1979) and include such concepts as distribution of vertebrate hosts and arthropod vectors and feeding preferences of the arthropod vectors. Intrinsic factors,on the other hand,are those that determine if,once virus has been ingested by an arthropod,it can initiate an infection,reach the salivary glands and be transmitted (Hardy et al,1979). It is beyond the scope of this thesis to discuss all these extrinsic and intrinsic factors in detail and therefore only a brief review is given. Temperature as an intrinsic factor is however discussed in more detail for several viruses.

1.1.1.Extrinsic factors.

A study conducted on western equine encephalitis(WEE) virus in Kern County,California,revealed those extrinsic factors which favoured epidemics of the virus there,both in 1952 and 1958 (Reeves et al,1964). These factors were:

1) An unusually large population of the mosquito Cx.tarsalis,the major vector of WEE virus,brought about by unusually heavy rains,
2) an abundance of susceptible avian hosts and

3) the consistent occurrence of high densities of Cx.tarsalis during the summer in Kern County.

These extrinsic factors have also been associated with epidemics of other mosquito-borne viruses and are discussed below.

A.Rainfall.

Epidemics of several arboviruses have been correlated with high rainfall which brought about much larger populations of mosquito vectors. Examples of this correlation between rainfall and arbovirus epidemics include those of Rift Valley fever(RVF) virus in Kenya (Davies et al,1985) and in South Africa (McIntosh and Jupp,1981);WN virus in South Africa (McIntosh et al,1976);SIN virus in South Africa (McIntosh et al,1976;Jupp et al,1986) and dengue(DEN) virus in Malaysia (Foo et al,1985). Because of the breeding habits of mosquitoes this correlation between large numbers of mosquitoes and epidemics of arboviruses is not difficult to understand,and more such correlations between high rainfall and epidemics of arboviruses will most likely be found.

B.Susceptible hosts.

A sufficiency of susceptible vertebrate hosts is also critical in transmission of arboviruses and is always included as a variable in models that predict epidemics of arboviruses (Scott et al,1983;and De Moor et al,1970),where the larger the number of susceptible hosts the larger the epidemic predicted.

C.Temperature.

Coupled with the global distribution of arboviruses is the distribution of the natural arthropod vectors. Many experiments have proved that arthropods (including mosquitoes) have temperature tolerancies

and therefore are only found in certain types of climatic conditions.

For example,some tropical mosquitoes belonging to the genus Haemagogus(vectors of yellow fever (YF) virus) live optimally at temperature ranges higher than most other species of mosquito,with greater longevity at 30°C (Bates and Roca-Garcia,1945). Mosquitoes living in temperate regions have lower optimal temperature ranges and Aedes triseriatus,a vector of eastern equine encephalitis(EEE) virus,lives longer at 21°C(32-42 days) than at higher temperatures of 32°C(16 days) (Chamberlain and Sudia,1955).Some mosquitoes such as Cx.quinquefasciatus,collected in even colder climatic regions,are able to live up to 110 days at 10°C but only 45 days at 26°C(LaMotte, 1963). Increased breeding and longevity of mosquito vectors in their natural environments would therefore help to maintain a consistent population of mosquitoes during summer periods and thus favour transmission of arboviruses and increase the chances of epidemics. The situation as regards temperature is,however, complicated as temperatures are not constant but differ between seasons and from year to year. Some years are more favourable for flight range,growth, reproduction and certain other functions that affect the survival of mosquitoes. Furthermore,levels of metabolism of mosquitoes are largely controlled by ambient temperatures so that mosquito activities fundamental to virus transmision,such as blood feeding,would also be affected and vary between seasons.

D.Seasonal source of virus.

Seasonal climatic changes cause fluctuations in arthropod numbers,and when their numbers drop to low levels in response to adverse conditions(ie. winter months in temperate regions and dry seasons in the tropics),transmission cycles between man,arthropod

vectors and vertebrate hosts become interrupted. Associated with this interruption in transmission,other pathways of virus maintenance,and in particular overwintering,have had to be investigated to explain how viruses remain endemic with continued seasonal appearance (Tesh,1984). Studies have shown that such overwintering pathways for arboviruses do exist. These include maintenance of virus in vertebrate hosts that develop chronic latent infections,long-living and persistently infected primary arthropod vectors and through infected arthropods capable of vertical transmission (transovarian transmission-TOT) of virus to progeny(Reeves,1974). Definitive examples of TOT in mosquitoes are relatively few and those that have been firmly established have originated from both field and laboratory investigations. These field studies have involved recoveries of arboviruses from naturally infected immature mosquitoes such as larvae(Andrews et al,1977) and eggs(Chamberlain et al,1964). Laboratory studies successfully demonstrating TOT of several mosquito-borne viruses (Watts et al,1973;Berry et al,1977;Tesh,1984) are increasing as an understanding of the mechanisms of TOT and laboratory methods improve so that the importance of TOT as an overwintering mechanism is rapidly being realized.

Field studies in North America and Europe have confirmed that some species of mosquitoes are capable of hibernating through the winter as adults in a state of diapause(Bennington et al,1958;Schaefer and Washino,1969; Buffington,1972;Sulaiman and Service,1983). Laboratory studies have also demonstrated hibernation of mosquitoes,and the environmental conditions which induce the physiological changes to trigger diapause are reasonably well understood for some species of mosquitoes(Tekle,1960;Bellamy and Reeves,1963; Spielman et al,1973;Washino,1977;Mitchell,1981;Bailey

et al,1982;Wilton and Smith,1985). In South Africa where winter temperatures are not as cold as those in North America and Europe,adult mosquitoes have been collected during the winter season in a quiescent state(Jupp,1969). As opposed to diapause,which is true hibernation viz. a genetically determined state of suspended activity and suppressed development (Wilton and Smith,1985), quiescence is a more temporary suspension of activity in direct response to an unfavourable influence,the removal of which will promptly permit the mosquito to return to normal activity(Bellamy and Reeves,1963). Since adult mosquitoes are capable of surviving cold winter months,the question arises as to whether infected overwintering mosquitoes could serve as another overwintering reservoir of mosquito-borne viruses. Few isolations of arboviruses have been made from field collected diapausing or quiescent mosquitoes(Bailey et al,1978). However,several laboratories have subjected infected adult mosquitoes to simulated winter conditions to demonstrate that diapausing and quiescent mosquitoes can serve as overwintering reservoirs(Hurlbrut,1950;AspÖck and Kunz,1970;Hayashi et al,1976),and transmit virus to vertebrate hosts in the spring. Finally,the endemic persistence of mosquito-borne viruses has also been explained by the repetitive arrival at the beginning of each summer season of infected migratory hosts,such as birds. This hypothesis has been supported by the isolation of several mosquito-borne viruses from migratory birds world wide (Calisher,1971;Taylor et al,1956;Scherer et al,1959; Work,1958).

1.1.2. Intrinsic factors.

Mosquitoes ingest virus particles via infected blood meals,and the sequential infection and multiplication of the virus in the organs of the mosquito determine whether it becomes a competent vector or not(Hardy et

al,1979). Such sequential multiplication of virus is reviewed,followed by the effects of various intrinsic factors upon it.

A.Virus replication.

The sequence of viral replication within competent mosquito vectors was summarized by Hardy et al(1983) as follows:

a)The virus must initiate an infection in the mesenteron of the mosquito,

b)multiply productively in the mesenteronal epithelial cells,

c) escape from the mesenteron and finally

d)infect and multiply in the salivary glands and ovaries whence it can be transmitted orally to vertebrate hosts and perhaps transovarially to progeny.

Furthermore,Hardy et al(1983) summarised and divided the replication of arboviruses within mosquitoes into three major amplification stages. These are stages when the virus actually replicates within mosquito cells resulting in an overall increase in titre. These amplification phases are as follows:

1)Infection first occurs in the mesenteron,wherafter it spreads or disseminates to other tissues via the haemocoel.

2)Infection in cells and tissues associated with the haemocoel.

3)Infection of the salivary glands.

There is much evidence supporting the above summary for the sequence of infection in the mosquito and some of the more convincing studies are discussed below.

Pionering work on the sequence of viral replication was done by LaMotte(1960) with Japanese B encephalitis(JBE) virus in Cx.pipiens. La Motte's results have since been confirmed and extended by immunofluorescence studies on JBE virus in other

species of mosquitoes(Doi,1970),DEN-2 virus in Ae.albopictus(Kuberski,1979),electron microscopy of Venezuelen equine encephalitis virus in Ae.aegypti (Larsen et al,1971),California encephalitis in Ae.aegypti and Culiseta inornata(Mclean et al,1975) and St.Louis encephalitis (SLE) in Cx.pipiens pipiens(L)(Whitfield et al,1973).

To be more specific,after virus particles have entered the mosquito via an infective blood meal,the virus moves to the midgut where virus penetration of host parenchyma and active infection of epithelial cells occurs (Chamberlain and Sudia,1961;Kuberski,1979). The virus then gains access to other cells of the mosquito through the haemolymph(LaMotte,1960;Danielova,1962), such cells being the fat body cells,nerve ganglia,ovarial cells,malpighian-tubules and the salivary gland cells. Salivary glands produce saliva which is secreted as the mosquito imbibes blood and,if the salivary gland is infected,virus is secreted into the host. Therefore,transmission of virus occurs when the salivary glands are persistently infected with virus(Thomas,1963;Chamberlain,1968; Miles et al,1973;Kramer et al,1981). This transmission of virus via infected salivary fluid is termed biological transmission and should be clearly distinguished from mechanical transmission. Arthropods mechanically transmitting virus only act as carriers and transmit virus from infected to uninfected hosts via contaminated mouthparts(Chamberlain and Sudia,1961). For biological transmission,however,a period must elapse to allow imbibed virus (ingested in an infected blood meal) to reach the salivary glands of the mosquito before virus can be biologically transmitted. This time period is known as the extrinsic incubation (EI) period(Hardy et al,1983). The EI period may therefore be defined as the interval between ingestion of virus and subsequent ability to transmit virus by the mosquito(Turrell et al,1985). There

are,however,some exceptions to this where transmission of virus did not correlate precisely with the presence of virus in the salivary glands. In a study done on Cx.theileri infected with SIN virus (Jupp,1985),it was found that even though its salivary glands were infected,the mosquito did not always transmit virus. Therefore one should be carefull not to assume that transmission rates will equal salivary gland infection rates. Considerable viral replication occurs in the salivary glands and it is of interest to mention the areas in the salivary gland where viral replication occurs. Each salivary gland of a female Ae.aegypti is made up of three lobes,two lateral and a smaller median or middle lobe. The lateral lobes are further divided into proximal,intermediate and distal portions,and the middle lobe into intermediate and distal parts. Salivary glands of Cx.univittatus appear to have a similar structure and are shown in Figure 1.

Fig.1.External structure of a salivary gland from Cx.univittatus.MAG.100X.



A study on the replication of CHIK virus in salivary glands of Ae.aegypti (Janzen et al,1970) showed that the site of greatest potential for virus replication was in the proximal portion of the lateral lobes. Immunofluorescent studies (Kuberski,1979) also demonstrated that DEN-2 viral antigen appeared initially in the proximal portions in Ae.albopictus salivary glands. Perhaps of more significance from the transmission point of view, was that the appearance of fluorescence in the salivary glands was more granular than in any of the other parts of the mosquito. Kuberski suggested that this type of fluorescence confirms that salivary glands play an integral part in secreting infectious virons necessary for effective transmission of virus.

B.Genetics as a basis for controlling vector competences of mosquitoes.

Variation in susceptibility to oral infection of a mosquito-borne virus commonly occurs between populations of the same species of mosquito. For instance,geographic strains of Ae.albopictus vary in susceptibilty to infection with DEN virus (Gubler and Rosen,1976),strains of Ae.aegypti vary in their susceptibility to and transmissibility of Tahyna (TAH) virus (Labuda et al,1985) and infection and transmission rates for La Crosse (LAC) virus differ quite considereably between different strains of Ae.triseriatus(Grimstad et al,1977). Cross-breeding between strains with different vector competence has been done and some factors controlling the competence seem to be inheritable. Different strains of Ae.albopictus also vary in susceptibility and in the quantity of virus present in infected individuals after a standard incubation period with CHIK virus. Crossing strains with high and low CHIK virus thresholds resulted in offspring with infection rates and mean viral titers intermediate between those shown

by the parent colonies (Tesh et al,1976). Strains of Cx,tarsalis resistant (WR) and susceptible (WS) to oral infection with WEE virus have also been selected. Hardy et al(1978) crossed these strains and the F1 progeny were almost 100% susceptible indicating susceptability may have been dominant. However, backcrossing with WR and WS strains and F2 progeny gave inconclusive results,which led Hardy et al (1983) to suggest that inheritance of susceptibility to WEE virus was probably multifactorial.

More recent cross-mating experiments with strains of Cx.tritaeniorhynchus(Giles) also showed that the susceptibility to WN virus could be inherited(Hayes et al,1984). These experiments indicated that the trait of oral susceptibility is dominant over resistance to viral infection,and both male and female mosquitoes of the susceptible strains are carrying factors for increased susceptibility. Back crossing however showed that increased susceptibility is partially dominant,since the F1 progeny developed intermediate titres of WN virus when compared to titres of the parental strains.

Due to this variation in vector competence,and because factors affecting vector capability seem to be inheritable traits,genetics must play a vital role in controlling the vector competence for arboviruses. Still further evidence for this comes from vector competence studies of colonized mosquitoes of different filial generations. It has been reported that mosquitoes,like many other arthropods,once having been removed from the field,undergo drastic genetic selection with rapid alteration of the gene pool due to colonization in response to the conditions in the laboratory. Several studies have demonstrated that vector capability of many mosquitoes gradually changes with increase in filial generation,eg,with RVF virus(Gargan et al,1983) and YF virus (Lorenz et al,1984). These changes have even been correlated with loci of specific isoenzymes (Lorenz et al,1984).

C.Barriers to replication and dissemination of arboviruses im mosquito bodies.

A further set of intrinsic factors affecting interrelationships between arboviruses and mosquitoes is the "barrier" effect. These barriers are also genetically determined physiological phenomena involving membranes,enzymes and cell surfaces. Due to their properties they may either prevent viral replication or prevent virus particles from leaving one area and entering another (dissemination) within the mosquito body. Since these barriers are genetically determined traits,their properties and the subsequent effects they have on viral replication are species specific. This helps explain why some species of mosquitoes are better vectors for some viruses than others. This whole concept of barriers has received much attention in the last few years and has been reviewed by Hardy et al(1983).

D.Temperature.

Mosquitoes are cold-blooded animals so that their level of metabolic activity is directly proportional to ambient temperature. As a consequence of this,it follows that a virus within a mosquito would also be affected and multiply best in mosquitoes within certain temperature ranges. Studies on the effect of temperature on vector potential of mosquitoes with various viruses do indeed support this hypothesis and have demonstrated that short EI periods and maximum transmission and infection rates are only attained at certain temperatures. This optimal temperature range is,however,not only favourable in terms of viral multiplication but also in terms of mosquito longevity and activities such as blood feeding. When all these factors combine in a certain manner,optimal conditions for transmission of viruses are created. For instance,lengthened EI periods expected at lower

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temperatures due to lower rates of viral multiplication (Danielova,1975;Hardy et al,1983),can be extremely important to the epidemiology of the arbovirus. This is because the period the mosquito must survive after an infective meal until transmission occurs will determine if the mosquito can still be a competent vector.

Studies have also indicated that incubation temperatures affect dissemination and infection barriers within the mosquito and very often gives rise to rather inconsistent results (Hardy et al,1983; Kramer et al,1983). Virus multiplication varies within different species of mosquito and the same virus often has different EI periods in different species of mosquito even if the mosquitoes are held at the same temperature (Chamberlain et al,1955;1959). It is therefore clear that each virus has its own unique relationsip with a mosquito vector,and the effects of temperature on vector competence should be studied for each virus and vector. Many such studies have been done and the discussion of these studies is divided into three main groups according to the endemic climatic distribution of the virus concerned.

D.1.

<u>Viruses occuring in the cooler regions of the world.</u>

a)Snowshoe hare virus (Bunyaviridae).

Snowshoe hare (SSH) virus,a subtype of CE virus has been isolated from several species of <u>Aedes</u> mosquitoes,particularly <u>Ae.canadensis</u>(Mclean et al,1971) in North America as far north as Alaska. These mosquitoes are active and feed on humans in the field at temperatures of 10°C and lower(McLean et al,1974). Higher temperatures of 13°C and 26°C did not change the EI period for SSH virus in orally infected <u>Ae.aegypti</u> mosquitoes and virus was transmitted after 28 days at both these temperatures (Mclean et

al,1974). However, Ae.aegypti is neither a natural vector of SSH virus nor an efficient laboratory vector. In spite of this,studies with Ae.aegypti do indicate that SSH virus multiplies and can be transmitted at low temperatures.

b) Whataroa virus (Togaviridae).

Whataroa virus(Alphavirus) is endemic to New Zealand which experiences diurnal average monthly summer temperatures of 16°C,with maximum temperatures of 21-22°C,and diurnal average monthly winter temperatures of 6°C. Main vectors of WHA virus are the mosquitoes Culex pervigilans and Culiseta tonnori (Miles et al,1973). Since the workers were not able to keep these species alive sufficiently long enough in the laboratory,the multiplication of WHA virus was studied in another mosquito,Aedes australis,which also survives best at cool temperatures with maximum survival at 20°C. Most Ae.australis became infected and transmitted virus 17 days after imbibing the infective blood meal at 20°C indicating that WHA virus is adapted to replicate in mosquitoes at relatively low EI temperatures.

D.2.

Viruses from warm regions.(tropical to sub-tropical).

Most studies on the effects of temperature on vector competence with viruses endemic to the tropics,have been conducted with the following flaviviruses:YF virus;DEN virus and JBE virus. The results obtained from a few studies on these viruses are summarized in Table 1.

These viruses are clearly strongly influenced by EI temperature,with infection and transmission rates rapidly declining at temperatures below 30°C. Reduction in rates of replication of these viruses within the mosquito due to lower temperatures not only

reduces infection and transmission rates,but also lengthens the EI period. At temperatures less than 15°C virus multiplication is so low that the mosquitoes no longer seem to be infected,particularly with YF and DEN viruses. Nevertheless,virus infection at these lower temperatures still persists as rapid virus replication occurs when mosquitoes held at low temperatures are transferred to higher temperatures (Davis,1932;Bates and Roca-Garcia,1945;1946; LaMotte,1963). JBE virus seems to have an unusually broad temperature tolerance,as it is transmitted by different mosquito species in different climatic regions(Huang,1957). It is probably due to this greater temperature tolerance of JBE virus that periodic epidemics of the virus have occurred during summer periods in temperate climates in Asia (Schichijo et al,1972;Hayashi et al,1976), although the virus is endemic to tropical Asia (Bhattacharya et al,1986).The main vector of JBE virus in tropical Asia is Cx.vishnui (Battacharya et al,1986),while in temperate areas it is Cx.tritaeniorhynchus (Hayashi et al,1976).

D.3.

Viruses from temperate areas.

a) western equine encephalitis virus (Togaviridae)

Strains of Cx.tarsalis resistant or susceptible to oral infection with WEE virus have been selected and female F1 progeny from crosses between these strains exhibit mesenteronal or salivary gland infection barriers (Kramer et al,1983). The F1 females were orally infected with WEE virus and transmission and infection rates determined at different temperatures. The mosquito was found to be a less competent vector after 2-3 weeks extrinsic incubation at 32°C than after incubation at 18°C or 25°C (Kramer et al,1983). These authors thus reported they had shown a case

Table 1.

Summary of results obtained from temperature vector competence studies on viruses occuring in the tropics.

VIRUS	SPECIES OF MOSQUITO	ROUTE OF INFECTION	INCUBATION TEMPERATURE °C	INFECTION RATE %	TRANSMISSION RATE %	EXTRINSIC INCUBATION PERIOD (DAYS)	REFERENCE
DEN-2	Ae.aegypti	Oral	32-35	65	N/D (+)	7	Watts et al,1987
"	"	"	32	N/D	N/D	6	Mclean et al, 1974
"	"	"	30	65	N/D	12	Watts et al,1987
"	"	"	27	N/D	N/D	13	Mclean et al,1974
"	"	"	13	N/D	N/D	32	Mclean et al,1975
YF	Ae.aegypti	Oral	36-37	N/D	N/D	4-5	Davis,1932
"	"	"	31	N/D	N/D	6	"
"	"	"	21	N/D	N/D	18	"
"	"	"	8-18	0	0	0	Davis,1932
YF	Haemagogus capricornii	Inoculated	30	68(10-19)	N/D	N/D	Bates + Roca-Garcia,1945
"	"	"	24-27	0-10 (10-22)	N/D	N/D	"
"	"	"	20 for 20 hrs and 35 for 4hrs	35-65 (2-12)	N/D	N/D	",1946
"	"	"	20 for 6 hrs 30 for 8 hrs	31(19-22)	N/D	10	"
"	"	"	25	83-86 (2-10)	59-83 (8-18)	N/D	"
"	"	"	20	0(22)	10(8-18)	8	",1945;1946
JBE	Culex tritaenio-rhynchus	Oral	26-30	N/D	85	N/D	Huang,1957
"	"	"	28	N/D	100(14-23)	9	Takahashi,1976
"	"	"	20	N/D	40-60(20-35)	20	"

where an inverse relationship existed between the vector competence of a mosquito for an arbovirus and the temperature of EI period within the range 18 to 32°C. They believed the phenomonen was related to the mosquitoe's ability to limit multiplication and/or dissemination of the virus which was in turn controlled by various dissemination,transmission and infection barriers within the mosquito body. The vector competence of wild strains of Cx.tarsalis orally infected with WEE virus responds similarly to incubation temperature although perhaps not as markedly as that of the F1 progeny from crosses between WR and WS strains of Cx.tarsalis (Kramer et al,1983). Maximum transmission rates of WEE virus by Cx.tarsalis is therefore only obtained at certain temperatures and this could account for the endemic temperate distribution of WEE virus. Even so it cannot be stated what these optimal temperatures are since there is also geographical genetic variation in the mosquito with respect to vector competence. For example,certain strains of Cx.tarsalis are adapted to cooler temperatures and therefore occur further north in the colder Saskatchewan region of North America which experiences diurnal average summer temperatures between 21-24°C (Hayles et al,1972). Vector competence studies on these mosquitoes have shown that they effectively transmit a local strain of WEE virus at both 21°C and 24°C,4 to 44 days after an infective blood meal.

b) St.Louis encephalitis virus (Flaviviridae)

Temperature vector competence studies have been conducted on Cx.quinquefasciatus(Say) mosquitoes infected with SLE virus (Hurlbrut,1973) and as with WEE virus,the maximum transmission of SLE virus was only obtained within a certain temperature range,with maximum transmission reached at 30°C. This study was perhaps unique in that Hurlbrut was able to

demonstrate that EI period and blood digestion time were approximately linearly related to temperature between 20-30°C. Furthermore,survival of this species of mosquito to completion of virus incubation was also shown to be optimal at a mean incubation temperature of 30°C.

If transmission of SLE virus by Cx.quinquefasciatus is compared to transmission of WEE virus by Cx.tarsalis it seems that Cx.quinquefasciatus is better adapted to transmit SLE at higher temperatures than Cx.tarsalis is able to transmit WEE virus. This could explain why environmental temperatures usually need to be slightly higher for epidemics of SLE virus than for WEE virus. Evidence has in fact accumulated suggesting activity of WEE and SLE viruses in Kern County,California, usually follow half month periods of mean daily temperatures exceeding 27°C and 31°C respectively (Reeves et al,1964;Kramer et al,1983). Furthermore,SLE virus activity follows unusually warm spring temperatures,while high WEE viral activity most frequently occurs after abnormally cool spring temperatures. Associated with this,some workers even go so far as to state that epidemics of WEE virus usually occur at or above the 25°C June isotherm,whereas those of SLE virus usually occur in areas below this isotherm (Hess et al,1963).

c) eastern equine encephalitis virus (Togaviridae)

The mosquito Ae.triseriatus was shown to transmit EEE virus mechanically for periods of up to 3 days after an infective feed at temperatures of 21°C (Chamberlain and Sudia,1955). Mechanical transmission was however completely eliminated at 32°C and Chamberlain and Sudia(1955) considered that the more rapid inactivation of virus and greater cleansing of mouthparts by the mosquito at higher temperatures accounted for this. A similar trend in reduction of mechanical transmission at higher temperatures was

also shown with JBE virus and Cx.tritaeniorhynchus mosquitoes (Hayashi et al,1976). In Ae.triseriatus, however,an increase in temperature from 21°C to 32°C had no effect on infection rates or titres of EEE virus reached in individual mosquitoes,although higher transmission rates and shorter EI periods were observed at the higher temperature. The subjection of mosquitoes to temperatures of 32°C for 4 hours and 21°C for 20 hours affected vector competence,causing intermediate transmission rates and EI periods as compared to the mosquitoes held at a constant 32°C and 21°C.

d) Rift Valley fever virus (Bunyaviridae)

Incubation temperatures were shown to influence the vector competence of Cx.pipiens and Ae.taeniorhynchus with RVF virus (Turrell et al,1985). The virus replicated more rapidly at higher temperatures in both species after oral ingestion of virus,so that there was an inverse relationsip between EI period and incubation temperature. Infection rates in the 2 species,however,differed in response to temperature, with rates in Cx.pipiens significantly higher at 33°C(95%) than at 13°C(38%),whereas they remained unchanged in Ae.taeniorhynchus. Of further significance is that the low infection rate obtained at 13°C(38%) in Cx.pipiens increased in mosquitoes that were transferred from 13°C to 26°C. It appears that at low temperatures the virus is not lost,but rather falls to undetectable levels or enters some sort of latent infectious phase. This has been shown to occur with several other arboviruses already mentioned in Section 1.1.2.D.2.

e) Tahyna virus (Bunyaviridae)

TAH virus is endemic to Central Europe where it is transmitted by Ae.vexans. In an effort to study the

effect of temperature on the virus,Malkova and Marhoul(1976) inoculated it into an Ae.albopictus cell culture line to observe viral multiplication at 28°C and 20°C. These temperatures correspond to the average temperatures experienced by the mosquito vector during it's summer activities in Central Europe. Titres were slightly lower at 20°C.Hence it seems that TAH virus is not markedly affected by temperature,but that it's distribution is restricted to temperate regions where the natural vector Ae.vexans occurs.

1.2.Epidemiology of WN and SIN viruses in South Africa.

Two arthropod-borne viruses,namely WN and Sindbis(SIN) viruses are enzootic in the highveld region of South Africa which experiences a temperate climate. These viruses have also been isolated in many other regions of the world including parts of Africa,Asia and Europe (Smithburn et al,1940;Woodall et al,1961;Weinbren, 1955;Taylor et al,1956;Goldblum et al,1958;Shah et al,1960;Pavri and Singh,1965; Filipe,1972;Hayes et al,1982;Mcintosh,1986) and SIN virus only from Australia (Doherty et al,1969). Both WN and SIN viruses are epidemiologically similar in that they are avian-borne viruses with the mosquito Cx univittatus as the main vector. Serological surveys done on birds in Egypt (Work et al,1953;1955;Taylor et al,1955), South Africa(Kokernot et al,1956;Mcintosh,1969) and Asia(Shah et al,1960) have shown that several species of birds play a major role in the epidemiology of these viruses. Many of these birds migrate between northern and southern Africa and Asia and could therefore serve as important seasonal sources of the viruses resulting in the widespread distribution of these viruses. Many isolations of these viruses, particularly WN virus have been made from Culex mosquitoes in Egypt(Work et al,1953),Pakistan(Akhter et al,1982),India(Pavri et al,1965),Arabia(Wills et

al,1985) and South Africa (Weinbren et al,1956).In South Africa most isolations of both WN and SIN viruses have been made from the mosquito Cx.univittatus Theobald (Mcintosh et al,1967;1978;Jupp and Mcintosh,1967;Jupp et al,1986) from the inland plateau region. Cx.univittatus is mainly ornithophilic,but will feed on man and probably more so when abundant after heavy rainfall.The mosquito plays a major role in maintaining the avian-borne cycles of the viruses due to its ornithophilic feeding preferences,but on ocassions bites and could infect humans,who then become secondarily involved. These viruses both cause a mild illness in man,an account of which is given by McIntosh(1986).

In the past,isolations of both WN and SIN viruses have also been made from 2 other species of mosquitoes which also commonly occur in the inland plateau region. These are Cx.pipiens L.and Cx.theileri Theobald (McIntosh et al,1978). Isolations from these two species of mosquitoes have been far fewer than from Cx. univittatus which may be because these two species of mosquitoes feed preferentially on birds that could be relatively resistant to infections with the viruses (Jupp et al,1972;Jupp,1976).

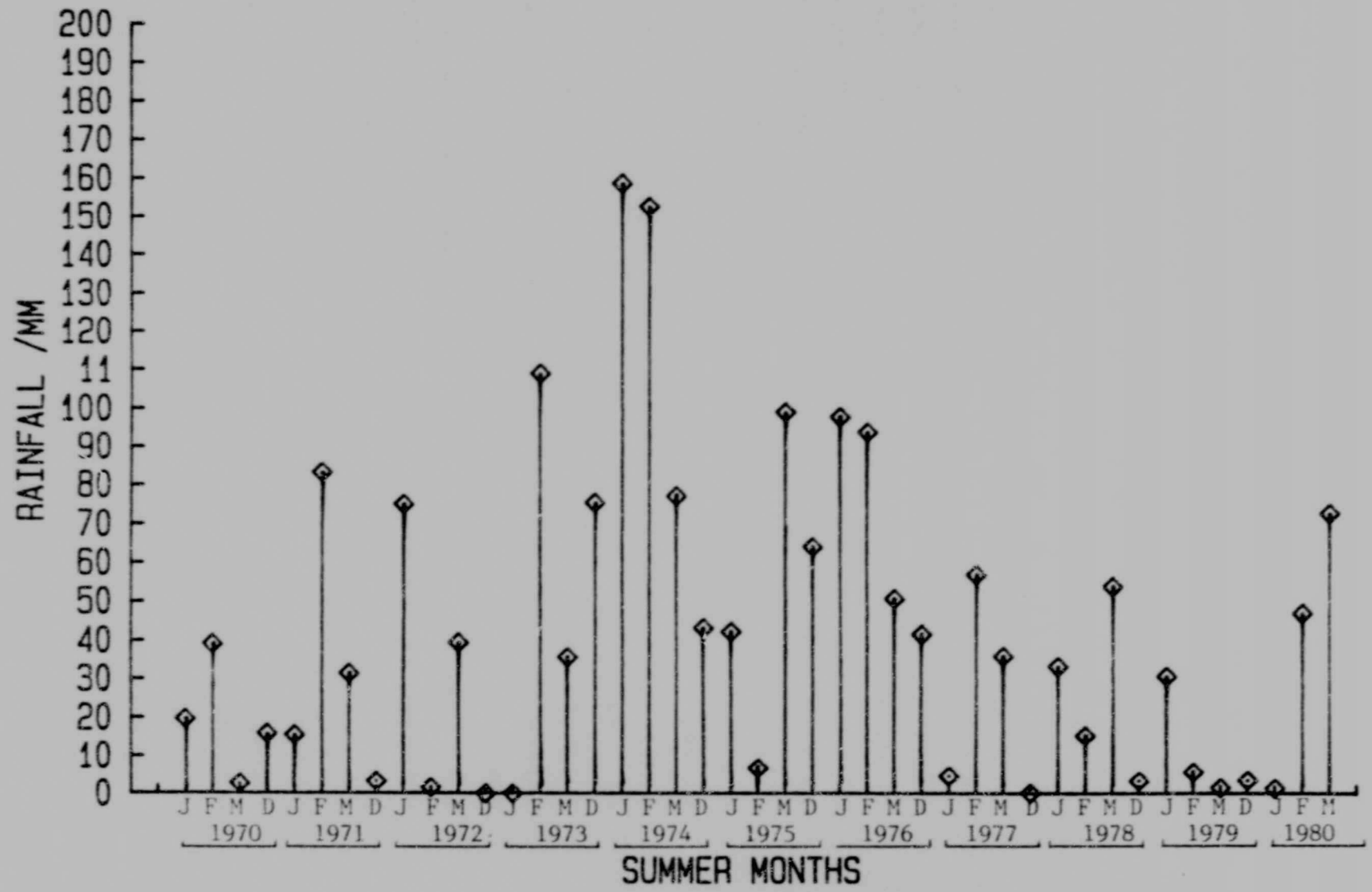
Although all 3 species are highly susceptible to both viruses when orally infected by feeding on viraemic chicks,only Cx.univittatus is capable of transmitting the virus efficiently (Jupp,1976). Serological surveys on the inland plateau and in the Natal coastal lowlands have shown that human immune rates to both WN and SIN viruses are much higher in the inland plateau region (Jupp et al,1986). Cx.univittatus is mainly restricted to the higher inland plateau and is replaced by a similar but more anthrophilic species of mosquito called Culex neavei(Theobald) in the lower coastal regions.Cx.neavei is,however,less susceptible to WN and SIN viruses than Cx.univittatus with 50% infection thresholds 2 logs higher in the case of WN virus and 1 log in the case of SIN virus. Titres of

both viruses often develop below the infectivity thresholds of Cx.neavei in birds,which could account for the poor vector capability and hence the lower human immune rates and fewer incidences of clinical infections in the Natal coastal lowlands (Jupp et al,1986).

A few cases of WN and SIN viruses occur every summer particularly in the moist regions of the Transvaal and Orange Free State(Jupp et al,1986). However,in early 1974 the largest epidemic of both these viruses ever recorded in South Africa occurred in the usually arid Karoo and Northern Cape Province with thousands of human infections estimated to have occurred(Mcintosh et al,1976). That summer,the region received exceptionally high rainfall(Fig.2) which resulted in large populations of mosquitoes. This together with the custom of many of the local people of sleeping outside because of the summer heat is thought to have led to the higher number of cases. A higher than usual number of human cases of SIN virus and to a lesser extent WN virus also occurred in the Witwatersrand-Pretoria region from December 1983 to April 1984 (Jupp et al,1986). The above two incidents are unusual for viruses that are normally enzootic with sporadic human cases. During these epidemics Cx.univittatus infection rates were markedly higher than usual which confirmed the important role that this species plays both in maintainence of the virus and in infecting humans during epidemics. Jupp et al(1986) reported that the higher than normal temperatures recorded during the summer of 1983 and 1984 (Fig.3a) and higher than usual early summer rainfall (Fig.3b),might have favoured viral infections in the mosquito and subsequently enhanced transmission of the viruses in the Witwatersrand-Pretoria region. Secondly,earlier preliminary studies by Jupp(1974)on the transmission of WN virus at different temperatures demonstrated that a lowered temperature reduced the viral transmission rate 17 days after the infective feed,but

Fig.2.

Total monthly rainfall figures in mm during the summer months from 1970-1980 in Upington.


RAINFALL /MM
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SUMMER MONTHS

did not reduce the infection rate. Jupp also mentioned that in the plateau region,particularly the Highveld,under normal summer conditions,daily temperatures fluctuate markedly which is typical of temperate climates. Therefore to get a better overall understanding of the dynamics of viral transmission by Cx.univittatus it seemed important to investigate the effects of fluctuating temperatures on vector competence,and to compare this with similar studies carried out at constant temperatures.

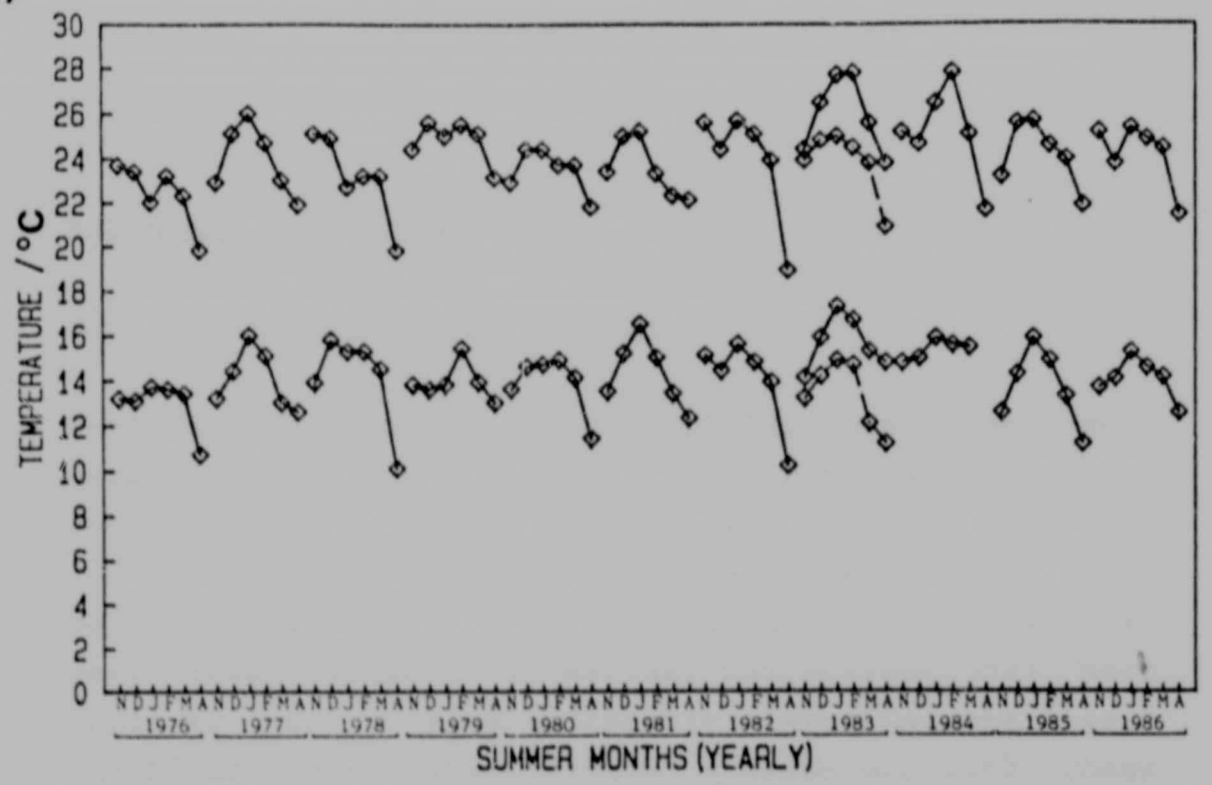
The present study further investigated the effect of temperature on the vector competence of Cx.univittatus for WN and SIN viruses respectively,"vector competence" being the susceptibility to virus infection and ability to transmit virus. Groups of infected mosquitoes were held at constant temperatures of 14,18,26 and 30°C and another group at the fluctuating ambient temperature outdoors in the late summer in Johannesburg. Limited studies were also conducted on the longevity of uninfected mosquitoes at different temperatures,and a comparison was made between the longevity of uninfected and SIN and WN virus-infected mosquitoes held at 26°C. Combined with knowledge of the geographical location,temperature and rainfall figures of past epidemics of both viruses,the results of these temperature vector competence and longevity studies contribute towards a better understanding of the factors that influence outbreaks of these viruses in South Africa. Replication and the distribution of each virus in Cx univittatus has not yet been studied,and hence this was investigated by fluorescent antibody tests on the various organs of the mosquito.

Fig.3

a)Average maximum and minimum temperatures in °C experienced during the summer months in Johannesburg from 1976-1986.

b)Total monthly rainfall in mm in Johannesburg during the summer months of 1978-1985.

a)


TEMPERATURE /°C
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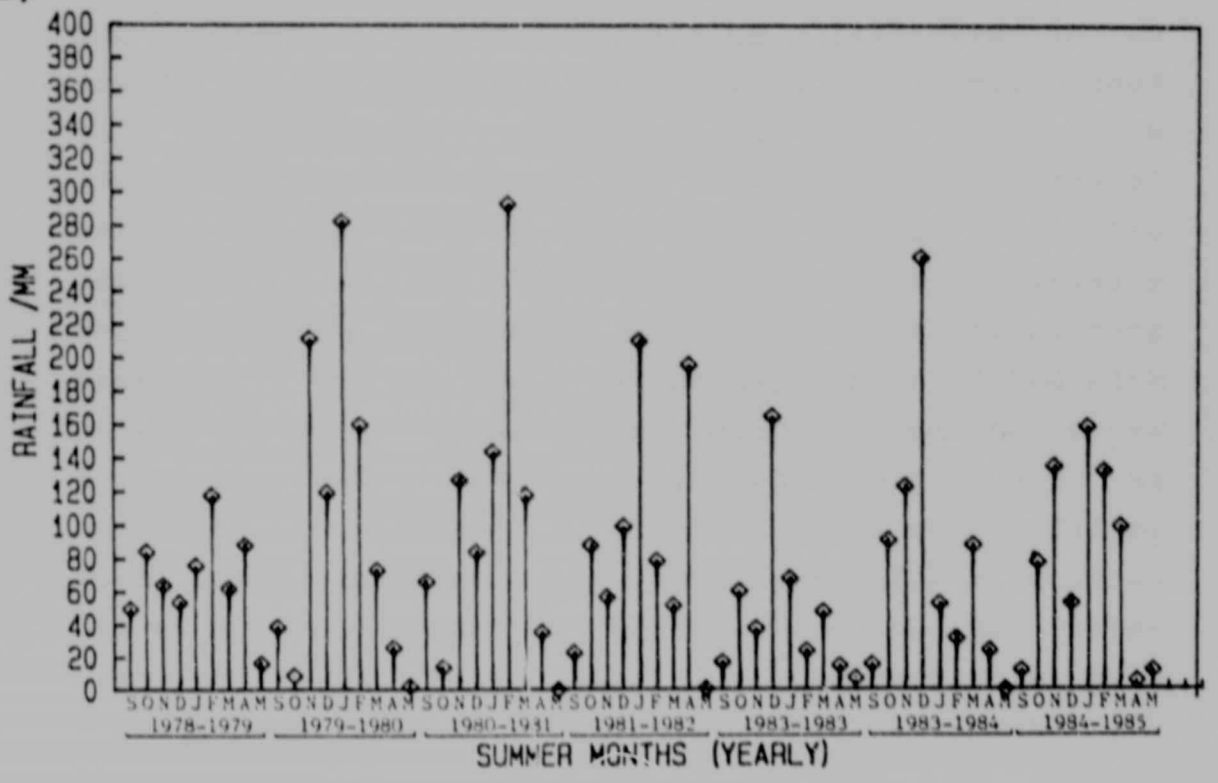
b)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SUMMER MONTHS (YEARLY)

CHAPTER 2

MATERIALS AND METHODS.

2.1.Mosquito colony and rearing.

Methods for rearing Cx.univittatus mosquitoes were essentially the same as those described previously by Jupp (1967).Mosquitoes were collected in the summer of 1985 near the Klip river in Johannesburg using portable baited traps which were based on the lard-can bait trap designed by Bellamy and Reeves(1952). Bait employed was a single domestic pigeon(Columba livea) which was restrained within a nylon stocking. These traps were suspended from trees about 1-2 meters above the ground and left overnight. The following morning the traps were brought back to the laboratory,where the engorged Cx.univittatus mosquitoes were removed and placed in cages measuring 35x35x35cm in an insectary. Conditions in the insectary were maintained at 26±2°C,75-80% relative humidity(RH) and a photoperiod of 11hrs complete darkness and 11hrs of maximum light with a 1hr crepuscular period in the late afternoon and early morning. Eggs were initially obtained from individual gravid females by placing them in 2.5cm diameter glass tubes half filled with grass infusion. This method was only used for a few generations,and afterward eggs were just as efficiently obtained by placing black dishes filled with grass infusion into the cages. The mosquitoes deposited egg rafts on the surface of the water overnight,which were carefully removed the next morning and placed in beakers of distilled water for hatching. The larvae were placed in enamel pans(27*37cm) in water with a few pieces of dried Kikuyu grass and given a daily diet of Brewers yeast (500mg potency manufactured by Vita force) and

"Nestum"(mixed baby • cereal manufactured by Nestlé),and airated. The newly emerged progeny of field mosquitoes were subsequently added to cages containing recently emerged adults from an older established laboratory colony(F8) of Cx.univittatus that also originated from individuals collected in Johannesburg. This mixed colony of wild and colonized mosquitoes provided the initial material for the vector competence studies. Before any experiment,large numbers of young adult moquitoes were reared using the techniques described above,of which half the adults reared were used for vector competence studies and the other half for maintaining the colony and providing the next generation of mosquitoes for the next experiment. All adult mosquitoes were maintained on a diet of a 4% sucrose solution. In the summer of 1986 more field mosquitoes were collected and their offspring also added to the colony.

The initial establishment of a colony only incorporates a portion of the field population's genetic variability,and fluctuations in colony size during colonization sometimes reaching very low levels could also add to genetic change due to stochastic processes of genetic drift (Lorenz et al,1984). For this reason and for the reasons discussed earlier in chapter 1,viz.,colonization resulting in a change in vector competences of mosquitoes,the colony was kept as large as possible by rearing offspring from every egg raft obtained,and wild mosquitoes were added to the colony during the summer months.

Such mosquitoes were used throughout the project.

2.2.Virus.

The H442 strain of WN virus used was originally isolated from a human being at Ndumu,South Africa,in 1959 (Kokernot and McIntosh,1959).

The AR86 strain of SIN virus used was originally isolated in 1954 from a pool of mixed Culex mosquites collected in Springs,east of Johannesburg (Weinbren et al,1956).Both viruses had been passaged five times in mice and four times in Vero cells.

2.3.Oral infection of Culex univittatus.

2.3.1.Membrane feeding.

Two different artificial feeding techniques and four different membranes were evaluated to establish a method whereby a large number of mosquitoes could be be fed efficiently on defibrinated blood. It was hoped that such an artificial feeding method could be used to infect the mosquitoes by feeding them on blood-virus mixtures,permitting the control of the viral titre in the mixtures. One of the artificial techniques used was developed by Davis et al (1983). Here the blood was citrated,kept at 32°C and constantly stirred. The other feeding technique was that of Jupp and McIntosh(1970) where defibrinated blood was maintained at 32°C without stirring.The four membranes tested were the Badruche membrane,stretched Parafilm (American Can Company,Greenwich,U.S.A.), freshly harvested skin from 2 week-old chickens and a silicon membrane(Davis et al,1983). Each of these membranes was evaluated with both feeding techniques.

2.3.2.Viraemic chicks.

The experiments required large numbers of mosquitoes infected wiith a high dose of virus. To meet this need the method previously employed by Jupp (1976) was adopted. Maximal titres of SIN virus were obtained by inoculating 1-day-old chicks intramuscularly,and exposing them to mosquitoes 24 hours later.On the other hand,maximal titres of WN virus were obtained by inoculating 2-day-old chicks and exposing them to mosquitoes 36 hours later. Just prior to infecting the

mosquitoes,the concentration of virus in the blood of each chick was determined. For this,Ø.1ml of blood was removed from the wing vein,diluted with Ø.9ml of Leibovitz's medium (Gibco Europe Limited,Paisley, Scotland) to which streptomycin, neomycin,penicillin, geromycin and foetal calf serum were added and 1Ø fold serial dilutions then inoculated onto monolayers of Vero cells. Vero cells respond to both WN and SIN virus infections by visually displaying cytopathic effect. Plaques form after 2 days in the case of SIN virus and 4 days with WN virus. The 5Ø% cytopathic dose ($CPD_{5Ø}$) endpoints were calculated by the method of Reed and Muench(1938). Viral titres were expressed as $Log_{1Ø}$ $CPD_{5Ø}$/ml of chick blood. After the chicks had been bled they were restrained inside nylon stockings and left overnight in cages containing 1-week-old mosquitoes. The mosquitoes were housed in an insectary at 26°C and a RH of 75-8Ø% where all of the infective feeds were given. The mosquitoes were starved for 24 hours preceding each infective meal. The following morning the chicks were taken out of the cages and all fully engorged mosquitoes were placed into other cages to be held at the various incubation temperatures.

2.4.Temperature conditions.

For vector competence and longevity studies at 26°C, the infected mosquitoes were held in the insectary. However,only the insectary had a constant relative humidity under electronic control and therefore at all the other temperatures the mosquitoes were kept at high humidity,(see section 2.7),by placing a large pad of cotton-wool saturated with water on top of the cages and then covering the whole cage with plastic to retain the moisture. Incubation cabinets (14°C and 3Ø°C) and a temperature-controlled room (18°C) provided the different temperatures required. The lighting regime consisted of 12 hours light and 12

hours darkness. For exposure of mosquitoes to temperature conditions outdoors,a mosquito proof "walk-in" cage measuring 2x2x2 metres was constructed and positioned outside the laboratory away from direct sunlight. Mosquitoes within smaller 35x35x35cm cages provided with moist pads of cotton wool and covered with plastic,were placed on a table in the middle of the walk-in cage. The mosquitoes were thereby exposed to fluctuating ambient temperature while still held at a high RH within the plastic,and were prevented from escaping by 2 barriers. Thermohygrographs were placed with the mosquitoes at the various temperatures to record any fluctuations in temperature and RH.

2.5.Transmission.

2.5.1.Definitions of infection and transmission rates.

The infection rate(IR) is the proportion of mosquitoes feeding on the infective blood-meal which become infected.The transmission rate(TR) is the proportion of infected mosquitoes transmitting virus. Both are expressed as percentages.

2.5.2.Determination of method for transmission tests.

The method used at present at the National Institute for Virology for transmission studies with WN and SIN viruses and Cx.univittatus mosquitoes involves feeding individual infected mosquitoes on susceptible laboratory animals,for example,either individual chicks or individual hamsters. Golden Syrian hamsters,the more convenient animals,respond to SIN virus infections by developing high antibody levels that can be demonstrated after 21 days by the haemagglutination inhibition test (Casals and Brown,1954). West Nile virus on the other hand is lethal,the hamsters dying 7-10 days after the

bite of an infected mosquito. Livers are removed from the dead hamsters and tested for virus in infant mice. Although hamsters serve as good laboratory animals for transmission studies with these viruses,very low feeding rates are obtained with ornithophilic Cx.univittatus mosquitoes. As transmission tests needed to be done at regular intervals in the present study the hamster method would have been both time consuming,cumbersome and costly. Hence two other in vitro transmission methods,"droplet and capillary", previously utilized for other viruses,were compared to the transmission rates obtained using hamsters for both WN and SIN viruses. The droplet method developed by Gubler and Rosen(1976) had been found as sensitive as engorgement upon suckling mice for detection of YF virus transmission by Ae.aegypti (Beaty and Aitken,1979) and for transmission of DEN virus by Ae. albopictus, (Gubler and Rosen,1976). Using this method,each infected mosquito is placed in a tube covered with fine-mesh nylon material,so that it can feed from a droplet of fluid placed on the material(Fig.4). While feeding,the insect secretes saliva and virus into the droplet and the unconsumed part of the droplet is then tested for virus to determine whether transmission has occurred. The "capillary" method developed by Aitken(1977) was used successfully for demonstrating transmission of YF virus by Ae.aegypti mosquitoes(Beaty and Aitken,1979). In this technique a capillary tube is drawn to a fine point narrow enough to fit snuggly over the proboscis of a mosquito. The tube is filled with a predetermined amount of feeding suspension. A mosquito is anaethetized with carbon dioxide and the first two pairs of legs removed to immobilize the insect. The base of the wings are gripped with forceps and the proboscis of the mosquito forced into the narrow opening of the prefilled capillary tube. The tube with the mosquito dangling from it,(Fig.5)is suspended from a polystyrene rack until the insect has fed to

repletion. As with the droplet method,the mosquito secretes saliva and virus into the suspension which can then be tested for presence of virus to demonstrate transmission. In the present study,high feeding rates were obtained when a feeding suspension of equal parts of 10% sugar,foetal calf serum and washed goose erythrocytes was used in the droplet method,and a suspension of equal parts of 10% sugar and foetal calf serum was used in the capillary method. In the droplet method,25ul of the above solution was placed on the nylon material and after the mosquito had fed the remainder of the drop was diluted 10-fold in 250ul of Leibovitz's medium before inoculation onto Vero cells. In the case of the capillary method,only 4ul of feeding solution could be drawn up into the tube and after the mosquito had fed, only 2.5ul of fluid was left. Therefore the capillary droplet had to be dilutd 100-fold with Leibovitz's medium(250ul) before inoculation onto Vero cells. If the Vero cells developed cytopathic effect (CPE) due to the presence of virus in the suspensions then successful transmission had ocurred. Transmission rates were determined for Cx.univittatus with WN and SIN viruses respectively by each of the three methods described above to allow a comparison between them. In these experiments infection rates were determined by intracerebral inoculation of infant baby mice with 0.02ml of supernatant prepared from individually homogenized mosquitoes. The mice died 3 days after inoculation if the mosquito was infected with SIN virus and 6 days if the mosquito was infected with WN virus. The titre of infecting chicks, transmission and infection rates and P values calculated from chi square tests applied to the transmission rates are shown in table 3.

Fig.4.
Droplet method:mosquito feeding (life size).

Fig.5.
Capillary method:mosquito feeding (twice life life size).





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2.5.3.Capillary method.

In view of the results obtained from the comparison of the 3 methods for measuring the transmission rates(see chapter 3),it was decided to use the capillary method. Transmissions were attempted at regular daily intervals by several mosquitoes representing a particular incubation temperature. Each insect was fed by the capillary method. The numbers of mosquitoes tested each day are given in appendix A for the studies with WN virus and appendix B for studies with SIN virus. The capillary method used for the vector competence studies was the same as already described. The 2,5ul volume of the remaining feeding fluid was diluted in Leibovitz's medium and titrated by making serial 10-fold dilutions which were inoculated onto Vero cells in 96 well plates. The CPD_{50} end points were calculated according to the method described by Reed and Muench(1938),and the final results are represented as $Log_{10}CPD_{50}$/ml of capillary fluid. The capillary fluid corresponding to each mosquito was individually titrated,so that the virus transmitted by the mosquito at the temperature concerned could be determined. The titres of capillary fluids were expressed as $Log_{10}CPD_{50}$/ml to make comparisons with the titres of individual mosquitoes easier. Capillary fluid titre may,however be expressed as $Log_{10}CPD_{50}$/mosquito by subtracting 1.7 from the titre per ml. These viral titres were tabulated,and the daily average titres of virus transmitted at the various temperatures calculated and graphically represented to facilitate making comparisons. Transmission rates were also calculated from these results and are similarly represented graphically,so the EI periods can easily be seen for the various temperatures.

2.6.Mosquito infection rates.

After capillary feeding,each mosquito was stored at -70.C for 12hrs and then individually tested for virus. This was done by trituration in 1ml of Leibovitz's medium. The resulting suspensions were centrifuged at 900RPM for 5 min at room temperature and 10-fold dilutions of the supernatant fluids made and inoculated into already established monolayers of Vero cells.Adding the supernatent fluids to monolayers of Vero cells that had been prepared 12 hours beforehand was found to be more sensitve (Blackburn personal communication).
Titres of virus of individual mosquitoes that had fed on the capillary feeding suspension were thus determined and are represented as $Log_{10}CPD_{50}$/ml of mosquito suspension which can also be expressed as $Log_{10}CPD_{50}$/mosquito. These titres of individual mosquitoes were tabulated in the same way as with the titres of capillary fluids and the averages graphically represented.

2.7.Longevity studies.

Pupae of Cx.univittatus that had pupated within 3 days of each other were placed in 7 cages in the insectary.Adults were kept for a further 4 days after emergence to allow them to mate.In the afternoon of the 4th day the mosquitoes were starved to ensure a high feeding rate 24 hrs later. In 4 of the cages uninfected pigeons restrained inside nylon stockings were exposed to the insects, while 2 viraemic chicks were exposed to the insects in the 6th cage to infect them with SIN virus. The 7th cage of mosquitoes was similarly infected with WN virus by exposure of 2 chicks viraemic with that virus. All four vireamic chicks were bled prior to placing them in the cages so that the infecting doses could be determined. The viral of the chick blood,were determined in the same way as mentioned in section 3. The following morning all unfed mosquitoes were removed from the cages so that only female mosquitoes which had had one blood

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meal were used for the longevity studies. Cages of fully engorged females were set up as follows and held at the conditions indicated:

1 cage of uninfected mosquitoes,at 30°C±1°C,60-70RH,in an incubator.

1 cage of uninfected mosquitoes,in a room thermostatically controlled at 18-20°C,60-75%RH.

2 cages of uninfected mosquitoes outside in the walk-in cage,Fig.19,30-80%RH.

1 cage of uninfected mosquitoes in the insectary at 26°C±1°C,75-80%RH.

1 cage of mosquitoes infected with WN virus in the insectary at 26°C±1°C,75-80%RH.

1 cage of mosquitoes infected with SIN in the insectary at 26°C±1°C,75-80%RH.

As with the vector competence studies,all the cages of mosquitoes that were not kept in the insectary had to be kept at high humidity by placing a pad of moist cottonwool on top of the cage and covering the cage with plastic. All cages of mosquitoes were supplied with a 4% sugar solution for the duration of the experiment. Age specific results for females were obtained by removing and counting the dead female mosquitoes from each cage every day. The mean(average) longevity,standard deviation and variance were then calculated for both the uninfected and infected mosquitoes at each temperature from the age specific mortality results. The median value(50% alive) was read from graphs of percentage survival vs time,and since these values were very similar to the mean,normal distributions for the longevity studies were assumed. The Student Newman Keuls(SNK) test,an a posteriori significance statistical test (Sokal and Rohlf,1969) was used to establish the level of significant difference and similarity between the mean longevities of Cx univittatus at the various temperatures and between the uninfected and infected mosquitoes at 26°C. The calculations of the SNK test are shown in appendix C for uninfected mosquitoes and appendix D for infected mosquitoes.

2.8.Multiplication and distribution of WN and SIN viruses in mosquito organs.

In some of the transmission experiments,i.e.those done at 14°C and 18°C for both WN and SIN viruses,not all the mosquitoes that had fed through capillaries were triturated to determine their infectivity titres. Such mosquitoes were used to determine the sequence of multiplication and distribution of WN ard SIN viruses respectively within their bodies,by fluorescent antibody staining.

2.8.1.Conjugate and antibodies.

An affinity purified IgA+IgG+IgM(H+L) anti-mouse immunoglobulin fluorescein conjugate was used in this study (Cappel). Before staining the slides with the conjugate,2ml of Ø.ØØ1% Evans blue was added to Ø.Ø25ml of conjugate.
Hyperimmune ascitic fluids(HAF) prepared in the Arbovirus Unit against WN(H442) and SIN(AR86) produced from mice were used as the antibody source for the fluorescent antibody(FA) tests. The method used to produce the HAF in the Arbovirus Unit is based on the method described by Tikasingh,Spence and Downs(1966). The HAF fluids were diluted 1:1ØØ in phosphate buffer solution at a pH of 7(PBS) before use.

2.8.2.Fluorescent antibody staining.

Individual mosquitoes that had fed through capillaries were stored at -7Ø°C. These mosquitoes were later thawed and various organs,viz.,salivary glands,digestive tract,ovaries,malpighian tubules and abdominal ganglia were dissected out in PBS on a slide. These organs were then transferred to multi-well Teflon-coated slides (1 per well) and left at room temperature for two hours to dry. After they were completely dry and the organs securely stuck to

the slides, they were fixed in 100% acetone for 20 minutes at room temperature and then stored at -70°C. The following morning each slide was rinsed in distilled water and left to dry at 37°C before a 10ul drop of antibody solution was placed onto each well and the slides were incubated at 37°C for half an hour in an atmosphere of high humidity. After incubation, the slides were transferred to staining dishes and given two 5 minute washes in PBS. The slides were rinsed in distilled water to remove the PBS and left until dry whereupon 10ul of conjugate was placed onto each well and the slides incubated in a moist atmosphere at 37°C for half an hour. The slides were finally washed twice with PBS in a staining dish on a magnetic stirrer. Before viewing,cover slips were put over the specimens with 5% glycerol. The slides were examined with a Nikon Optiphot microscope using an epi-fluorecsence attachment with a 50 watt mercury vapour illuminator. Photographs were taken with the Nikon photographic attachment and camera.

2.8.Staining of salivary glands.

Mims et al(1966) demonstrated alteration of salivary glands infected with SF virus by haematoxylin(H) and eosin(E) staining of paraffin sections and it was decided that for an effective comparison,both uninfected and WN and SIN virus-infected glands of Cx.univittatus should be examined. As with the FA studies the source of infected glands was mosquitoes used for the temperature studies. Salivary glands studies were done on mosquitoes infected with WN virus that were incubated at 14,5°C for 90 days and mosquitoes infected with SIN virus and incubated at 18°C for 55 days. Unfortunately,it was necessary to use uninfected glands from 12 day old mosquitoes as controls,as these were the only uninfected mosquitoes available at the time. To avoid damaging the glands,complete thoracic sections were cut as appossed

to dissecting out individual glands. These thoracic sections were fixed in Bouin's solution for two hours,mounted in blocks of paraffin and bee's wax and 4ul sections were cut with a microtome. Finally, the sections were mounted onto slides and stained with H and E.

CHAPTER 3.

RESULTS.

3.1. **Evaluation of artificial feeding.**

Best feeding rates were obtained through chicken skin on stirred blood with Cx univittatus, but even so, the feeding rate was only 44% (Table 2). Due to the low feeding rates obtained, artificial feeding was therefore rejected as a means for infecting Cx.univittatus mosquitoes. Instead mosquitoes were infected by feeding on viraemic chicks.

Table 2. Feeding rates of Culex univittatus through four types of membranes on (A) Stirred blood and (B) unstirred blood.

Membrane	No.used	No.Fed	% Fed
Badruche.(A)	132	8	6.1
Parafilm.(A)	237	44	18.47
(B)	267	8	3
Silicon. (A)	26	Ø	Ø
Chicken (A)	111	28	25.23
skin. (B)	156	68	43.59

3.2. **Evaluation of methods for transmission tests.**

The results for a comparison of these methods for transmission tests, viz., hamster, droplet and capillary feeding by Cx.univittatus are shown in Table 3. It can be seen that although hamsters gave maximum transmission rates for both viruses, capillary feeding was a suitable alternative in vitro method for determining such rates, with each virus. This is indicated by low chi square P values between hamsters

and the capillary method. Transmission rates obtained with WN virus from the droplet method was similar to the rates obtained with the other two methods. Transmission rates with SIN virus from the droplet method was only 9% which was much lower than that obtained with the other methods. The blood of the geese used in the droplet method was always antibody-free and to try to explain this low transmission rate with SIN virus,a further experiment was conducted where mosquitoes infected with SIN virus were fed on a droplet of 10% sucrose,foetal calf serum and washed sheep erythrocytes. The different erythrocytes made no difference and very low transmission rates were still obtained. In another experiment,mosquitoes infected with SIN virus were first allowed to feed using the droplet method but when the mosquitoes were half engorged,they were removed and capillary fed to repletion. Both the droplet and capillary fluids were then titrated for virus in Vero cells. No virus was shown in any of the droplet fluids,but 4.5 to 5.35 $\log_{10}CPD_{50}$/ml of SIN virus was present in all the capillary fluids.This suggests that in most cases these mosquitoes secreted too little saliva into a droplet of feeding suspension,so that the highly diluted virus was undetectable by the assay system used. This is,however,still puzzling as the droplet method worked for the WN virus transmissions and further experiments remain to be done to explain this anomaly. On the basis of the above results and because high feeding rates were consistently obtained with the capillary method,it was decided to use this method for the transmission tests with individual mosquitoes. Furthermore,virus transmitted into the capillary fluid could be titrated and comparisons made at the different temperatures. It should however be noted,that use of the capillary method prevented the

mosquitoes from being used for further transmission studies. The second disadvantage of the method was that the capillary fluid had to be tested immediately after the mosquito had fed since no virus could be isolated from capillary fluids after they had been stored at -70°C for several days.

Table 3.Comparative transmission rates.

Virus	Titre of infective meal..	Days PI*	IR β	TR..	P value at 5% level
SIN	7-8.5	Ham 13	47/54 87%	34/47 74%	
SIN	7.3-8.5	Cap 14;19	66/74 89%	39/66 59%	-Ø.1-Ø.2
SIN	7.8-8.5	Drop 14;16	17/19 89%	3/35 9%	
WN	5-5.5	Ham 13	20/20 100%	20/20 100%	-Ø.1-Ø.05
WN	5-5.5	Cap 13	28/28 100%	22/28 78%	-Ø.05-Ø.01
WN	5-5.5	Drop 14	17/17 100%	12/17 71%	-Ø.05-Ø.01

(HAM)Hamster transmission,(CAP)Capillary transmission, (DROP)Droplet transmission.
.=$Log_{10}CPD_{50}$/ml of chick blood.
*=Days post infection.
β=Infection rate:No.mosquitoes infected/No.feeding.
.=Transmission rate:No.transmitting/No.infected.

3.3.Temperature vector competence studies.

Tables of data obtained from the vector competence studies are given in appendix A for WN virus and

appendix B for SIN virus. To make comparisons between the graphs of the temperature vector competence studies easier,transparencies of these graphs have been provided in the back cover.
Recordings of the daily ambient maximum and minimum temperatures taken outside during the vector competence studies are shown in Figure 6 for those mosquitoes infected with WN virus and in Figure 7 for those infected with SIN virus.

3.3.1.Effect of temperature on the competence of Cx.univittatus as a vector of WN virus.

Infection rates of 98%,96%,97%,96% and 92%,were obtained at 14°C,18°C,26°C,outside fluctuating temperatures and 30°C respectively,indicating that infection rates were not greatly affected by the temperature at which mosquitoes were held. Maximum titres of WN virus reached in individual mosquitoes (Figs.8c-12c) also showed little variation, i.e.6.4;6.3;6.1;7.0;and 6.4 at 14°C,18°C,outside fluctuating temperature,26°C and 30°C respectively with the titre being slightly higher at 26°C. Temperature did however have an effect on the time required to reach these maximum titres and took longest at 14.C(>110 days), shortest at 26°C and outside temperature(15 days) and intermediate at 30°C and 18°C(25 days). The patterns of infective titres of WN virus within the mosquitoes, exposed to outside summer temperatues(Fig.10c) seemed to follow most closely to those obtained in mosquitoes exposed to 18°C(Fig.11c),especially in terms of maximum and minimum titres. Minimal nightly temperatures probably had compromising effects on the higher maximum temperatures(fig 4 ...as reducing the overall rate of multiplication of the virus to a level similar to that at constant 18°C.
Titres of mosquito suspensions only declined at the higher temperatures,where the time before reaching

Fig.6

Daily maximum and minimum temperatures recorded in the outside walk-in cage housing mosquitoes infected with WN virus.

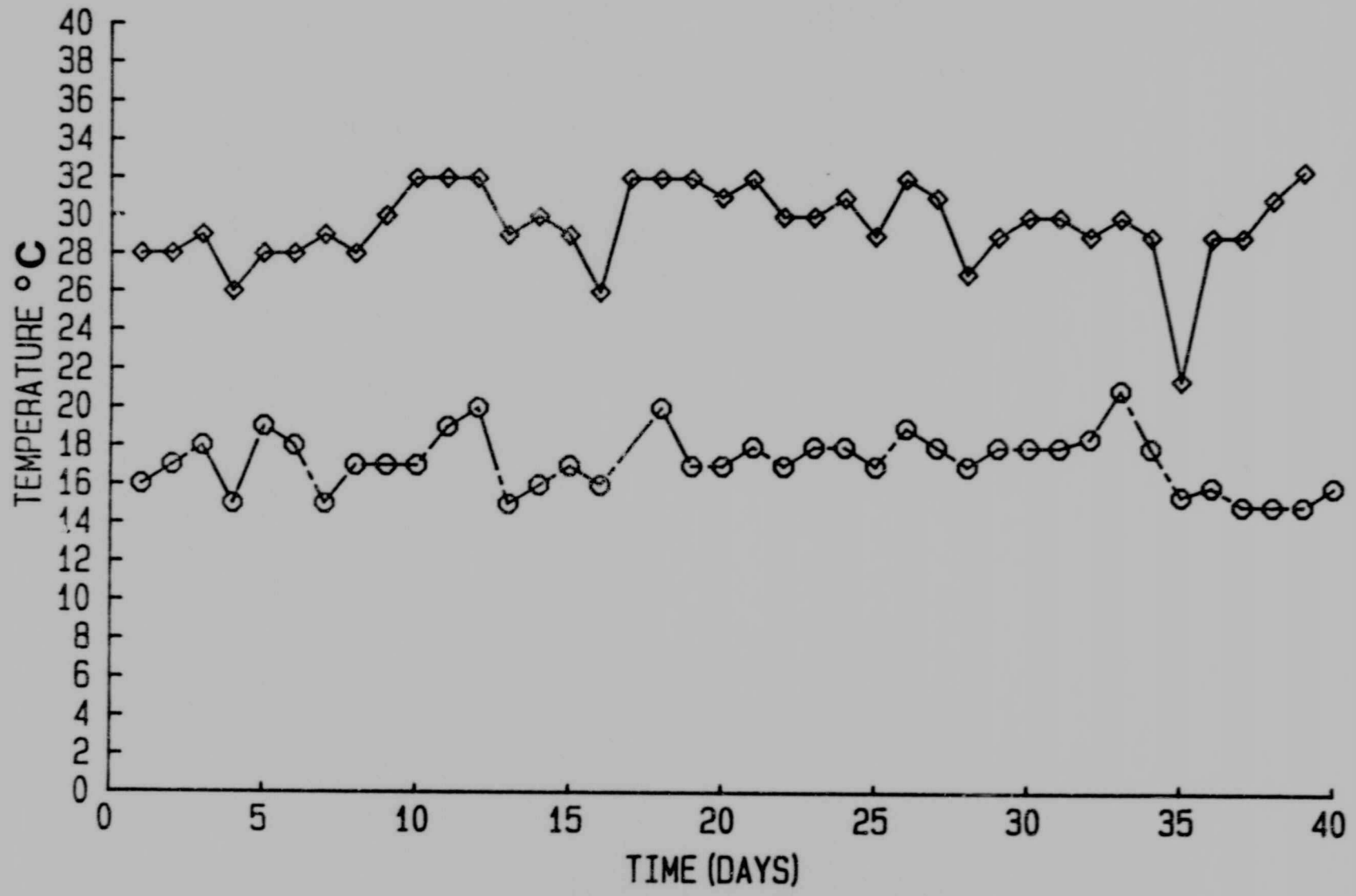
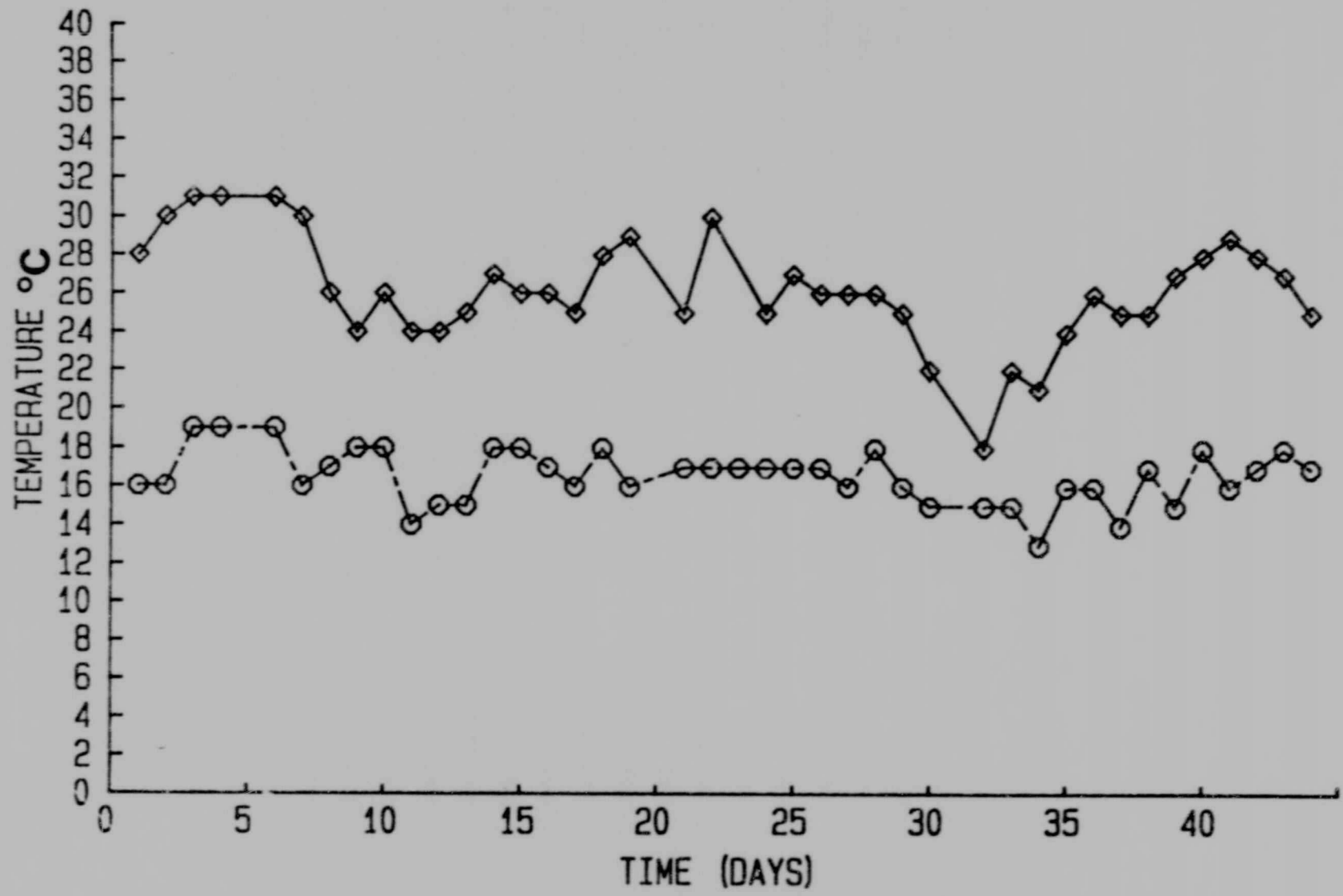

TEMPERATURE °C
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
40
TIME (DAYS)

Fig.7.

Daily maximum and minimum temperatures recorded in the outside walk-in cage housing mosquitoes infected with SIN virus.


TEMPERATURE °C
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
40
TIME (DAYS)

minimal titre was longest at ambient temperatures(8 days) and shortest at 30 and 26°C(4-6 days). The initial decline in titre at 14°C and 18°C with WN virus may have been masked by an overlap between loss of infectivity of imbibed virus and replication of new virus,both of which would be slowed down at these lower temperatures.

Since the capillary method is an artificial method for transmission tests,the amounts of virus secreted into capillary tubes might not neccessarily be identical to the amounts secreted into verterbrates during blood feeding. However, transmission rates obtained with the capillary method were similar to transmission rates obtained with hamsters in the experiment described above. Moreover,the capillary method could be carried out in the same manner for all the vector competence studies,thus facilitating comparisons. Transmission rates of 100% were obtained at all temperatures although it took much longer to reach this level at the lower temperatures,i.e. 15 days at 30°C,26°C and outside temperatures,22.5 days at 18°C and 79 days at 14°C(Fig.8a to 12a).

Maximum transmission rates and maximum titres of virus transmitted(secreted into capillary fluids) generally occurred simultaneously and responded similarly to different incubation temperatures. Maximum transmission once achieved was only maintained at that level for the duration of the mosquitoe's lifespan at 30°C. Whereas,at other temperatures,viral transmission seemed to fluctuate,with fluctuations becoming more pronounced at lower incubation temperatures.

Although WN virus multiplied much more slowly in the mosquitoes held at 14°C compared to those at the other temperatures,these mosquitoes lived almost twice as long (>110 days),still allowing time for the virus to multiply and reach infective titres similar to those reached at other temperatures. Therefore, Cx.univittatus was still an efficient vector of WN virus at 14°C,80 to 100 days after the infective feed

when both the titre of virus transmitted and transmission rate were similar to those at higher temperatures.

In the case of the experiment at 14°C,a few mosquitoes were transferred to 26°C,on day 48,to test the effects of the higher temperature on transmission(Fig.12a and 12b). This did not increase the transmission rate,but the rate might have increased if the mosquitoes had been transferred from 14°C to 26°C earlier since,when the mosquitoes were transferred to the higher temperature,those maintained at 14°C were already showing an upward trend in transmission rates. However titres of virus transmitted did increase when the mosquitoes were transferred to 26°C(Fig.12b;x----x) and by 16 days after being transfered had reached titres essentially the same as those attained in mosquitoes maintained solely at 30°C or 26°C. Furthermore,a few mosquitoes that were transferred to 26°C were placed in a separate cage with a black dish filled with river water. This was done to establish whether these mosquitoes which had been subjected to a cool temperature for up to 48 days could still lay eggs if temperatures were to rise. Of the 34 mosquitoes that were placed in this cage,20 had laid eggs by the 5th day(58%).On the 7th day after transfer the remaining 20 mosquitoes(14 having since died) were starved for 24 hours and fed overnight on a pigeon susceptible to WN virus. A 50% feeding rate was obtained and the pigeon developed a titre of 1:160 to the virus when tested 21 days later with the HI test,indicating that transmission of virus had occurred. In addition three mosquitoes were fed through capillary tubes 24 hours after the second blood meal,and all three transmitted exceptionally high titres of WN virus (Fig.12b;x-·-·-x). The second blood meal therefore seemed to enhance replication of virus in the salivary glands. Mosquitoes that were tranferred to the higher temperature were unfortunately not titrated. This

would prabably have confirmed wether viral replication was enhanced by the second blood meal.
Passive mechanical transmission only occurred in mosquitoes subjected to summer ambient temperatures(Fig.10c),and lasted for only 1-2 days after the infective feed. The extrinsic incubation period was reduced by subjecting mosquitoes to the higher temperatures of 30°C and 26°C as well as the summer outside temperatures when transmission first occurred 1-5 days after the infecting feed (Fig.8a,8b,9a,9b,10a and 10b),as compared to only 15 days afterwards at 18°C(Fig.11a and b) and 9-12 days at 14°C(Fig.12a and b).

3.3.2.Effect of temperature on the competence of Cx.univittatus as a vector of SIN virus.

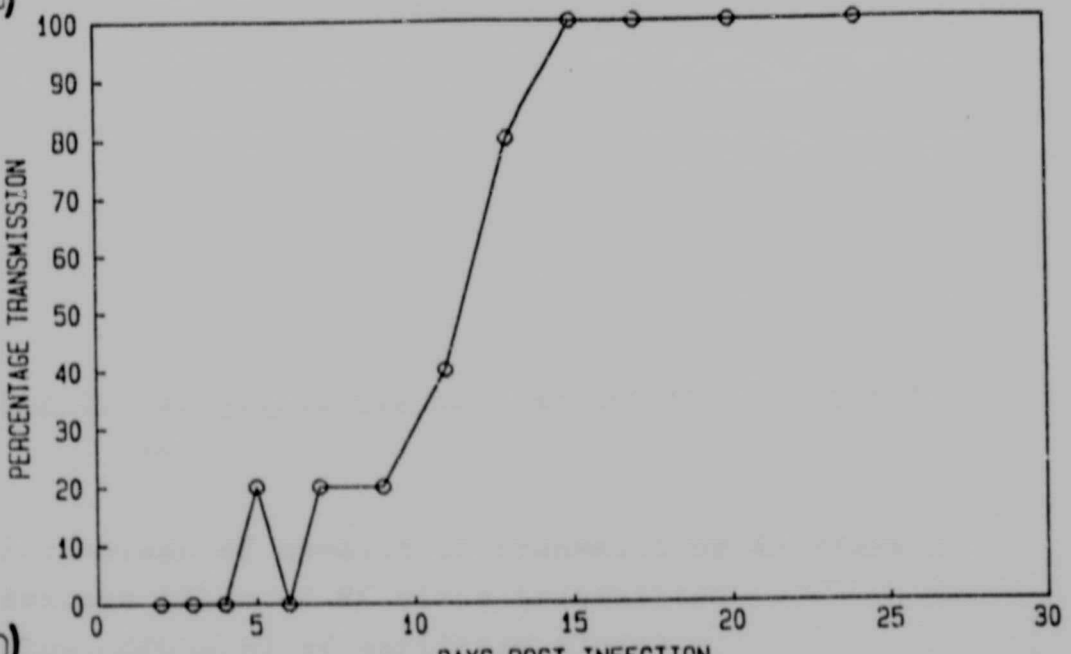
Transmission rates(Fig.13a to 17a) fluctuated markedly at all temperatures except at outside fluctuating summer temperatures. In the group of mosquitoes held at the latter temperature most mosquitoes (50-100%) were only capable of transmitting SIN virus for a limited period of 3 to 23 days,and already after 15 days there was a steady decline in the number of insects transmitting virus. However,transmission rates at all constant temperatures fluctuated and these fluctuations became larger at longer incubation periods.
Highest titres of virus transmitted were reached at 26°C and 18°C,ie.7.5logs,(Fig.14b and Fig.16b) and were almost 1.5 logs higher than that attained at 30°C and 14°C (Fig.13b and 17b) and 2 logs higher than that at fluctuating temperatures (Fig.15b).
The extrinsic incubation period was affected by the two extreme temperatures and most mosquitoes (>50%) were only transmitting virus after 5 days at 30°C and 14°C,whereas at the other temperatures,transmission rates as high as 80% were already reached 3-4 days after the infective feed. The period after the

Fig.8 Cx.univittatus held at 30:1°C and 60-70% RH:

a)Percentage of mosquitoes transmitting WN virus.
b)Average titre of WN virus transmitted
($Log_{10}CPD_{50}$/ml of capillary fluid).
c)Titres of WN virus in Cx.univittatus
($Log_{10}CPD_{50}$/ml of mosquito suspension)

Titre of viraemia of chicks used for infective meal=
5-5.5$log_{10}CPD_{50}$/ml of chick blood.

a)


PERCENTAGE TRANSMISSION
100
90
80
70
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25
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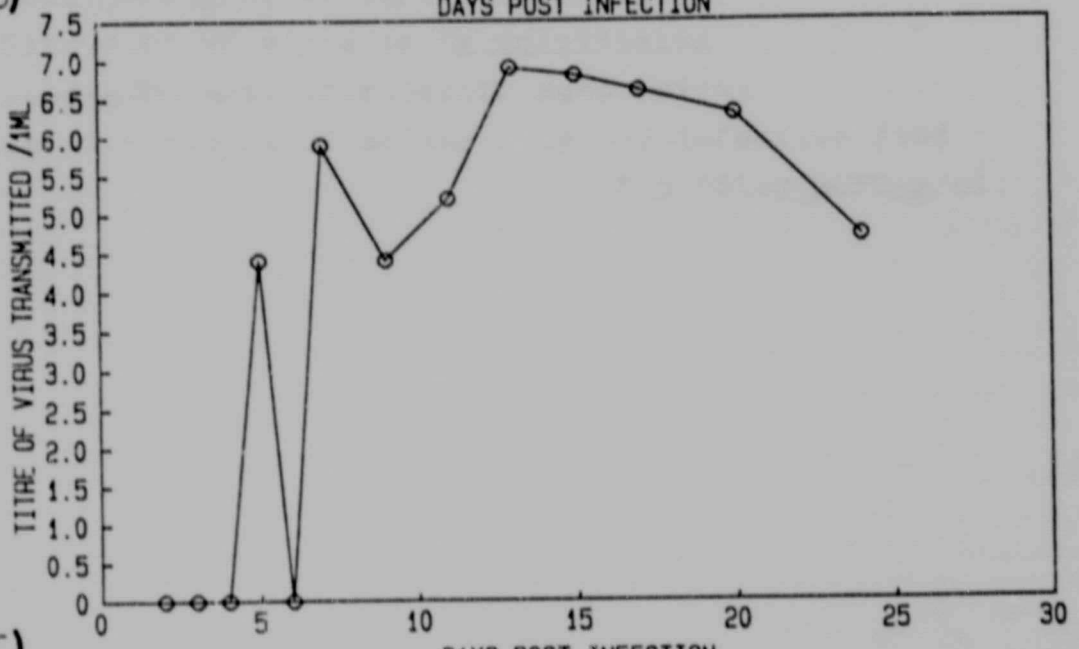
DAYS POST INFECTION

b)


TITRE OF VIRUS TRANSMITTED /1ML
7.5
7.0
6.5
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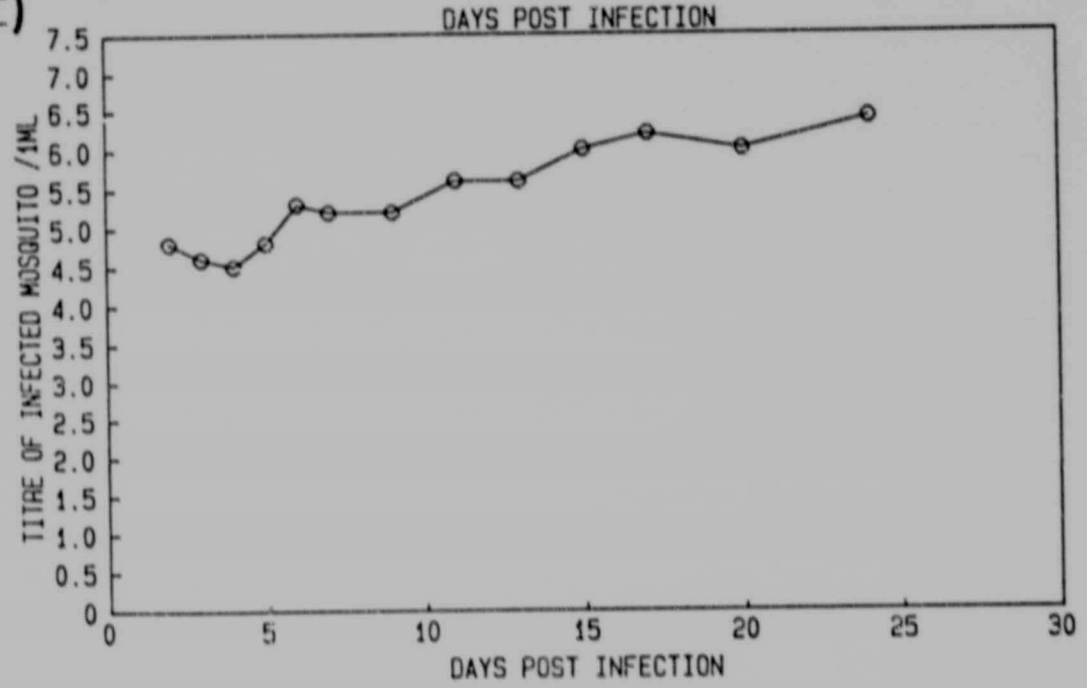
DAYS POST INFECTION

c)


TITRE OF INFECTED MOSQUITO /1ML
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7.0
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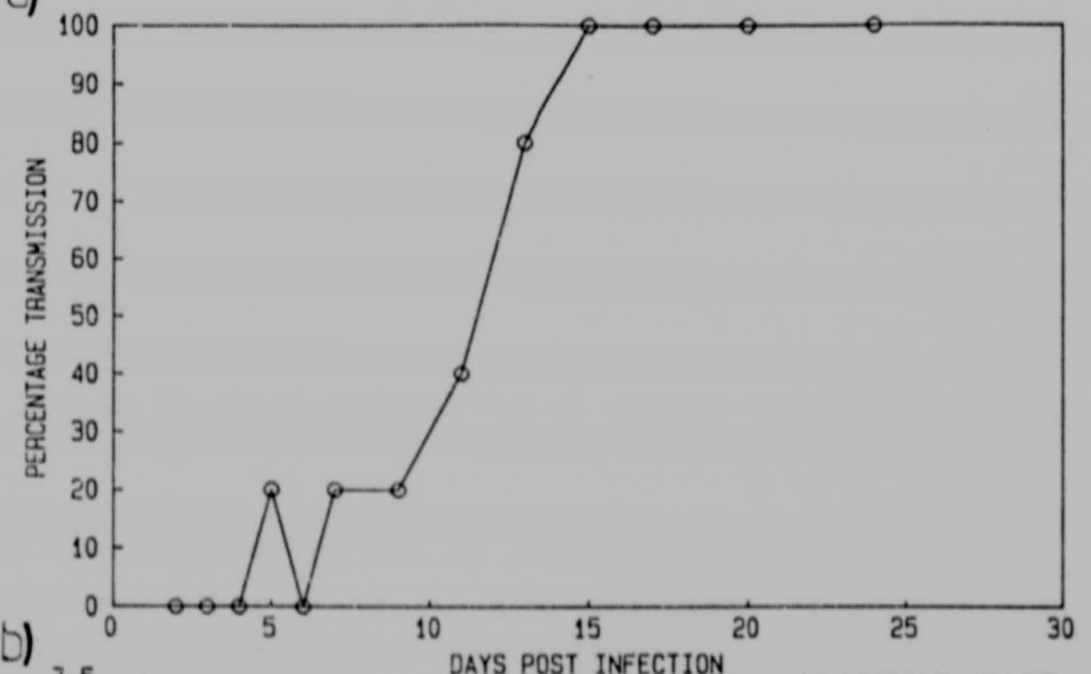
DAYS POST INFECTION

a)


PERCENTAGE TRANSMISSION
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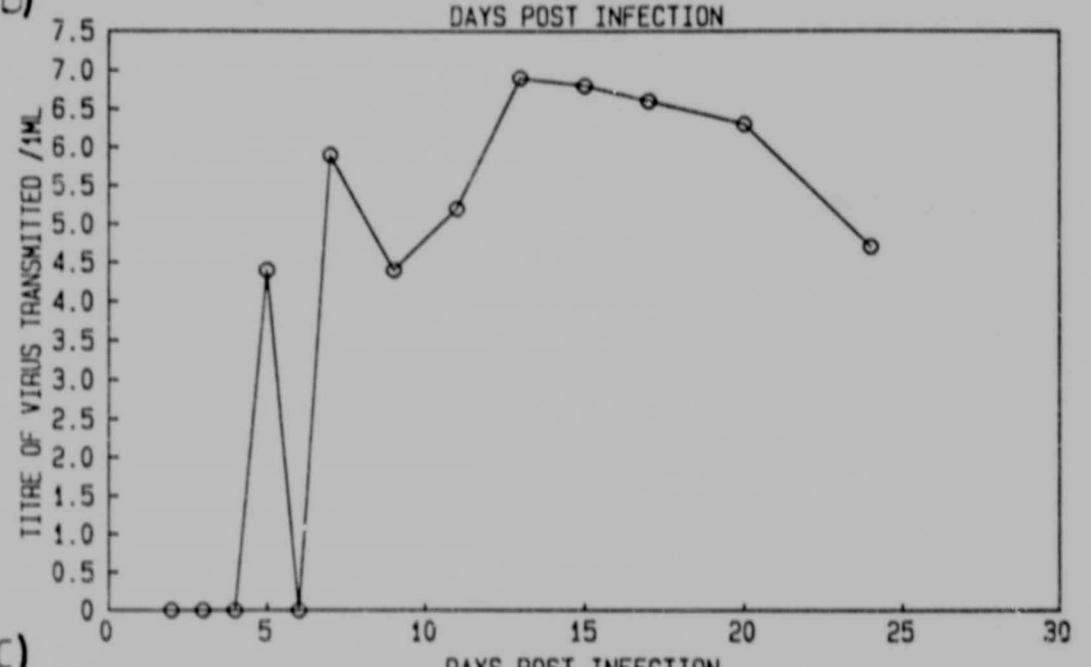
DAYS POST INFECTION

b)


TITRE OF VIRUS TRANSMITTED /1ML
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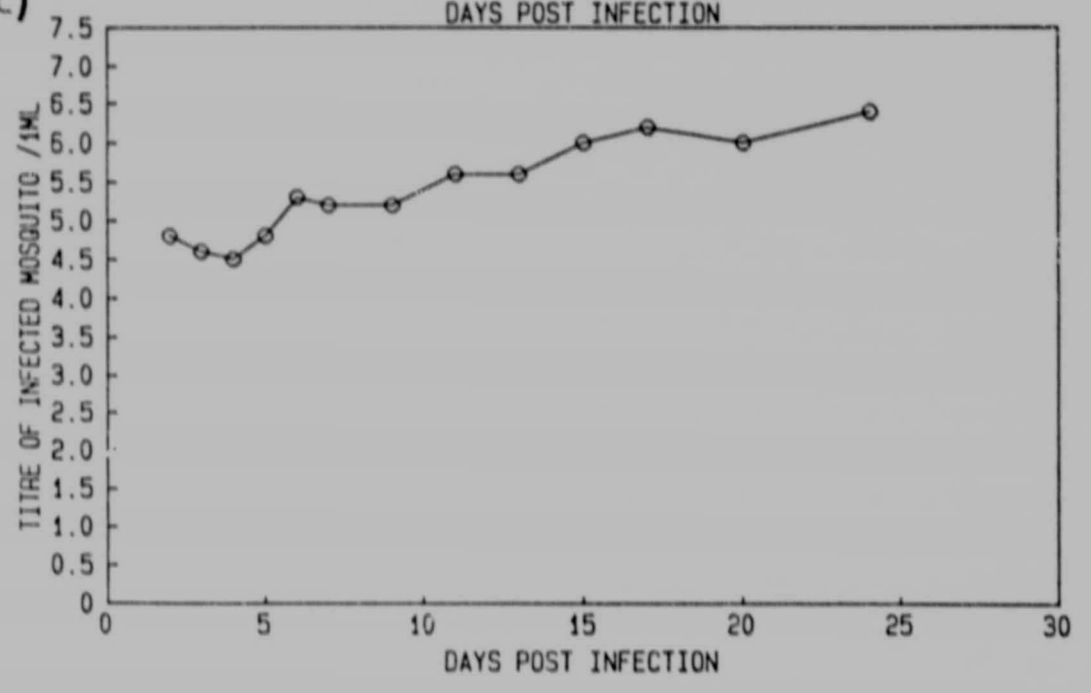
DAYS POST INFECTION

c)


TITRE OF INFECTED MOSQUITO /1ML
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6.0
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1.5
1.0
0.5
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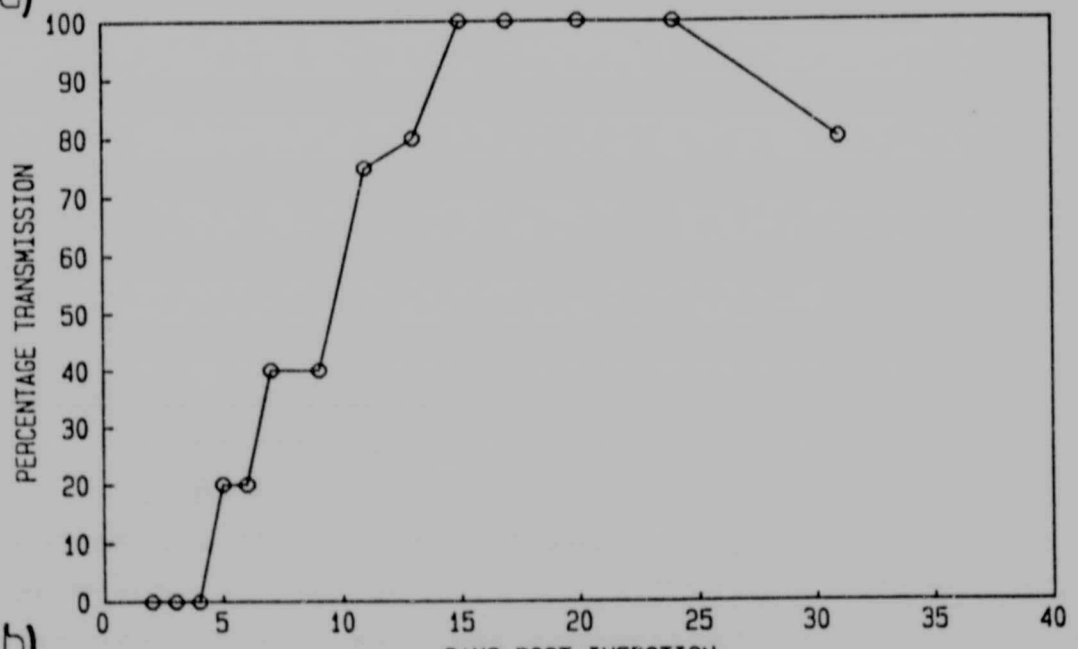
10
15
20
25
30
DAYS POST INFECTION

Fig.9. Cx.univittatus held at 26±1°C and 75-80% RH:

a)Percentage of mosquitoes transmitting WN virus
b)Average titre of WN virus transmitted
($Log_{10}CPD_{50}$/ml of capillary fluid).
c)Titres of WN virus in Cx.univittatus
($Log_{10}CPD_{50}$/ml of mosquito suspension)
Titre of viraemia of chicks used for infective feed = 5-5.55$Log_{10}CPD_{50}$/ml.

a)


PERCENTAGE TRANSMISSION
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10
20
30
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70
80
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30
35
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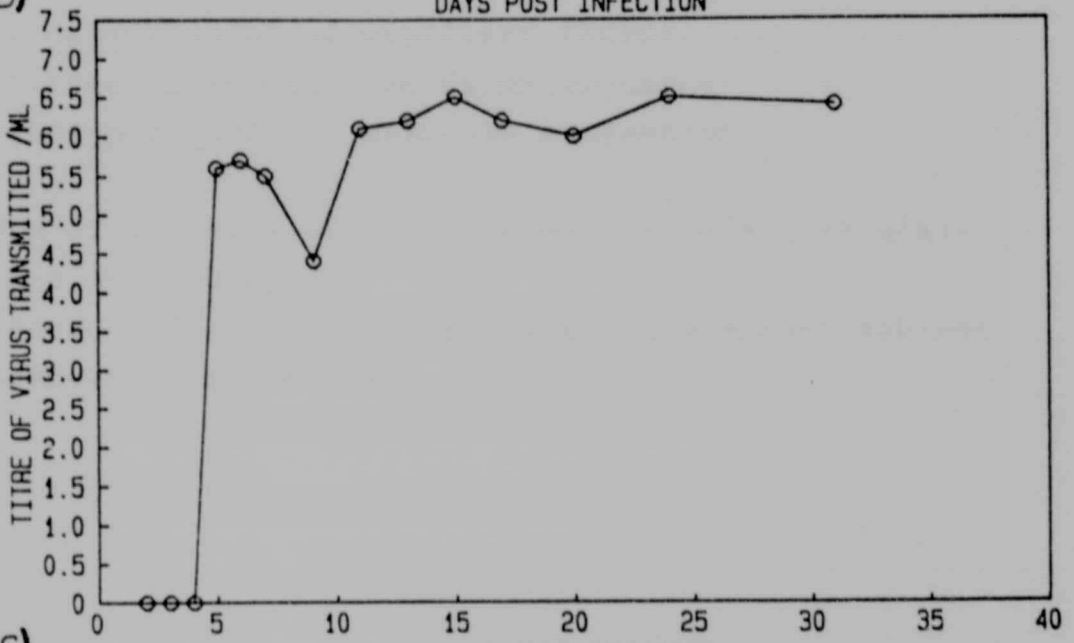
DAYS POST INFECTION

b)


TITRE OF VIRUS TRANSMITTED /ML
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
0
5
10
15
20
25
30
35
40
DAYS POST INFECTION

c)

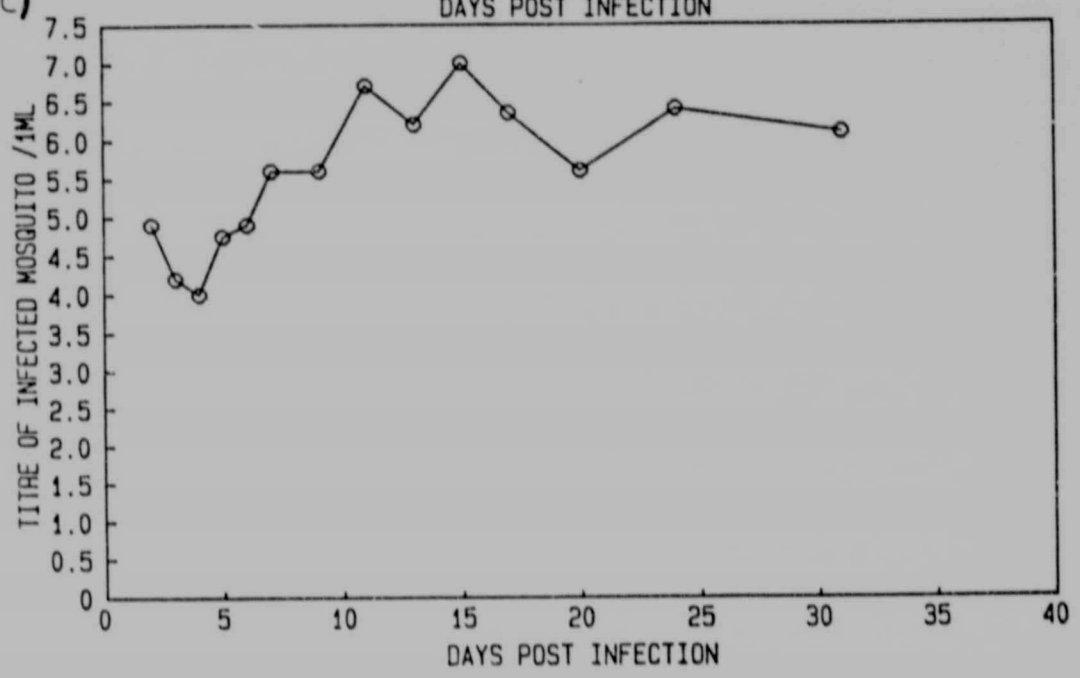

TITRE OF INFECTED MOSQUITO /1ML
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
0
5
10
15
20
25
30
35
40
DAYS POST INFECTION

Fig.10. Cx.univittatus incubated at outside fluctuating summer temperatures and 30-80% RH.

a)Percentage of mosquitoes transmitting WN virus.

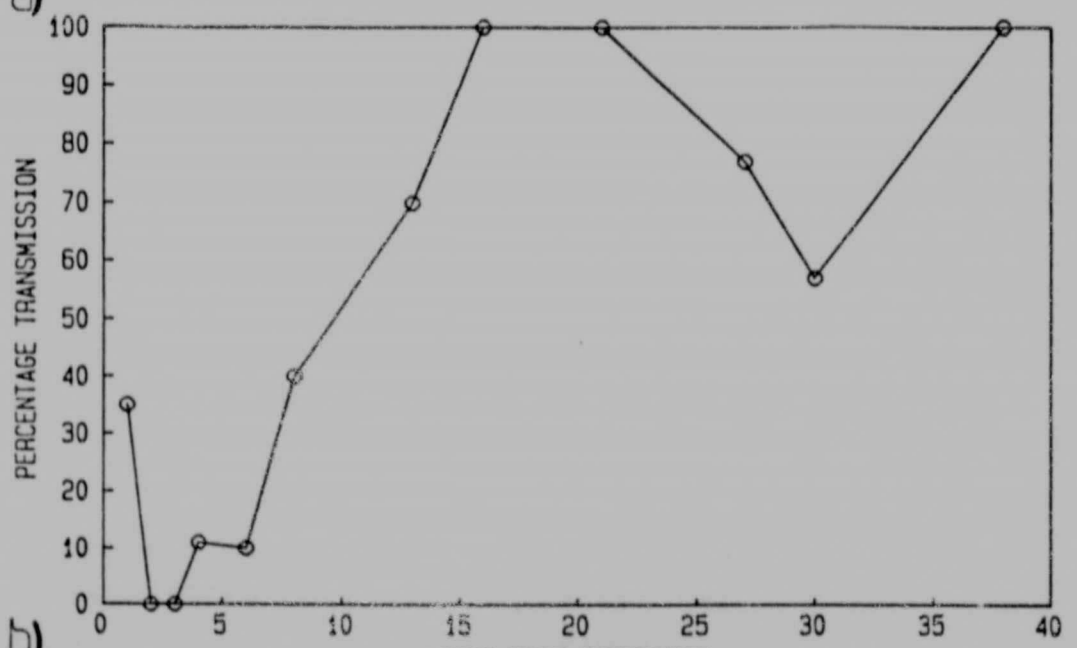
b)Average titre of WN virus transmitted ($Log_{10}CPD_{50}$/ml of capillary fluid).

c)Titres of WN virus in Cx.univittatus ($Log_{10}CPD_{50}$/ml of mosquito suspension).

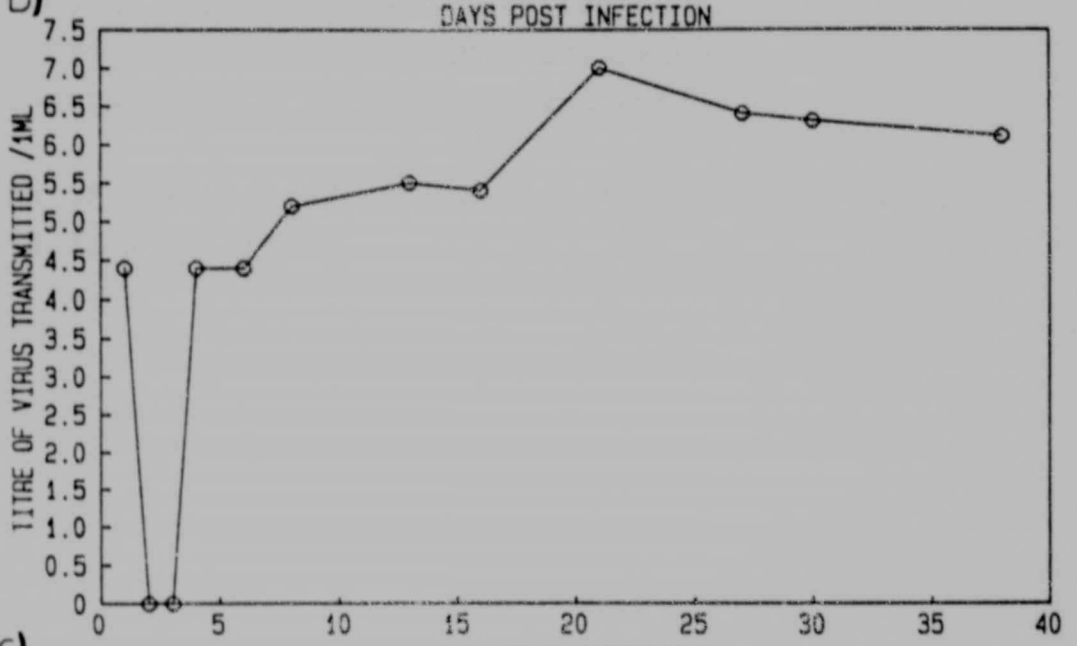
Titre of viraemia of chicks used for infective meal= 4.75-5.5$Log_{10}CPD_{50}$/ml

*=Records of daily maximum and minimum summer ambient temperatures are given in Fig.6.

a)


PERCENTAGE TRANSMISSION
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30
35
40
DAYS POST INFECTION

b)


TITRE OF VIRUS TRANSMITTED /1ML
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
0
5
10
15
20
25
30
35
40
DAYS POST INFECTION

c)

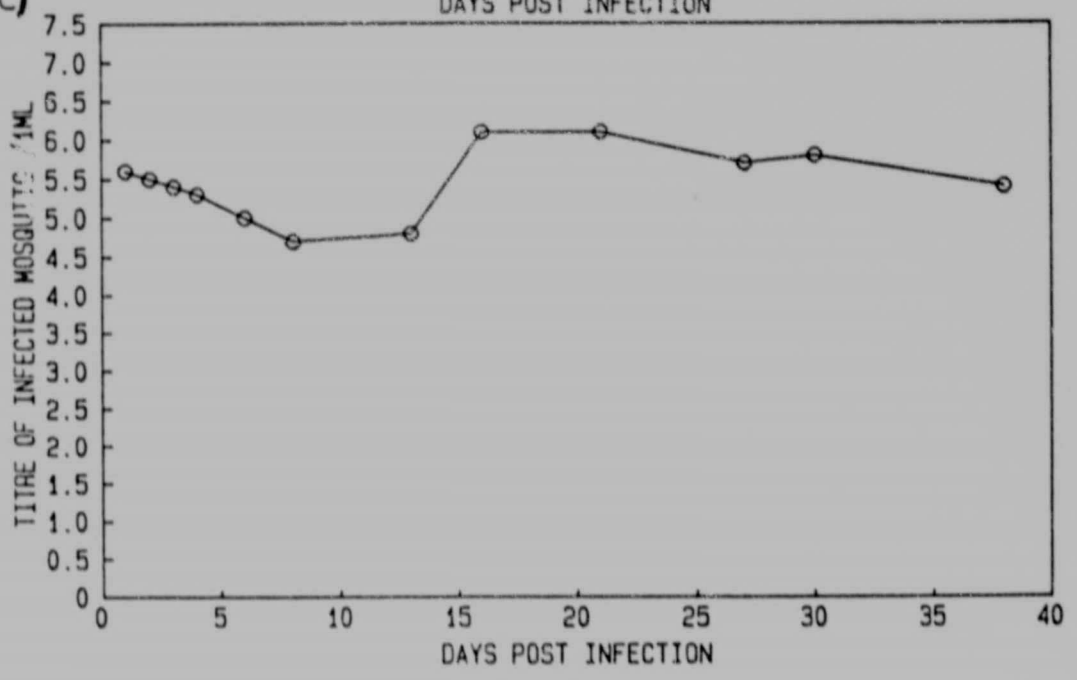
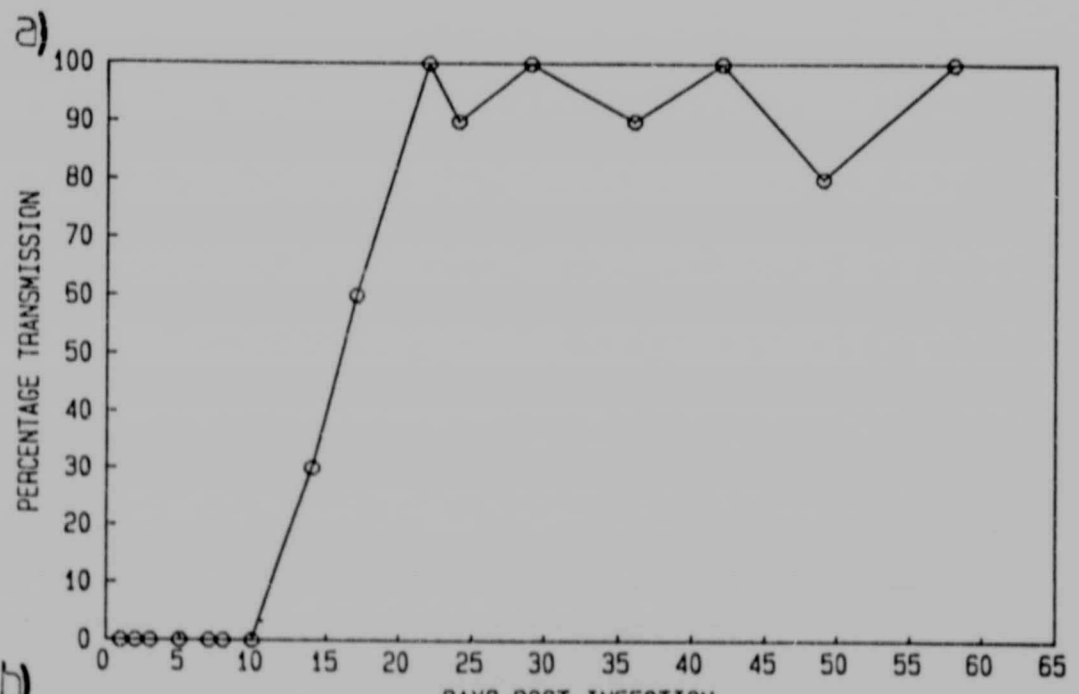

TITRE OF INFECTED MOSQUITO /1ML
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
0
5
10
15
20
25
30
35
40
DAYS POST INFECTION

Fig.11. Cx.univittatus held at 18±1°C and 60-75% RH:

a)Percentage of mosquitoes transmitting WN virus
b)Average titre of WN virus transmitted ($Log_{10}CPD_{50}$/ml of capillary fluid).
c)Titres of WN virus in Cx.univittatus ($Log_{10}CPD_{50}$/ml of mosquito suspension).

Titre of viraemia of chicks used for infective meal= 5.3-5.8$Log_{10}CPD_{50}$/ml.

b)


PERCENTAGE TRANSMISSION
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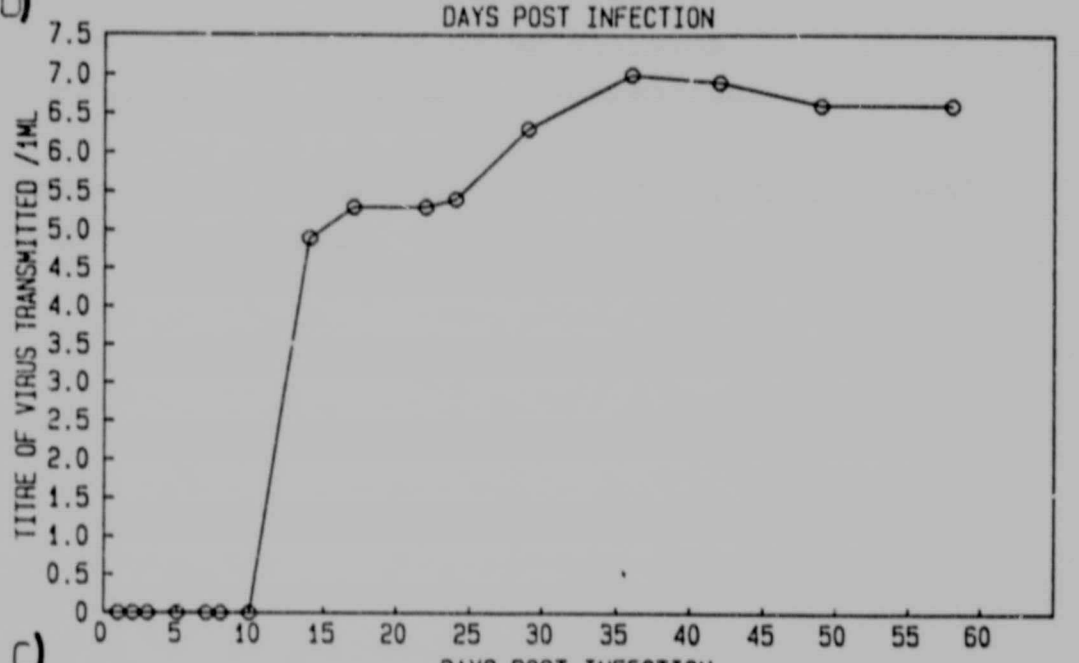
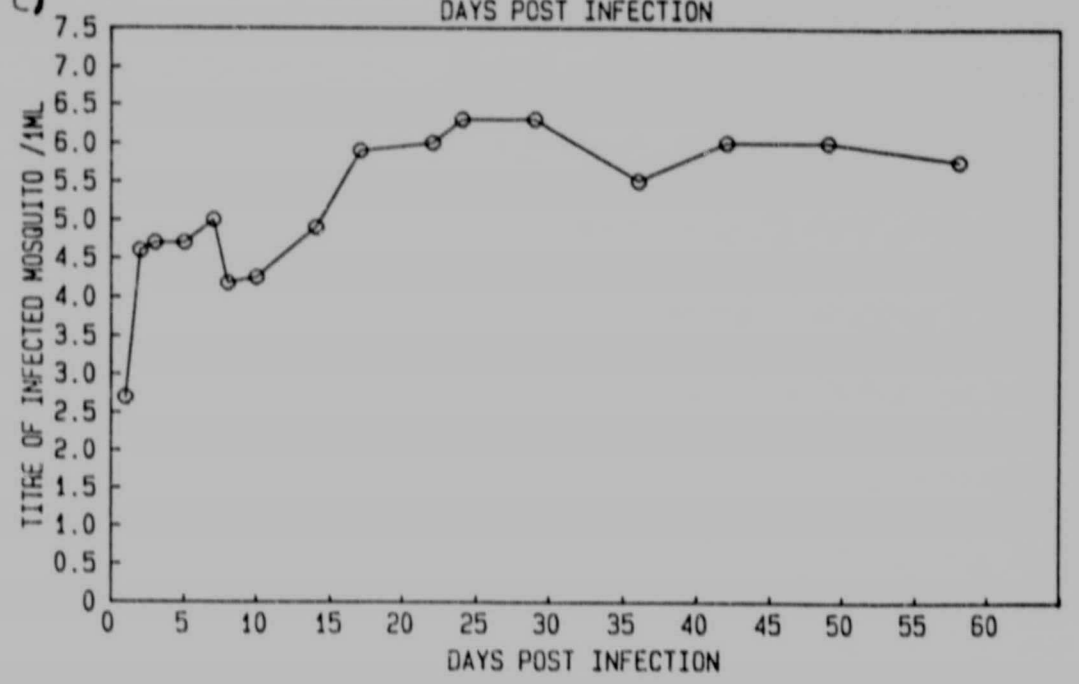
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30
35
40
45
50
55
60
65
DAYS POST INFECTION

c)


TITRE OF VIRUS TRANSMITTED /1ML
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
0
5
10
15
20
25
30
35
40
45
50
55
60
DAYS POST INFECTION

d)


TITRE OF INFECTED MOSQUITO /1ML
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0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
0
5
10
15
20
25
30
35
40
45
50
55
60
DAYS POST INFECTION

Author Cornel A J
Name of thesis The Effect of temperature on the Vector Competence of Culex Univittatus Theobald (Diptera: Culicidae) for West Nile and Sindbis Virusses 1987

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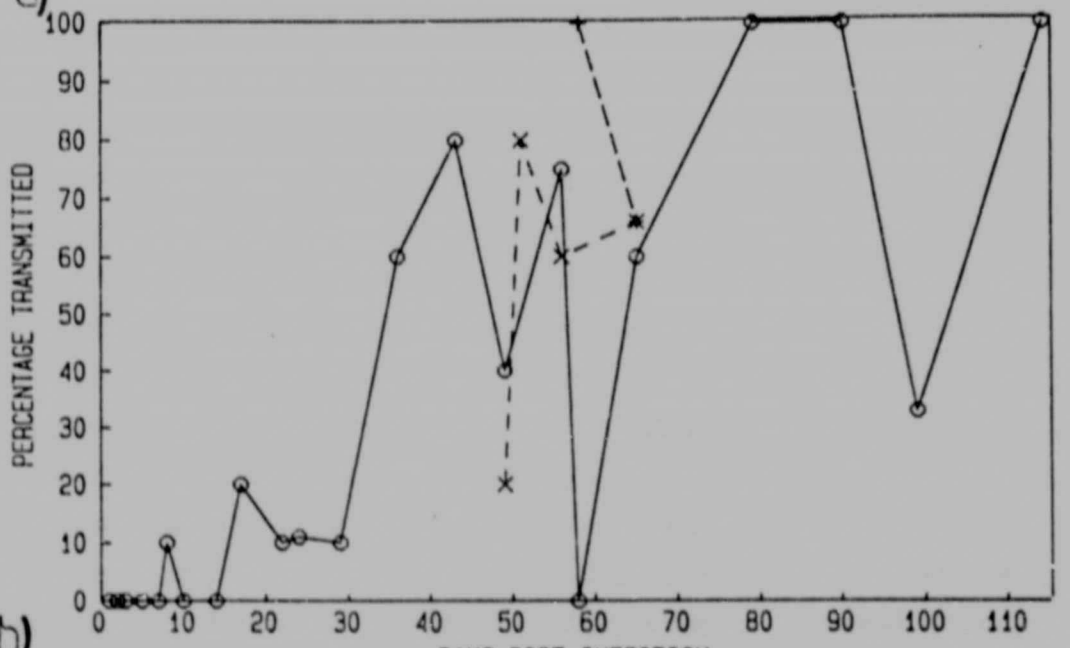
Fig.12. Cx.univittatus held at 14:0.5°C and 50-75%RH:

a)Percentage of mosquitoes transmitting WN virus.
b)Average titre of WN virus transmitted ($Log_{10}CPD_{50}$/ml of capillary fluid).
c)Titre of WN virus in Cx.univittatus ($Log_{10}CPD_{50}$/ml of mosquito suspension).

Titre of viraemia of chicks used for infective meal= 5.3-5.8$Log_{10}CPD_{50}$/ml.

a)


PERCENTAGE TRANSMITTED
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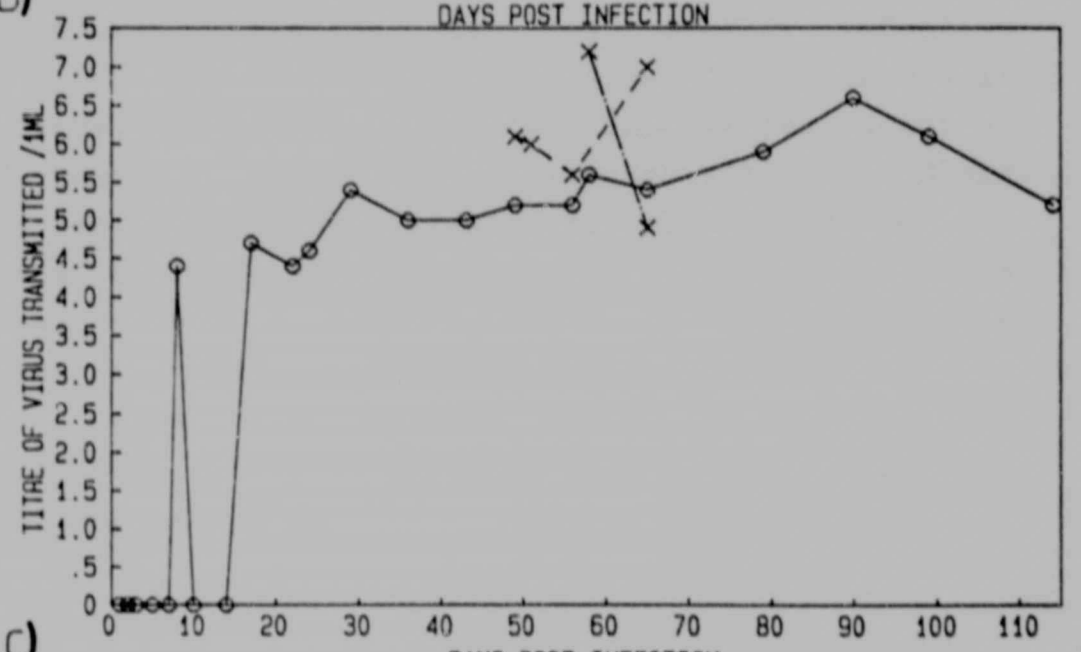
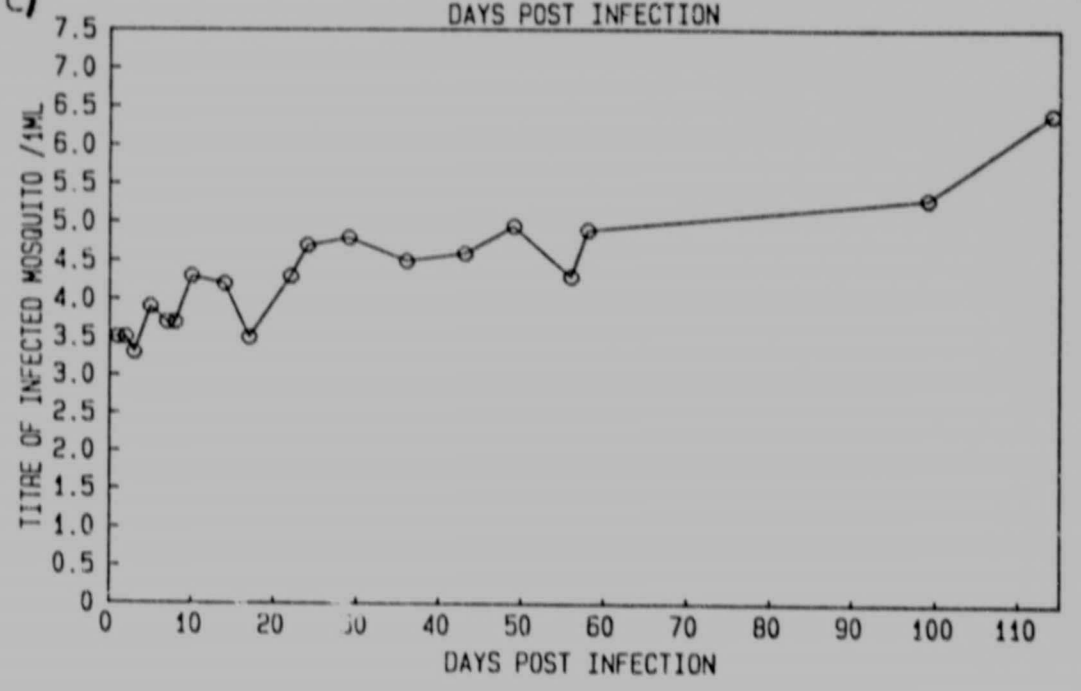
10
20
30
40
50
60
70
80
90
100
0
10
20
30
40
50
60
70
80
90
100
110
DAYS POST INFECTION

b)


TITRE OF VIRUS TRANSMITTED /1ML
0
.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
0
10
20
30
40
50
60
70
80
90
100
110
DAYS POST INFECTION

c)


TITRE OF INFECTED MOSQUITO /1ML
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
0
10
20
30
40
50
60
70
80
90
100
110
DAYS POST INFECTION

infective feed before maximum transmission rates were reached also followed a similar pattern in response to incubation temperature,and at the intermediate temperatures of 18°C and 26°C and fluctuating outside temperatures maximum transmission rates were reached after 6-7 days.(Fig.16a,14a and 15a). This period was considerably longer at the two extreme temperatures where it was doubled to 12 days at 14°C(Fig.17a) and quadrupled to 27 days at 30°C (Fig.13a).

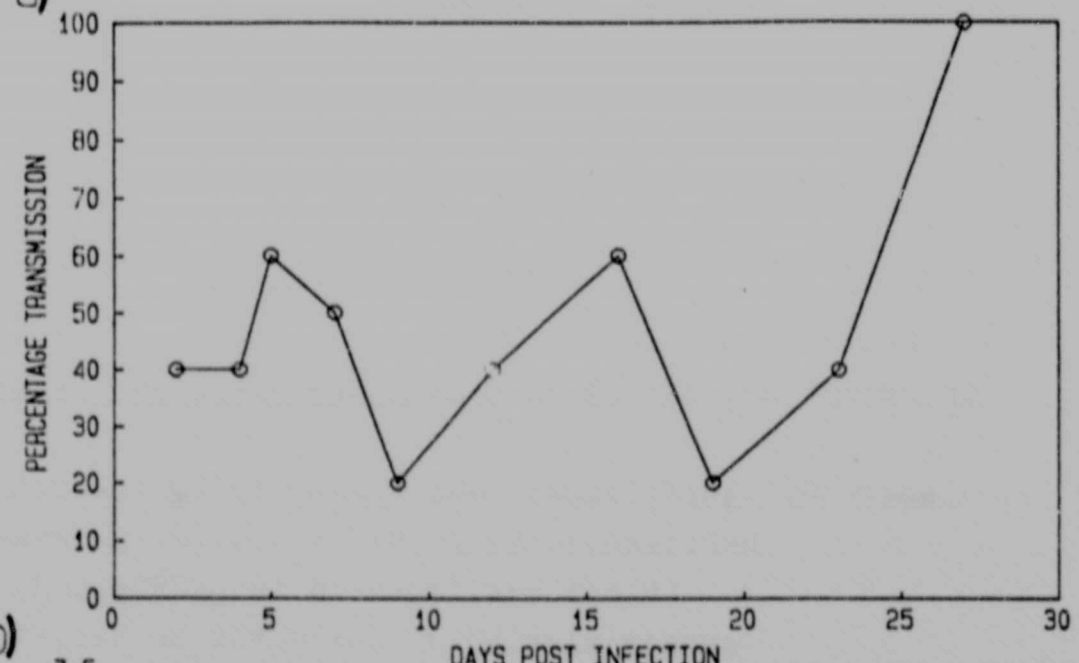
The average titre of virus in mosquitoes did not fluctuate nearly as much as the transmission rate and the titre of virus transmitted,and showed very little variation between the different incubation temperatures (Fig.13c to 17c). Average infective titres of SIN virus reached maximal levels of 6 logs and greater at most incubation temperatures except at 30°C where a lower maximum average titre of 5.6logs was reached. Interestingly,the period needed for maximum average infective titres of mosquitoes to be reached showed a similar trend to that of maximum titres of virus transmitted,where maximum infective titres were also reached sooner at intermediate temperatures,ie 10-12 days,and took 12-13 days at 14°C and 20 days at 30°C.

Fig.13.Cx.univittatus held at 30:1°C and 60-70% RH.

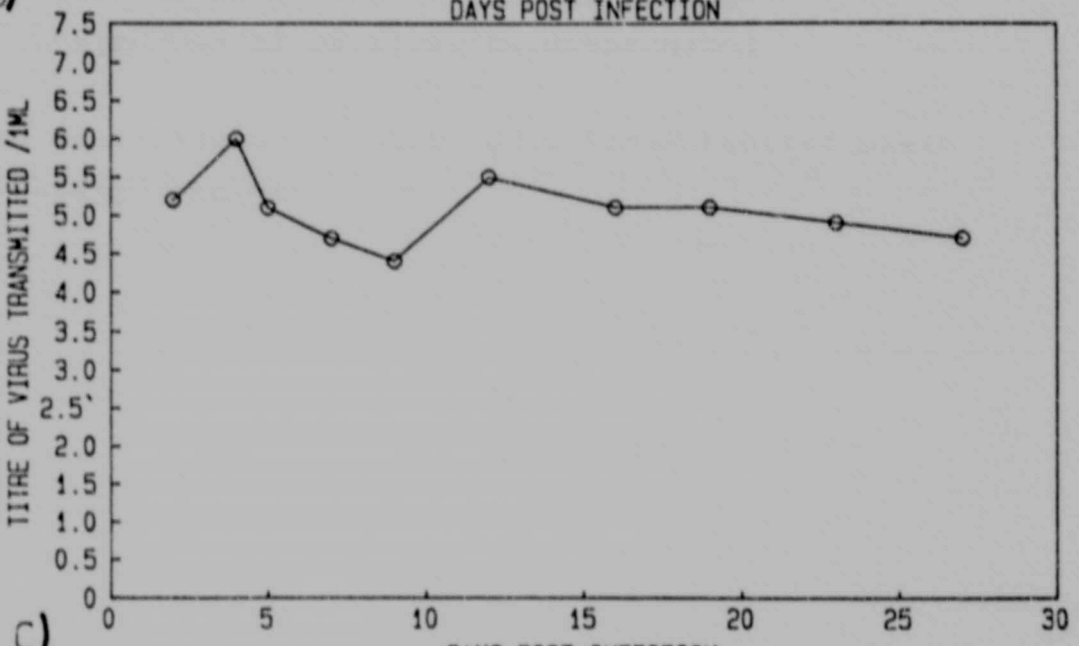
a)Percentage of mosquitoes transmitting SIN virus.
b)Average titre of SIN virus transmitted.
($Log_{10}CPD_{50}$/ml of capillary fluid).
c)Titres of SIN virus in Cx.univittatus
($Log_{10}CPD_{50}$/ml of mosquito suspension).

Titre of viraemia of chicks used for infective meal= 6-7.5 $Log_{10}CPD_{50}$/ml.

a)


PERCENTAGE TRANSMISSION
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
DAYS POST INFECTION

b)


TITRE OF VIRUS TRANSMITTED /1ML
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
5
10
15
20
25
30
DAYS POST INFECTION

c)

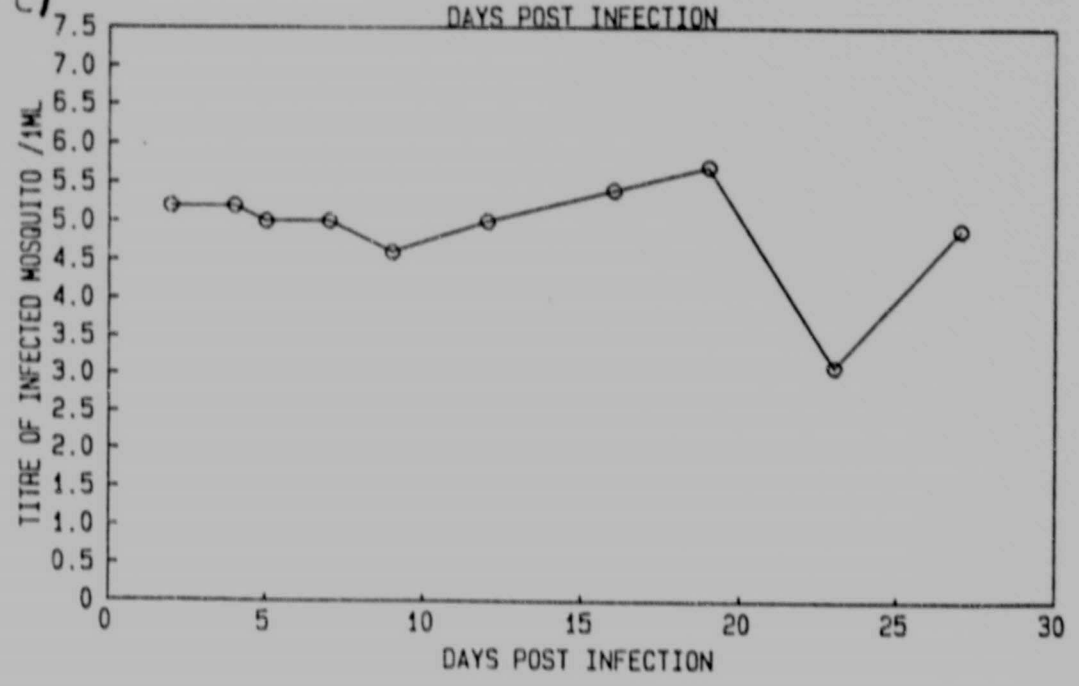
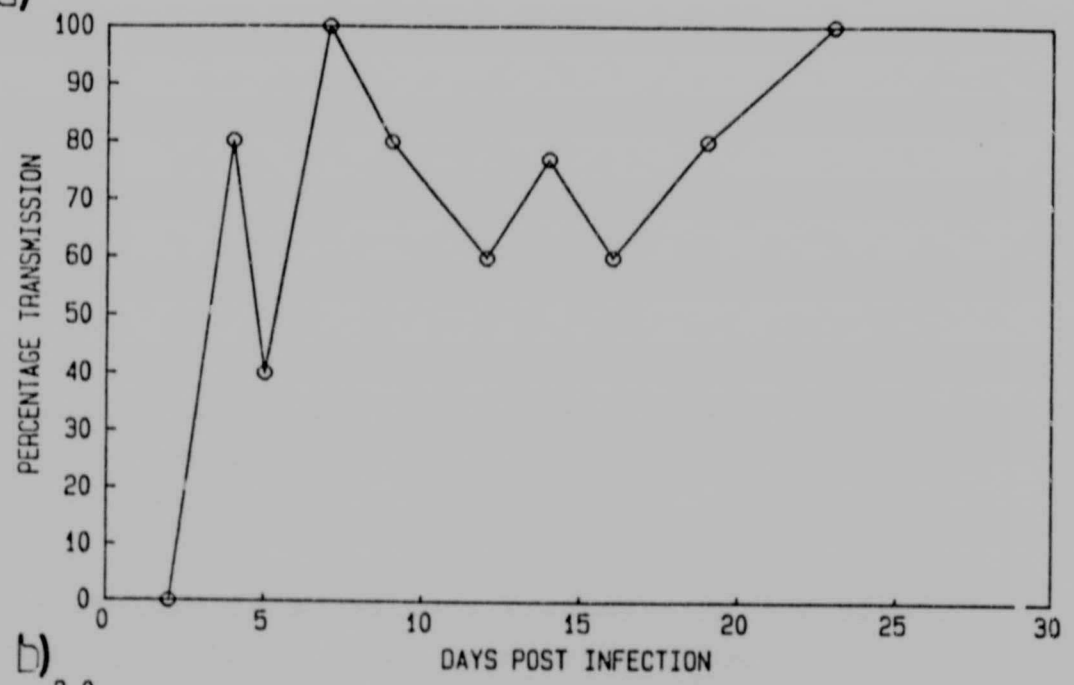

TITRE OF INFECTED MOSQUITO /1ML
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
5
10
15
20
25
30
DAYS POST INFECTION

Fig.14. Cx.univittatus held at 26:1°C and 75-80% RH.

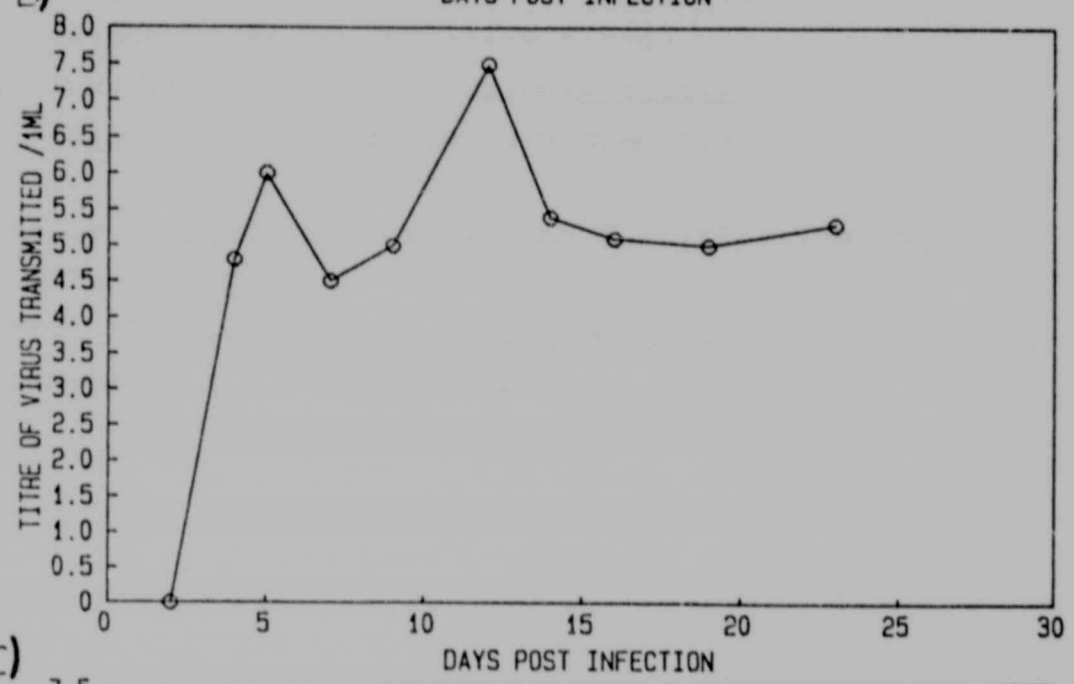
a)Percentage of mosquitoes transmitting SIN virus
b)Average titre of SIN virus transmitted.
($Log_{10}CPD_{50}$/ml of capillary fluid).
c)Titres of SIN virus in Cx.univittatus
($Log_{10}CPD_{50}$/ml of mosquito suspension).

Titre of viraemia of chicks used for infective meal= 6-7.5$Log_{10}CPD_{50}$/ml.

a)


PERCENTAGE TRANSMISSION
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
DAYS POST INFECTION

b)


TITRE OF VIRUS TRANSMITTED /1ML
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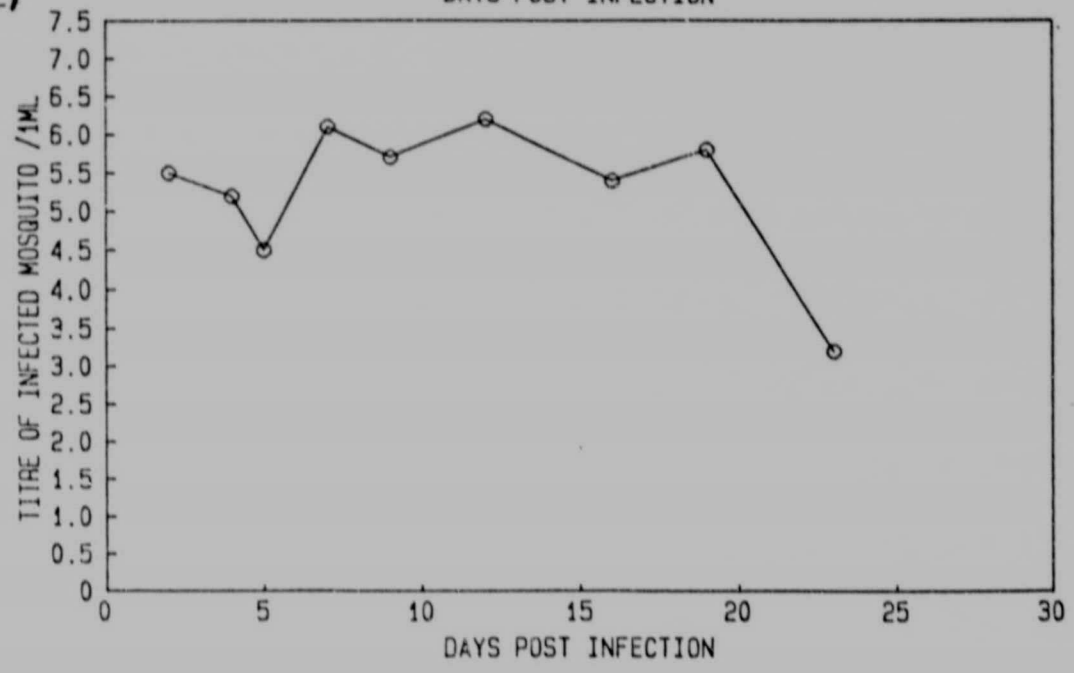
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
5
10
15
20
25
30
DAYS POST INFECTION

c)


TITRE OF INFECTED MOSQUITO /1ML
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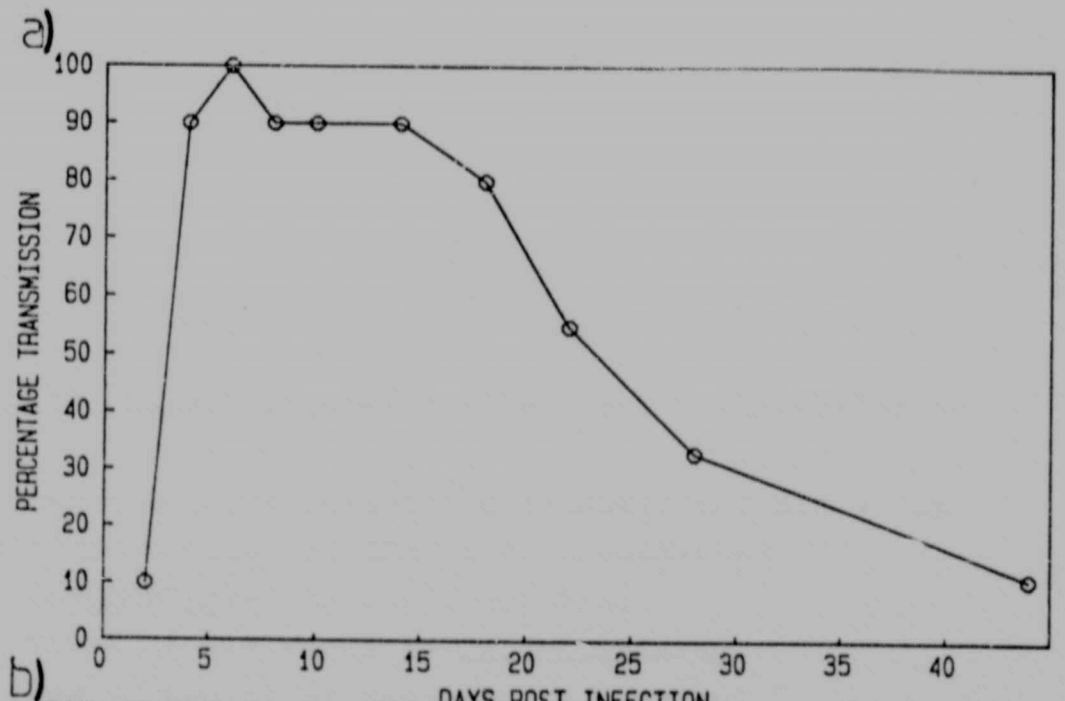
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
5
10
15
20
25
30
DAYS POST INFECTION

Fig.15. Cx.univittatus incubated at outside fluctuating summer temperature and 30-80% RH.

a)Percentage of mosquitoes transmitting SIN virus.
b)Average titre of SIN virus transmitted ($Log_{10}CPD_{50}$/ml of capillary fluid).
c)Titres of SIN virus in Cx.univittatus ($Log_{10}CPD_{50}$/ml of mosquito suspension).

Titre of vireamia of chicks used for infective meal= 7.8-8.7$Log_{10}CPD_{50}$/ml.
Records of daily maximum summer ambient temperatures are given in Fig.7.

a)


PERCENTAGE TRANSMISSION
0
10
20
30
40
50
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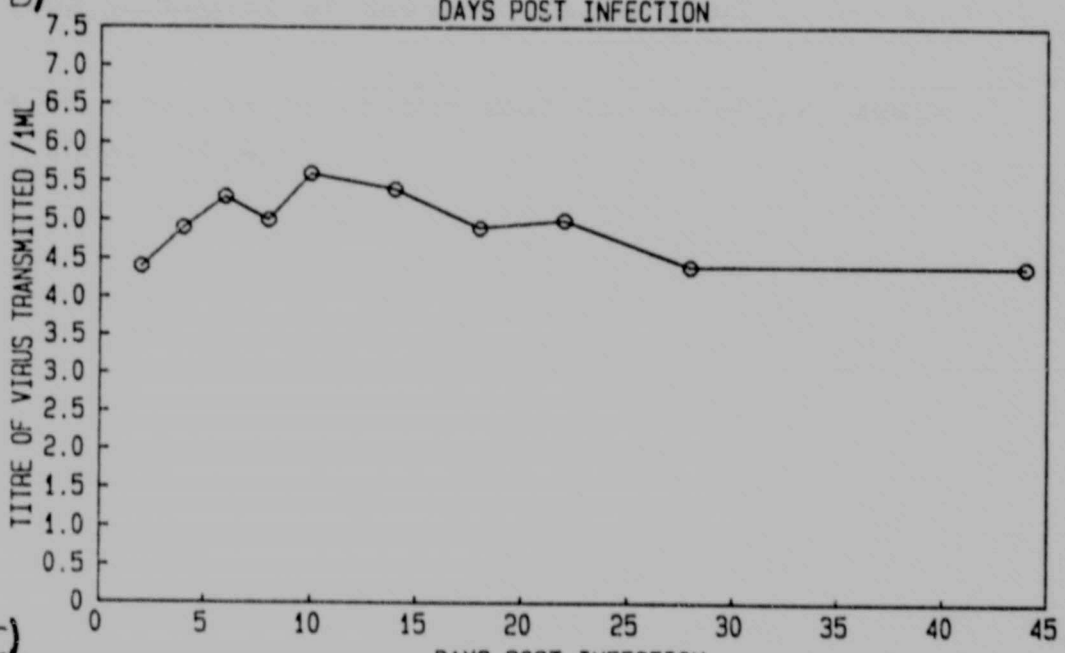
70
80
90
100
0
5
10
15
20
25
30
35
40
DAYS POST INFECTION

b)


TITRE OF VIRUS TRANSMITTED /1ML
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0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
0
5
10
15
20
25
30
35
40
45
DAYS POST INFECTION

c)

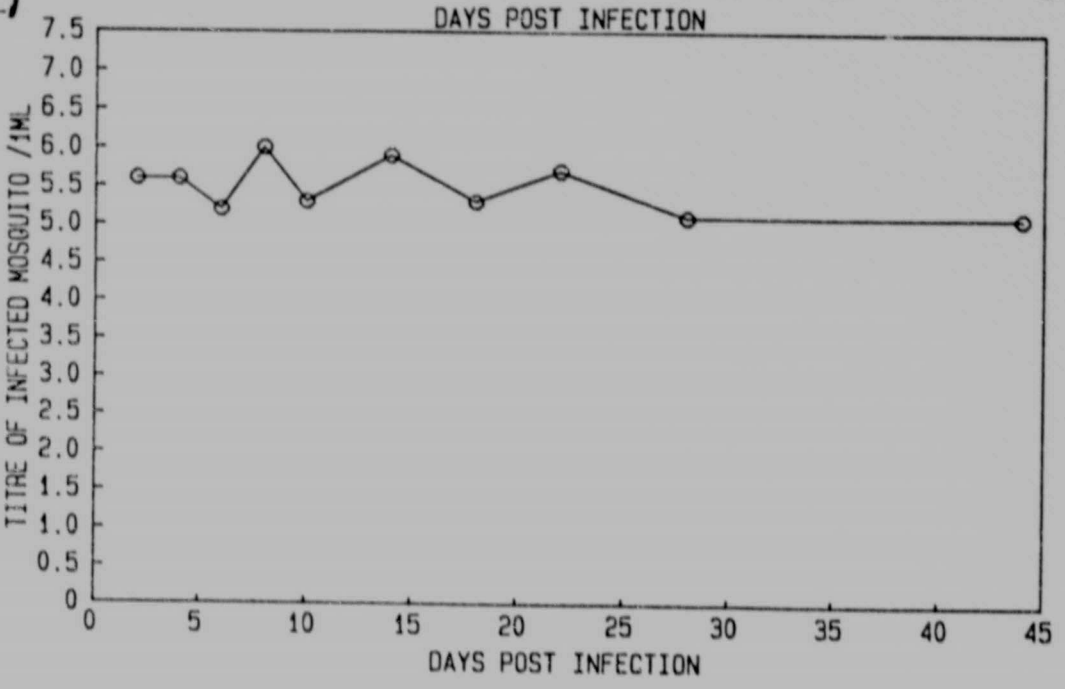
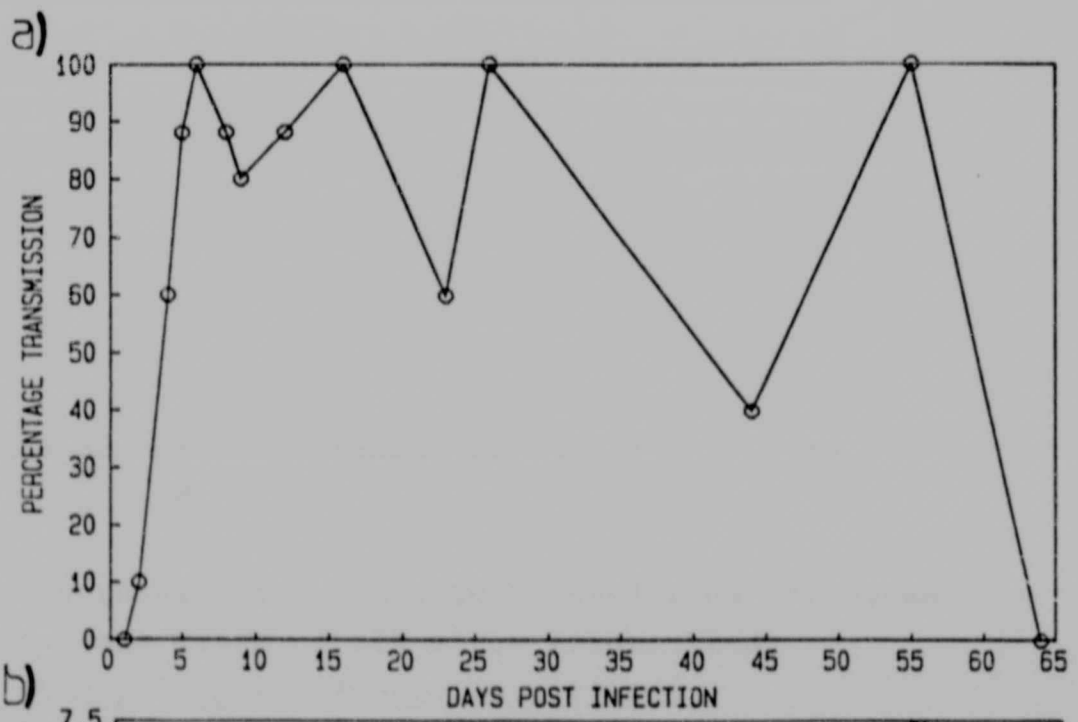

TITRE OF INFECTED MOSQUITO /1ML
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
0
5
10
15
20
25
30
35
40
45
DAYS POST INFECTION

Fig.16. Cx.univittatus held at 18±1°C and 60-75% RH.

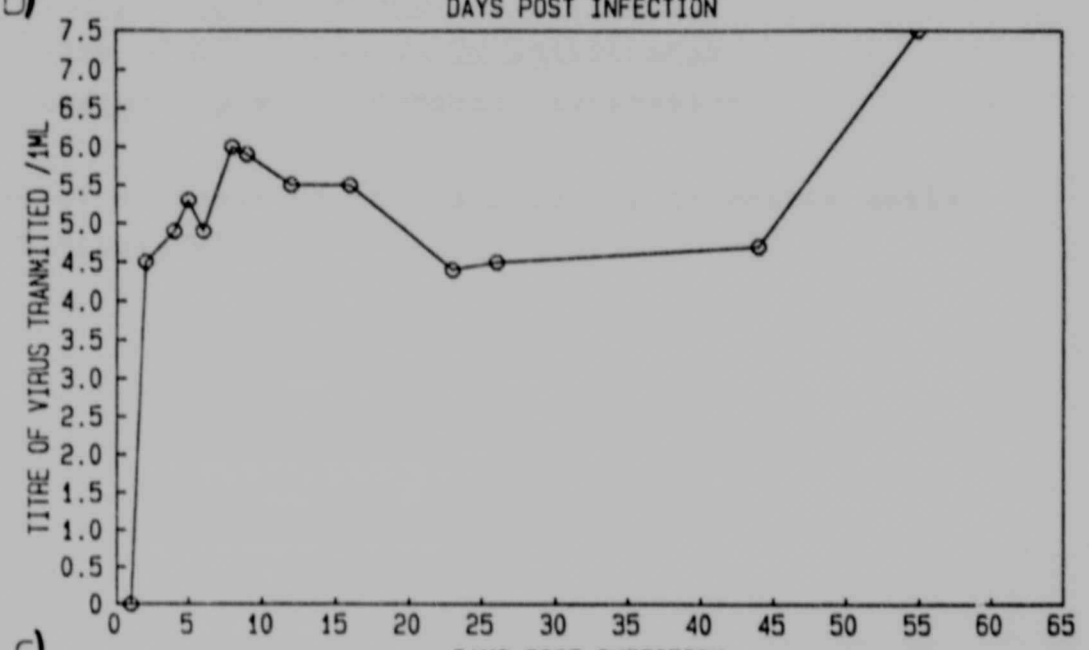
a)Percentage of mosquitoes transmitting SIN virus.
b)Average titre of SIN virus transmitted
($Log_{10}CPD_{50}$/ml of capillary fluid).
c)Titre of SIN virus in Cx.univittatus
($Log_{10}CPD_{50}$/ml of mosquito suspension).

Titre of viraemia of chicks used for infective meal=
7.3-7.5$Log_{10}CPD_{50}$/ml.

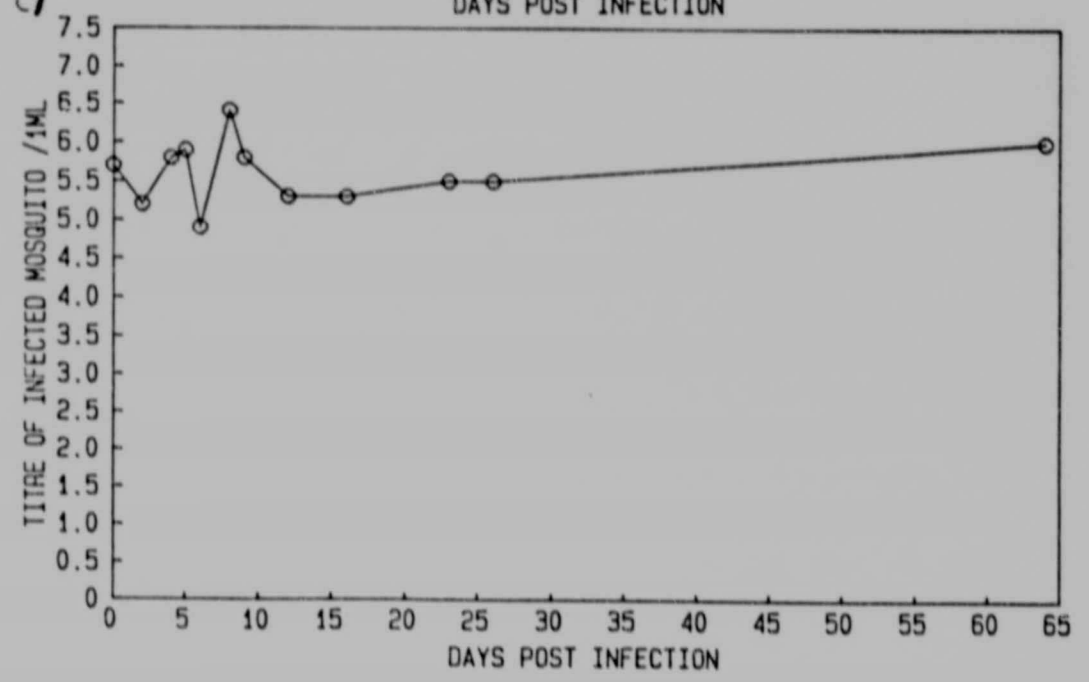
a)


PERCENTAGE TRANSMISSION
100
90
80
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65
DAYS POST INFECTION

b)


TITRE OF VIRUS TRANMITTED /1ML
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65
DAYS POST INFECTION

c)


TITRE OF INFECTED MOSQUITO /1ML
7.5
7.0
6.5
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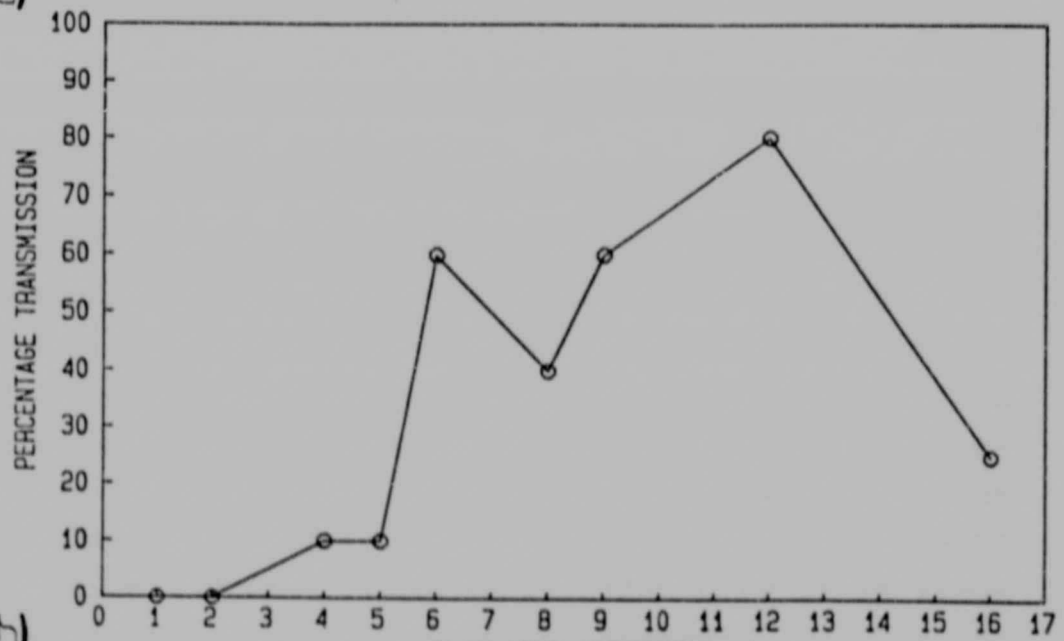
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65
DAYS POST INFECTION

Fig.17. Cx.univittatus held at 14±0.5°C and 50-75% RH.

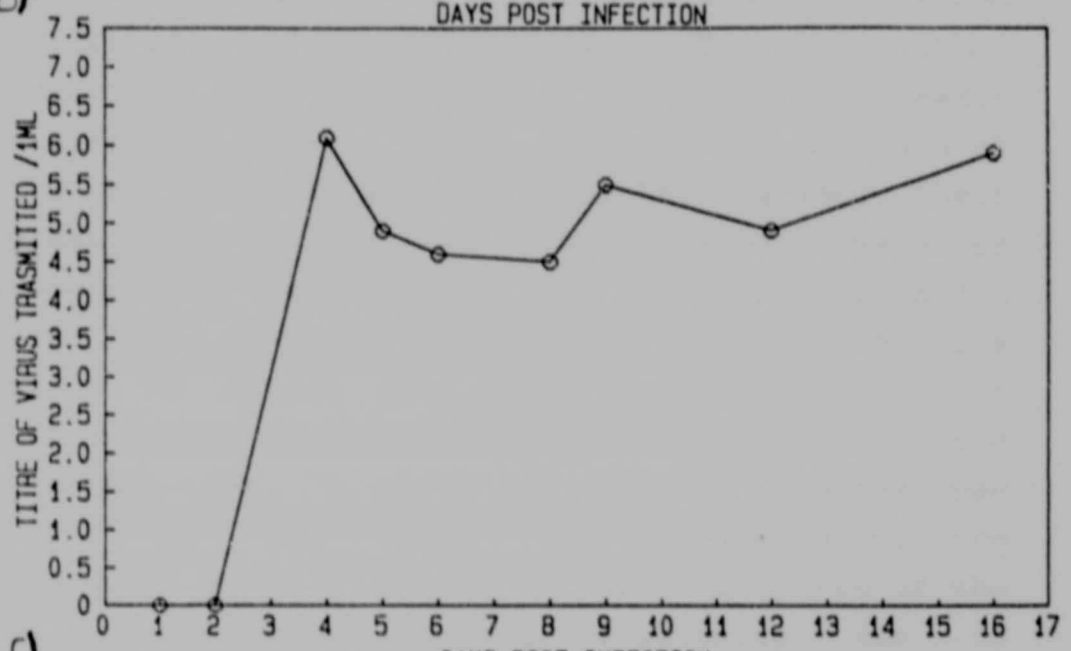
a)Percentage of mosquitoes transmitting SIN virus.
b)Average titre of SIN virus transmitted ($Log_{10}CPD_{50}$/ml of capillary fluid).
c)Titre of SIN virus in Cx.univittatus ($Log_{10}CPD_{50}$/ml of mosquito suspension).

Titre of viraemia of chicks used for infective meal= 7.3-7.5$Log_{10}CPD_{50}$/ml.

a)


PERCENTAGE TRANSMISSION
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
DAYS POST INFECTION

b)


TITRE OF VIRUS TRASMITTED /1ML
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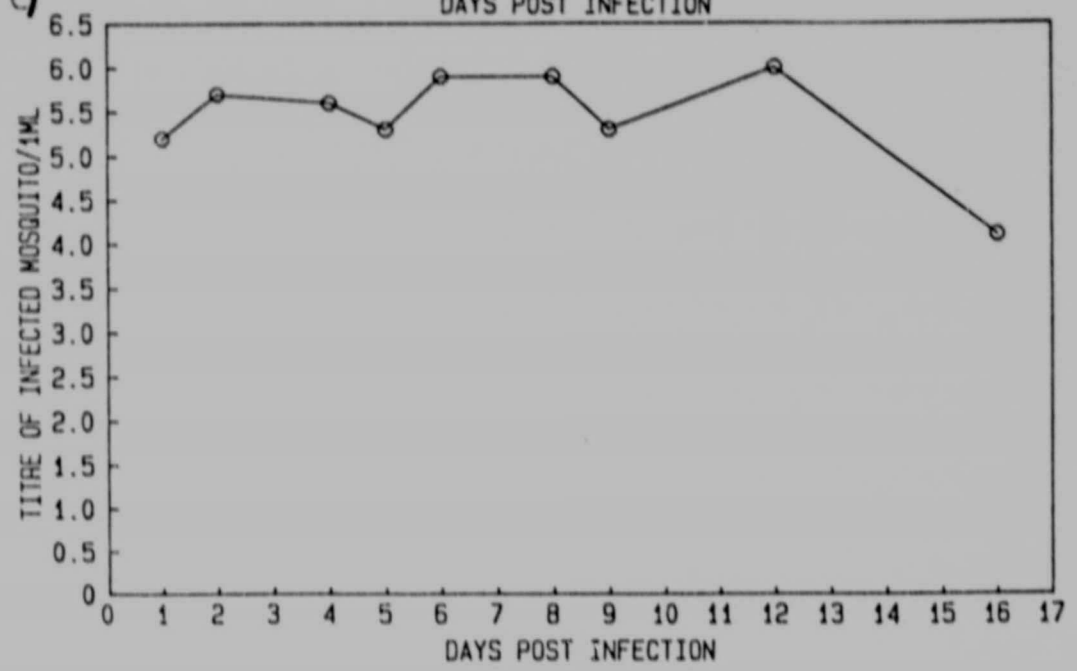
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
DAYS POST INFECTION

c)


TITRE OF INFECTED MOSQUITO/1ML
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6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
DAYS POST INFECTION

3.4.Histological examination of infected salivary glands of Cx.univittatus.

Both uninfected and WN virus infected salivary glands of Cx.univittatus appeared similar(Fig.18a and b) in that,the cytoplasm of the cells mainly took up E stain which is in agreement with observations made by Mims et al(1966),i.e."normal glands of Ae.aegypti had large eosinophilic expanses of secretary material extending from the flattened nuclei at the edge of the lobe to the central duct." Lack of pathogenicity of WN virus for salivary glands was also observed by Lam and Marshall(1968) in Ae.aegypti. On the other hand,salivary glands infected with SIN virus appeared very different.Their nuclei were larger and took up more H stain thus appearing darker(Fig.18c). Their cytoplasm differed in that there seemed to be a lot less secretory material as the E stain appeared a lot lighter and more patchy (Fig.18c). It was more difficult to obtain good sections of salivary glands from mosquitoes infected with SIN virus. This may have been either due to parts of the gland having been destroyed by SIN virus (as suggested by the fluorescence study Fig.25b) or because parts of the glands disintegrated during sectioning due to alteration of the cells by the virus.

Fig.18.

Section through salivary gland lobes of female Cx.univittatus,400x:

a)uninfected

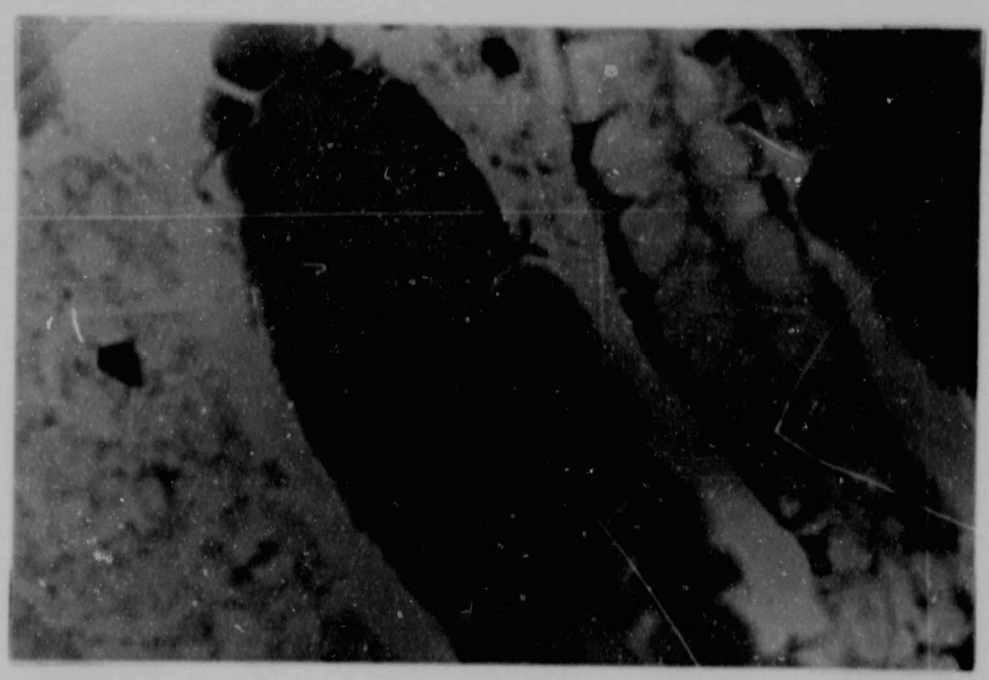
b)infected with WN virus.

c)infected with SIN virus.

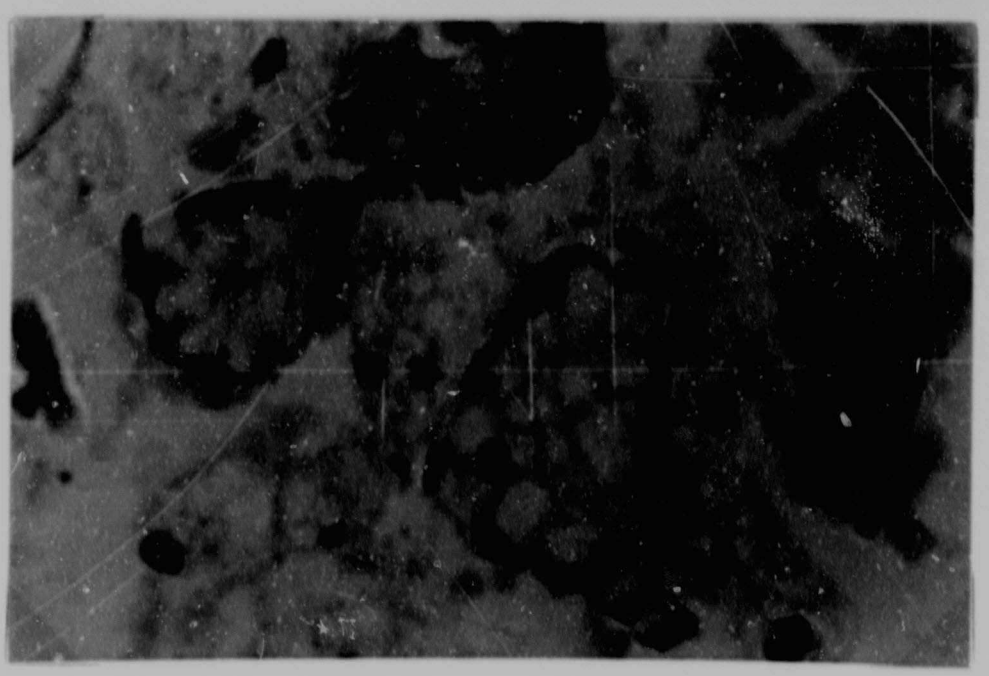
a)



b)



c)



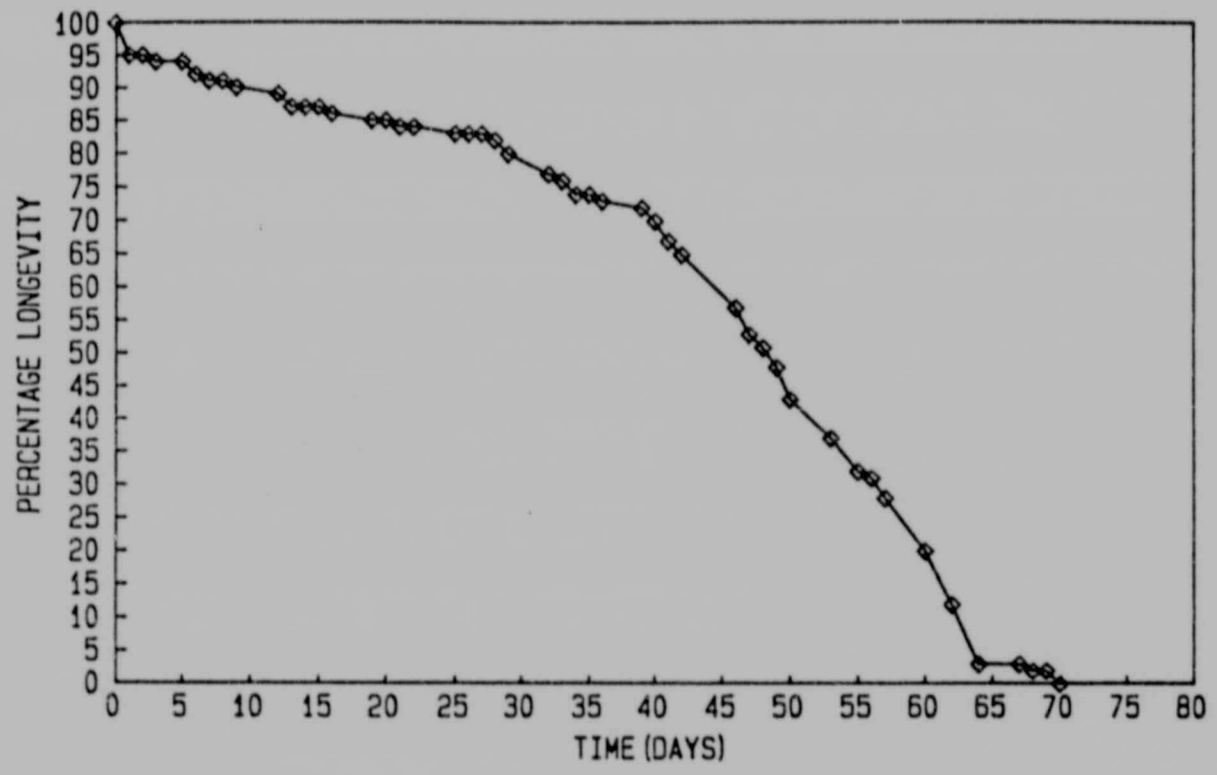
3.5.Longevity studies.

Average daily maximum and minimum temperatures in the highveld region of South Africa range between 18°C and 30°C,therefore constant temperatures of 18°C,26°C and 30°C were selected for the longevity studies. Longevity studies were also conducted at fluctuating summer temperatures outside the laboratory in the walk-in cage as a control and the daily maximum and minimum temperatures experienced during this period are represented graphically in Fig.19. Daily maximum temperatures in the mosquito cages within the walk-in cage were however,slightly higher than actual daily ambient maximum temperatures experienced at that time of the year. To facilitate a quantitative comparison between the longevity of uninfected female Cx.univittatus held at different constant temperatures and between uninfected mosquitoes and WN-infected and SIN-infected mosquitoes at 26°C,mean life expectancies were calculated. A summary of the mean,median and standard deviations for these longevity studies are represented in tablular form in appendix C for the uninfected mosquitoes and appendix D for infected and uninfected mosquitoes at 26°C. The SNK test was then used to test for significance of differences or similarities between these mean life expectancies. Mean life expectancies for uninfected mosquitoes were highest at fluctuating summer temperatures(51 days),intermediate at 26°C(40 days) and lowest at the extreme temperatures;19 days at 18°C and 14 days at 30°C. If the maximum lifespan of uninfected Cx.univittatus is compared at the different temperatures,a similar trend occurs,where the mosquitoes lived longest at 26°C and fluctuating outside temperatures(70-78 days),(Fig.19 and 20b),and shortest at the two extreme temperatures of 18 and 30°C(42 days),(Fig.20a and b).

Both WN virus-infected and SIN virus-infected mosquitoes had similar mean life expectancies(similar at the 5% SNK significance level)i.e.28 days with WN virus and 27 days with SIN virus. These life expectancies are much lower than the 40 days for uninfected mosquitoes at 26°C at the 5% SNK significance level (Fig 21). Furthermore, from the vector competence studies at 14,5°C,a striking difference occurs. Those infected with WN virus lived for as long as 120 days (Fig.12),whereas those infected with SIN virus only lived to 14 days(Fig.17). At these lower temperatures the mosquitoes seem to have a much slower metabolism and the digestion of blood and production of eggs took up to 3 weeks at 14°C. This slowing down of metabolism coupled with a SIN virus infection seemed to have a marked detrimental affect on the survival of Cx.univittatus,which was not apparent with the mosquitoes infected with WN virus. It should be stressed that at 14°5.C Cx.univittatus still produced eggs,thus gonotrophic dissociation had not occurred and the mosquitoes were therefore not in state of diapause.

Fig.19.

Percentage longevity of uninfected Cx.univittatus from 0-70 days subjected to fluctuating summer temperature shown in relation to daily maximum and minimum ambient temperatures in °C.


PERCENTAGE LONGEVITY
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
TIME (DAYS)

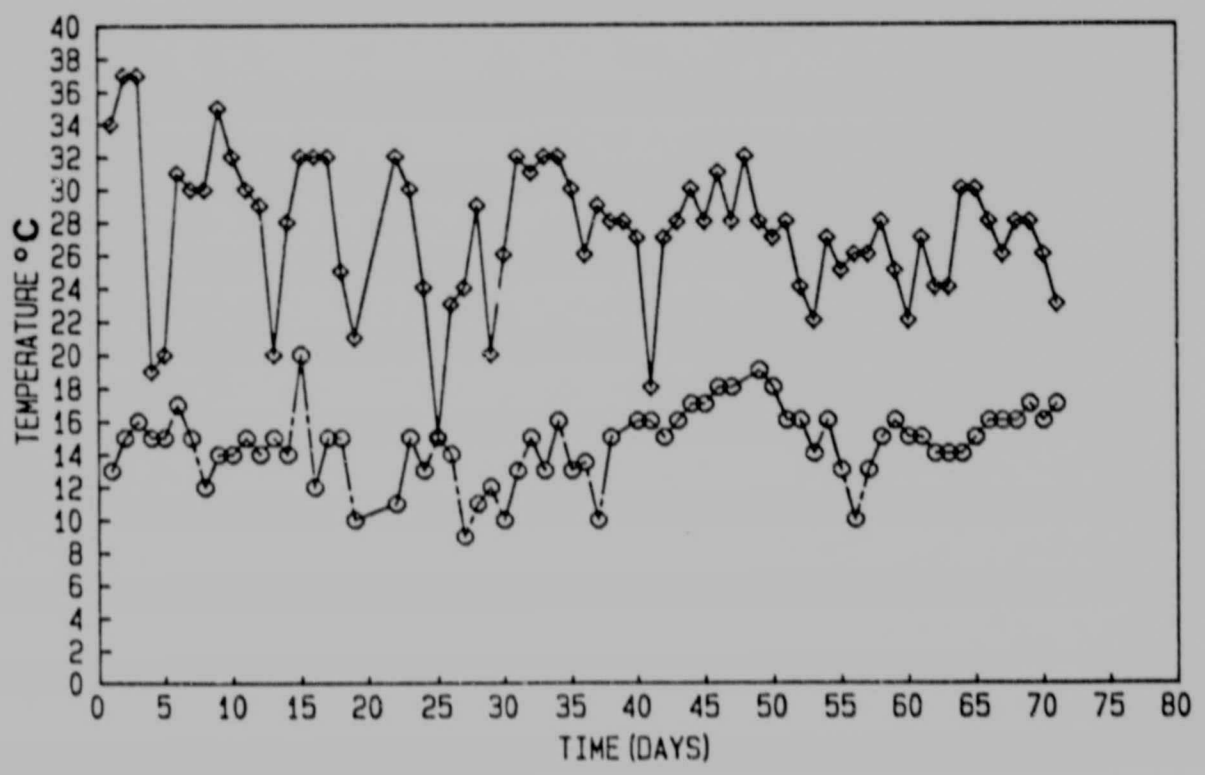

TEMPERATURE °C
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
TIME (DAYS)

Fig.20.

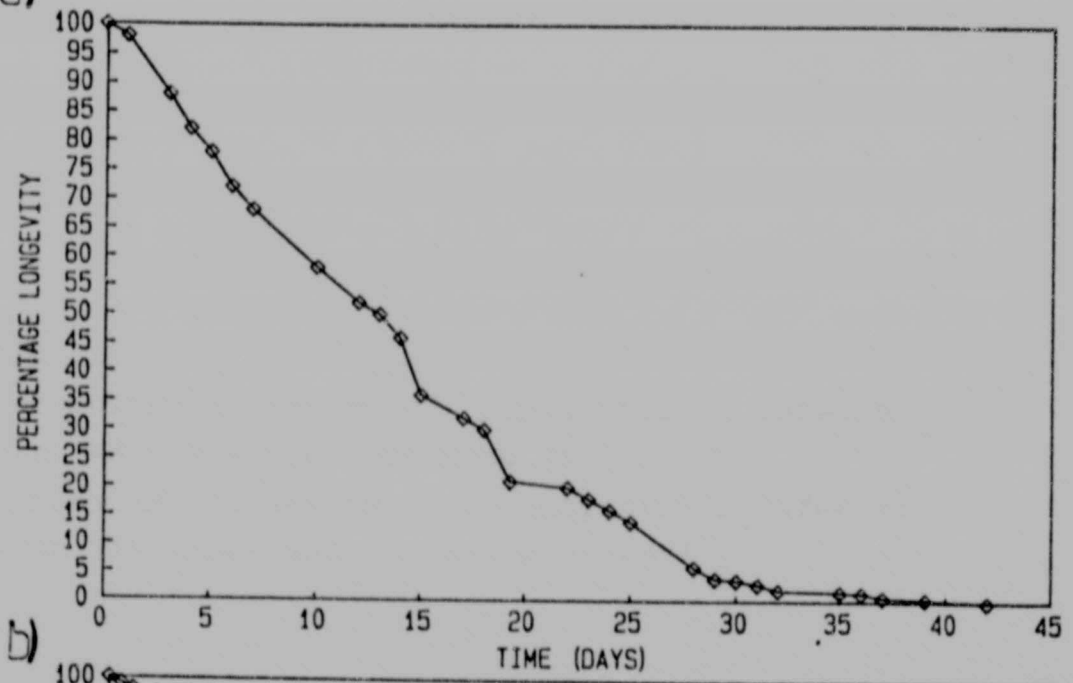
Percentage longevity of uninfected Cx.univittatus held at:

a)constant 30±1°C.

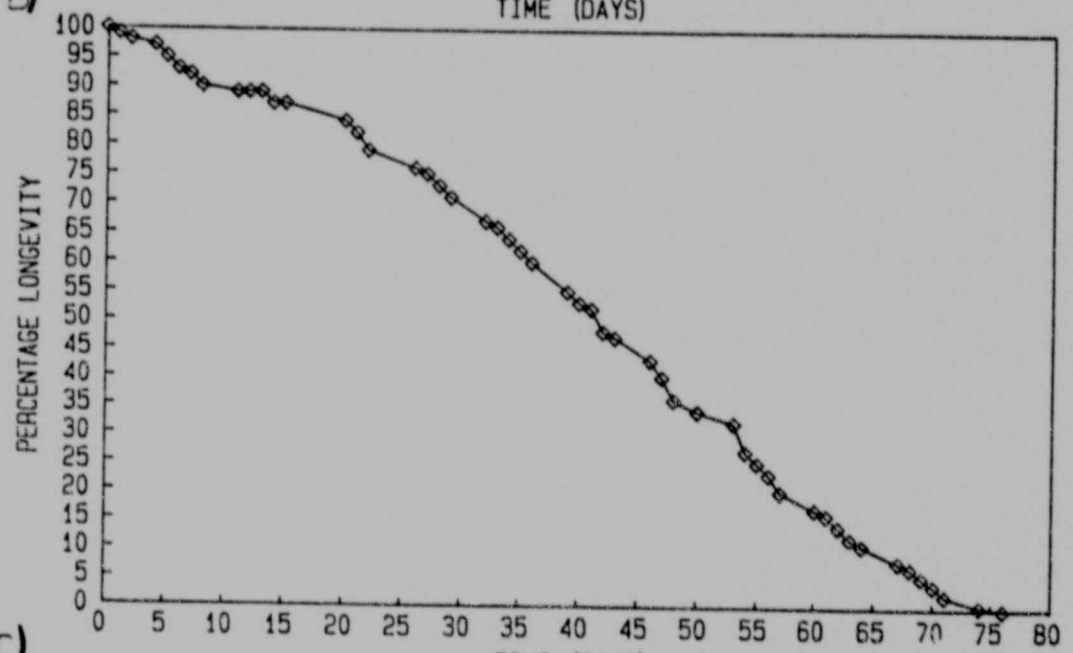
b)constant 26±1°C.

c)constant 18±1°C.

a)


PERCENTAGE LONGEVITY
100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5 0
0 5 10 15 20 25 30 35 40 45
TIME (DAYS)

b)


PERCENTAGE LONGEVITY
100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5 0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
TIME (DAYS)

c)

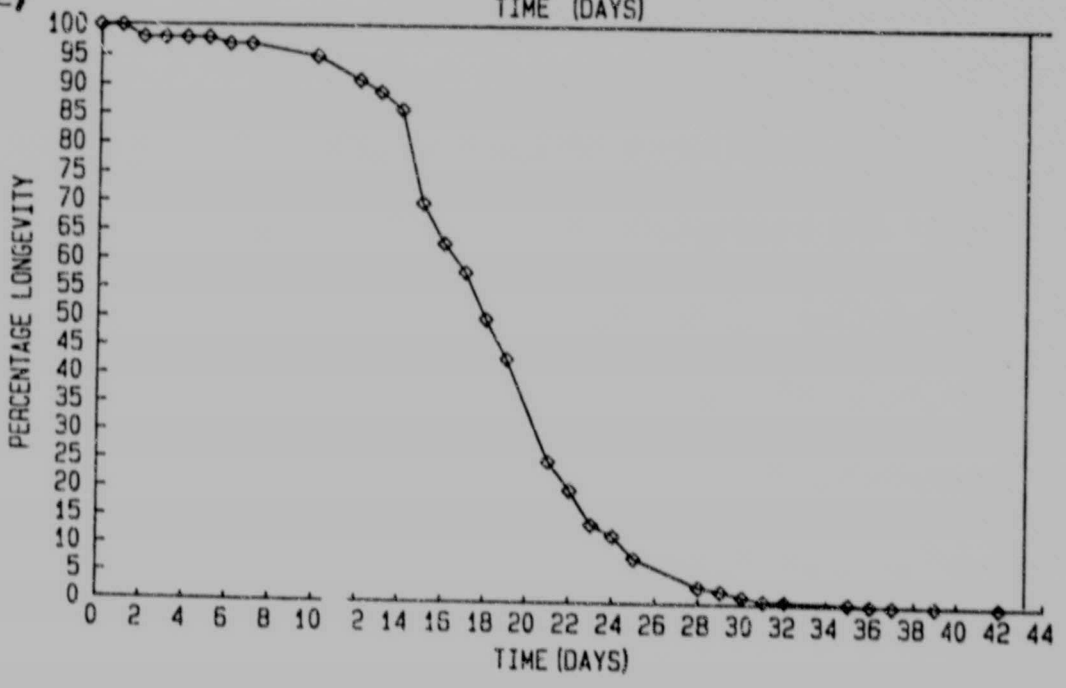
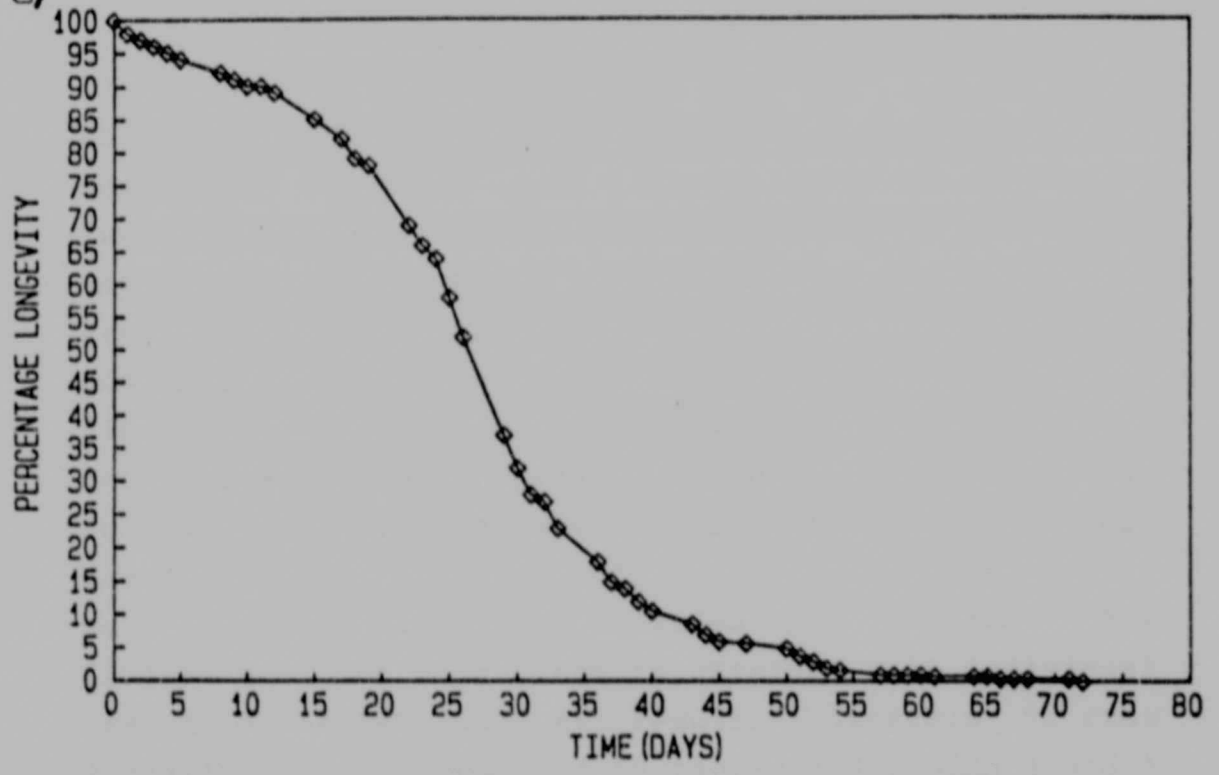

PERCENTAGE LONGEVITY
100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5 0
0 2 4 6 8 10 2 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
TIME (DAYS)

Fig.21.

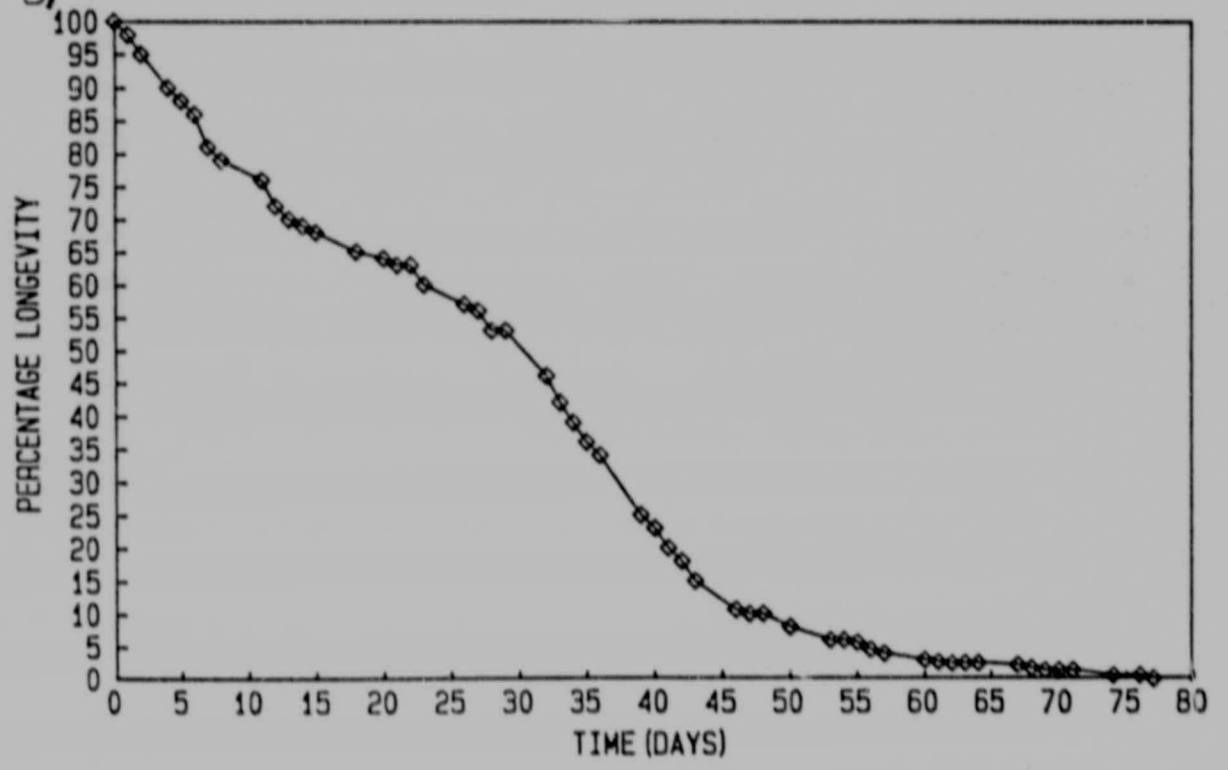
a)Percentage longevity of Cx.univittatus infected with WN virus and incubated at 26±1°C.

b)Percentage longevity of Cx.univittatus infected with SIN virus and incubated at 26±1°C.

a)


PERCENTAGE LONGEVITY
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
TIME (DAYS)

b)


PERCENTAGE LONGEVITY
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
TIME (DAYS)

3.6. Replication and sequential distribution of virus in the organs of Cx.univittatus.

When examining the different organs of infected mosquitoes which had been stained by the immunofluorescent technique to detect virus antigen,care was taken to distinguish characteristic green,granular fluorescence of the antigen from non-specific,yellowish fluorescence. Unfortunately the fluorescence shown in the photographs (Fig.22-26) does not appear in the original green colour since they had to be slightly overexposed to bring out the dark background. Photographs of uninfected organs showing no fluorescence are included for contrast. The fluorescent antibody results obtained with individual organs dissected out at regular intervals PI from mosquitoes are presented in tables 4-6.Tables 4 and 5 show the results for organs of mosquitoes infected with WN virus and held at 14°C and 18°C respectively and Table 6 shows similar results for organs of mosquitoes infected with SIN virus and held at 14°C and 18°C.

In the case of both WN and SIN virus-infected mosquitoes held at 14°C,fluorescence sometimes first occurred in small areas of the posterior midgut on day 1 after the infective feed. In mosquitoes held at 18°C,however,fluorescence only appeared on days 2-4. WN and SIN virus replication then became more extensive,spreading to most regions of the midgut by 2-4 days at both temperatures (Fig.22). SIN virus spread from the midgut to other regions of the body quicker than WN virus at both temperatures and by day 5 at 18°C fluorescence was observed in the ventral diverticulum and salivary glands(Fig.26 and 23),which correlated with transmission.

At 14°C,SIN virus took only slightly longer and by day 6 had reached the ventral diverticulum and salivary gland. Similarly,at 14°C first transmission also occurred when the salivary gland showed presence of

antigen. At this temperature SIN virus had also spread to the foregut by day 6 and only after 8 days at 18°C (Fig.24). SIN virus also appeared to replicate in more organs in the mosquito than WN virus since fluorescence also occurred in abdominal ganglia(Fig.25) as well as areas anterior to the dorsal diverticula in the digestive tract which is referred to as the oesophagus in the tables.

WN virus followed a similar pattern to SIN virus in that it took longer to disseminate from the midgut and infect other organs at the lower temperature of 14°C. In fact,the presence of WN antigen appear to lag by 10 days in the organs at 14°C when compared to the initial presence of antigen in organs of mosquitoes at 18°C,i.e.foregut became infected on day 7 at 18°C but day 17 at 14°C,and salivary glands on day 14 at 18°C but day 24 at 14°C. The only exception to this 10 day lag period at 14°C was the initial infection in the posterior portion of the midgut on day 3. This however,is understandable as all mosquitoes were infected at 26°C on viraemic chicks to obtain high feeding rates,and only 12 hours later were separated out into batches to be held at 14°C and 18°C.

TABLE 4. Replication of West Nile virus in various organs of Cx.univittatus at 14°C.

TEMP	DAYS	TRANS	DIGESTIVE TRACT			OE	DIV	GA	S GL
°C	PI		FORE	MID	HIND				
14*	1**	Ø"	-	-	-	ND	ND	ND	-
	1	Ø	-	+	-	ND	ND	ND	-
	2	Ø	-	-	-	ND	ND	ND	-
	2	Ø	-	-	-	ND	ND	ND	-
	3	Ø	+	+	-	ND	ND	ND	-
	3	Ø	-	+	-	ND	ND	ND	-
	5	Ø	-	+	-	ND	-	ND	-
	7	Ø	-	+	-	-	-	-	-
	8	Ø	-	+	-	ND	-	ND	-
	10	Ø	-	+	-	ND	-	-	-
	14	Ø	-	+	-	ND	-	ND	-
	17	3.6	+	+	-	ND	ND	ND	-
	22	Ø	-	+	-	ND	ND	ND	-
	24	3.6	-	+	-	ND	ND	ND	+
	29	Ø	-	+	-	ND	ND	ND	+
	36	Ø	-	+	-	ND	-	ND	-
	36	3.3	+	+	-	ND	-	ND	-
	36	5.1	-	+	-	ND	ND	ND	+
	42	3.8	-	+	-	-	-	-	+
	65	4.8	+	+	-	-	-	ND	+
14-26	1	Ø	+	+	-	ND	-	ND	-
14	79	4.6	+	+	-	ND	-	ND	+
14-26	3	Ø	-	+	-	ND	-	ND	+
14-26	3	Ø	+	+	-	ND	-	ND	-

Titre of infective blood meal=5.3-5.8 $Log_{10}CPD_{50}/ml$.

* Temperature at which the infected mosquitoes were held.

** Each horizontal line represents the results for one mosquito

" Titre of virus transmitted by the mosquitoes in $log_{10}CPD/ml$

Abbreviations:(PI)Post infection,(ND)Not done, (TRANS) Transmission,(FORE)Foregut,(MID)Midgut,(HIND)Hindgut,(OE)Oesophagus,(GA)Abdominal ganglia and S GL)Salivary gland.

Table 5.Replication of West Nile virus in various organs of Cx.univittatus at 18°C.

TEMP °C	DAYS PI	TRANS	DIGESTIVE TRACT			OE	DIV	GA	S GL
			FORE	MID	HIND				
18	1	Ø	-	-	-	ND	ND	ND	-
	1	Ø	-	-	-	ND	ND	ND	-
	2	Ø	-	+	-	ND	-	ND	-
	2	Ø	-	+	-	ND	ND	ND	-
	3	Ø	-	+	-	ND	ND	ND	-
	3	Ø	-	+	-	ND	ND	ND	-
	5	Ø	-	+	-	ND	ND	ND	-
	5	Ø	-	+	-	ND	ND	ND	-
	7	Ø	+	+	-	ND	ND	ND	-
	8	Ø	-	+	-	ND	-	ND	-
	10	Ø	-	+	-	ND	ND	ND	-
	14	3.35	+	+	-	ND	-	ND	+
	17	3.6	+	+	-	ND	ND	ND	+
	22	4.6	+	+	-	ND	ND	ND	+
	24	4.4	+	+	-	ND	ND	ND	+
	29	4.1	+	+	-	ND	-	ND	+
	36	4.6	+	+	-	ND	-	-	+
	36	5.4	+	+	-	-	-	-	+
	36	4.4	+	+	-	-	ND	ND	+
	36	7.1	+	+	-	-	-	ND	+
	43	6.6	+	+	-	ND	ND	ND	+
	43	6.4	+	+	-	ND	-	ND	+

Titre of infective blood meal=5.3-5.8$Log_{10}CPD_{50}$/ml.

Abbreviations:as in table 4

TABLE 6. Replication of Sindbis virus in various organs of Cx.univittatus at 14°C and 18°C.

TEMP °C	DAYS PI	TRANS	DIGESTIVE TRACT			OE	DIV	GA	S GL
			FORE	MID	HIND				
14	1	Ø	-	+	-	-	-	ND	-
	2	Ø	-	+	-	ND	-	ND	-
	4	Ø	-	-	-	-	-	-	-
	5	Ø	-	+	-	ND	-	ND	-
	6	3.6	+	+	-	+	ND	ND	+
	8	3.6	-	+	-	ND	+	ND	+
	9	4.1	-	+	-	+	+	+	+
	12	3.85	+	+	-	ND	ND	ND	+
	12	4.1	+	+	-	+	-	+	+
18	1	Ø	-	-	-	-	-	-	-
	2	Ø	-	-	-	-	-	-	-
	4	Ø	-	+	-	ND	-	ND	-
	5	3.85	-	+	-	ND	+	ND	+
	5	4.1	-	+	-	ND	-	ND	+
	6	3.85	-	+	-	ND	ND	ND	-
	8	4.6	+	-	-	-	ND	+	
	9	5.35	+	+	-	ND	ND	ND	+
	12	3.85	+	+	-	+	-	+	+
	12	3.85	+	+	-	+	+	ND	+

Titre of infective blood meal=
6.25-6.5 $Log_{10}CPD_{50}$/ml.

Abbreviations: See table 4.

Fig.22.

Fluorescent antibody studies on the midgut region of
a)uninfected Cx.univittatus
b)SIN-infected Cx.univitatus
Mag.200x.

Author Cornel A J
Name of thesis The Effect of temperature on the Vector Competence of Culex Univittatus Theobald (Diptera: Culicidae) for West Nile and Sindbis Virusses 1987

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a)



b)

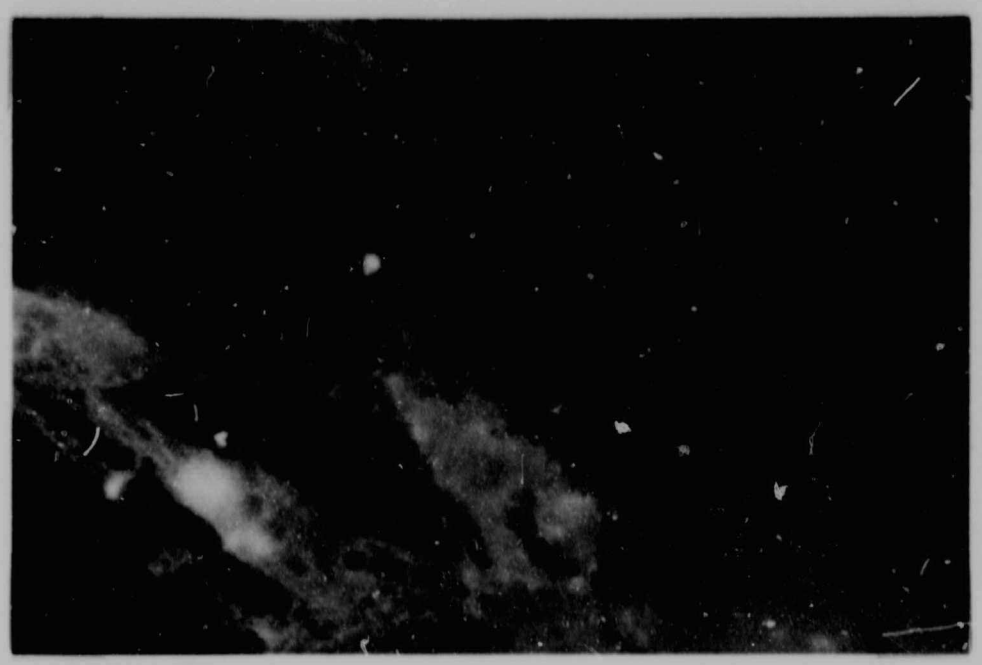
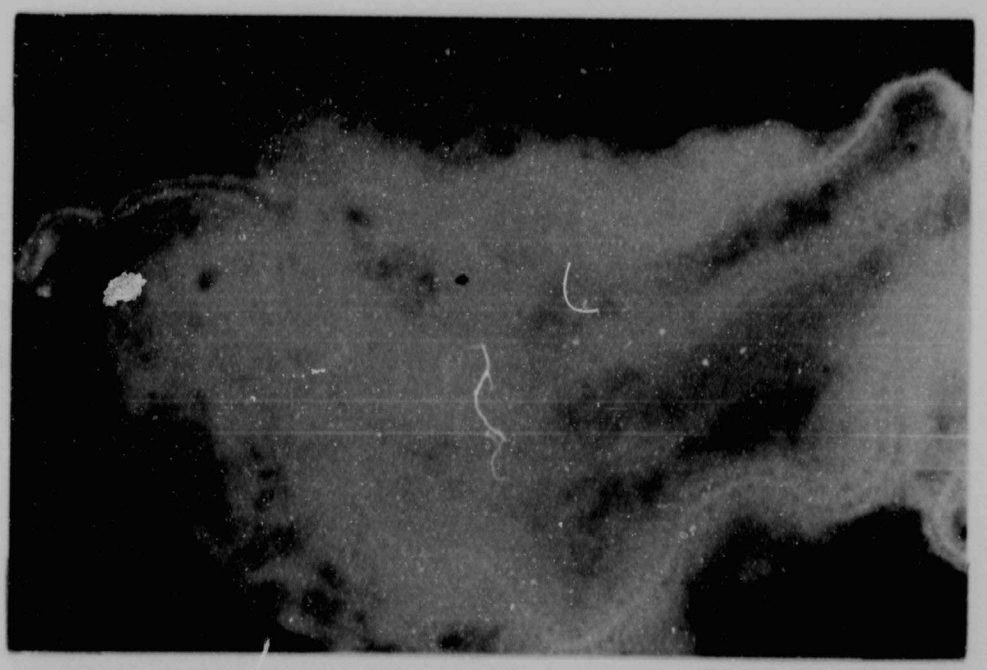


Fig.23.

Fluorescent antibody studies on salivary glands from
a)uninfected Cx.univittatus
b)Cx.univittatus infected with SIN virus
c)Cx.univittatus infected with WN virus
Mag.100x.

a)



b)



c)

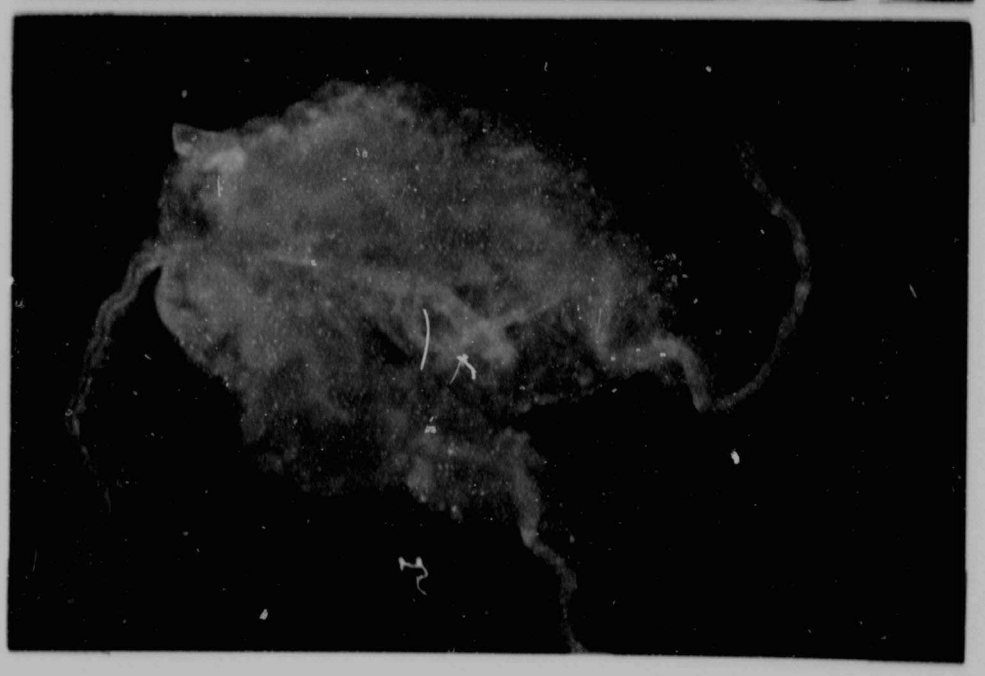


Fig.24.

Fluorescent antibody studies on the foregut region of
a)uninfected Cx.univittatus
b)Cx.univittatus infected with SIN virus.
Mag.200x.

a)



b)

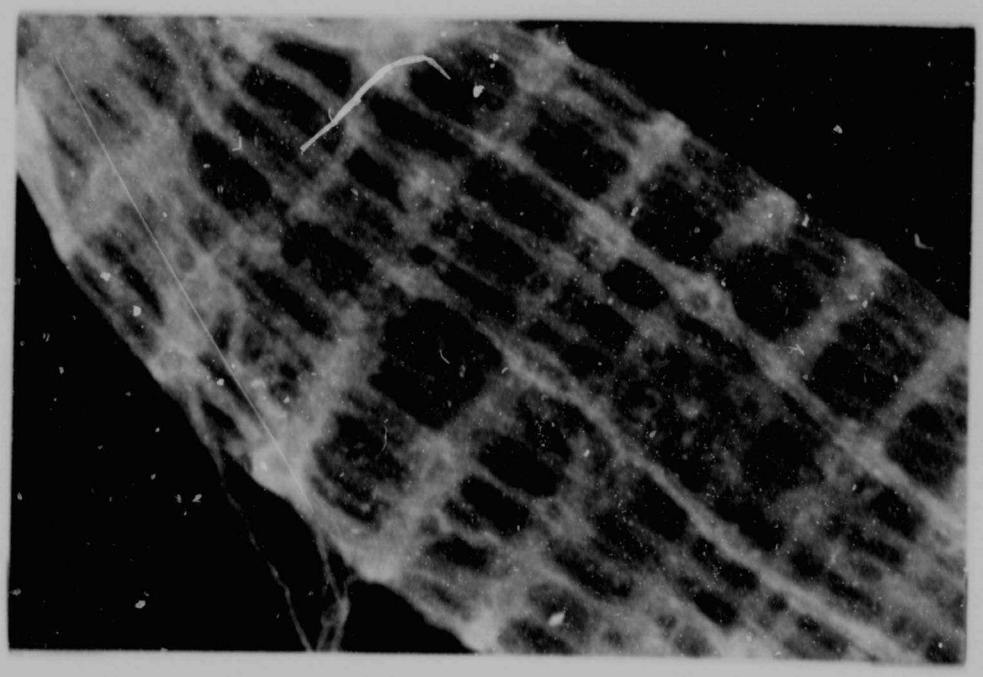
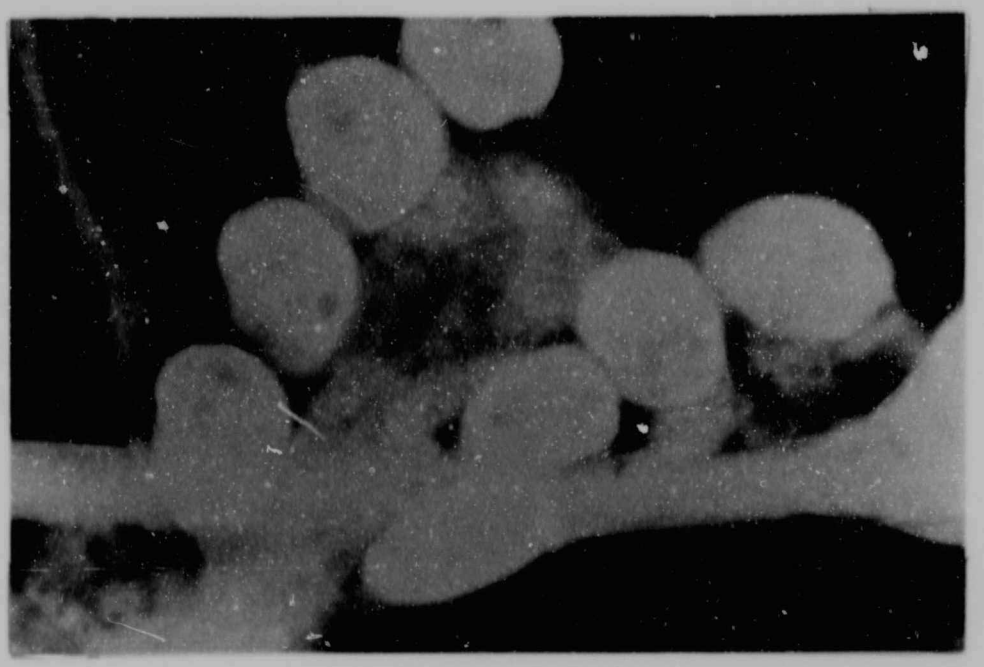


Fig.25.

Fluorescent antibody studies on abdominal ganglia from
a)uninfected Cx.univittatus
b)Cx.univittatus infected with SIN virus.
Mag.200x

a)



b)

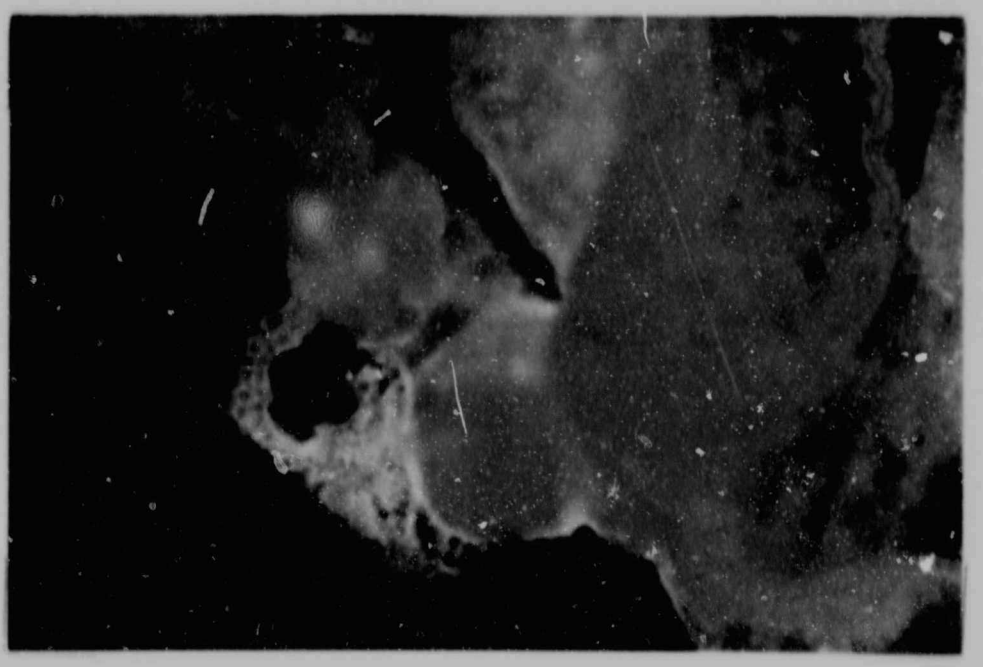


Fig.26.

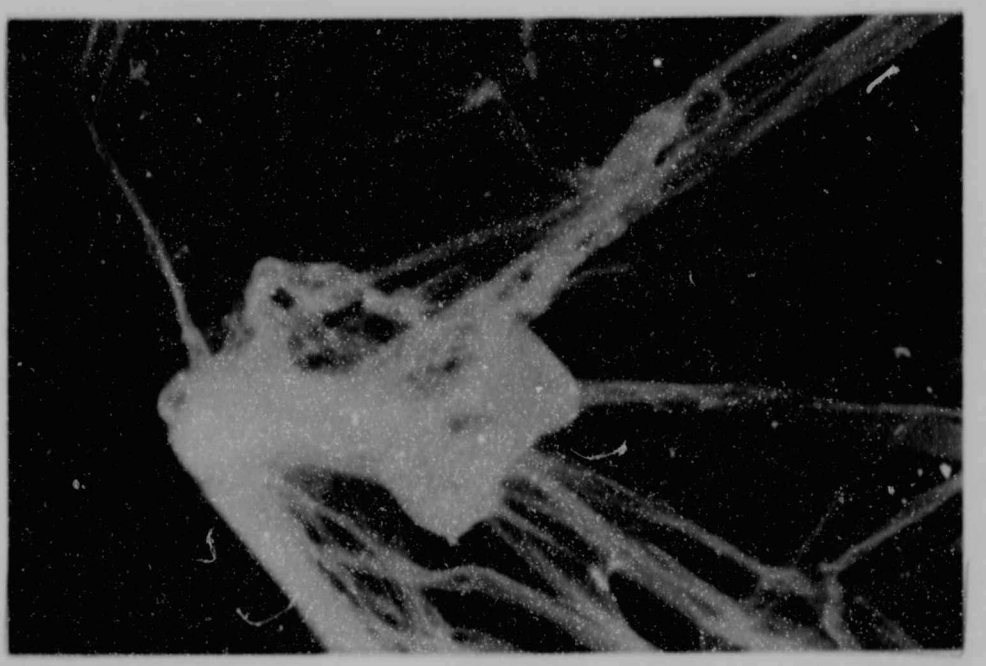
Fluorescent antibody study on the ventral diverticulum of

a)uninfected Cx.univittatus

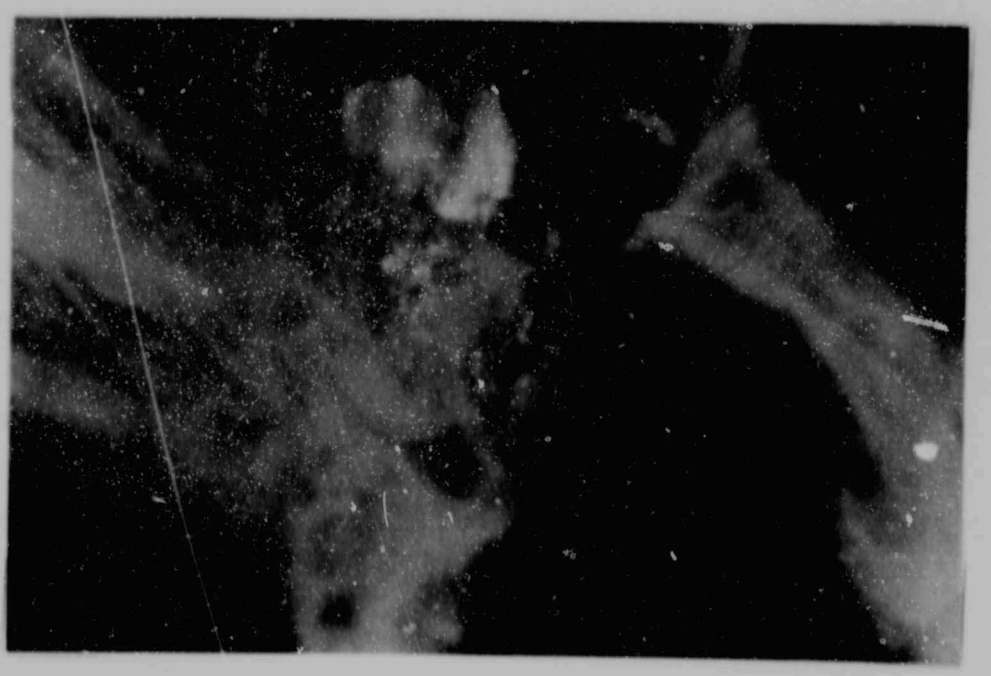
b)Cx.univittatus infected with SIN virus.

Mag.200x.

a)



b)



Chapter 4.DISCUSSION.

4.1. Replication and distribution of WN and SIN viruses in Cx.univittatus.

Higher incubation temperatures increased the rate of multiplication and spread of both WN and SIN viruses from the midgut to salivary glands in Cx.univittatus. The temperature vector competence studies and fluorescent antibody studies both provided evidence for this. This more rapid viral replication resulted in virus reaching maximum titres within the mosquitoes earlier. Hence both the EI periods and the period before maximum transmission rates were attained were reduced,showing that viral transmission was favoured by high temperatures. This inverse relationship between EI period and temperature has been shown to occur with several other virus-vector combinations (Davis,1932;Bates and Roca-Garcia,1946; Chamberlain and Sudia,1955;Hurlbrut,1973;Kramer et al,1983).

Incubation periods before maximum infective titres of SIN virus were reached in the mosquito followed the same pattern as with WN virus although they were consistently shorter,suggesting that SIN virus replicated more rapidly within the insect. Fluorescent antibody studies confirmed this by showing that SIN virus spread from the midgut to other regions of the body,particularly the salivary glands,quicker than WN virus at both 14°C and 18°C. The period before antigen was shown to be present in the salivary glands frequently correlated with the EI period determined by capillary transmission and this period was also consistently shorter with SIN virus at all temperatures.

The sequence of virus multiplication and distribution as shown by the fluorescent antibody studies appeared very similar for both WN and SIN viruses and followed

the pattern established for many other mosquito-borne viruses (Doi,1970;Kuberski,1979). SIN virus,however, did seem to infect more organs of the mosquito,such as the ganglia and oesophagus. Electron microscopic examination of tissues could be done to confirm that SIN virus replicates in these organs.

4.2.Temperature and competence of Cx.univittatus as a vector of WN virus.

WN virus multiplied and spread more rapidly in the insect at higher incubation temperature. As a result, the EI period was inversely related to incubation temperature and Cx.univittatus was able to transmit WN virus earlier at higher temperatures. Transmission rates of 100% were reached at all incubation temperatures but the period after the infective meal required before this transmission rate was attained followed a similar inverse relationship as the EI period. After maximum transmission rates were reached,they did however, fluctuate,particularly at the lower incubation temperatures. Concentrations of JBE virus were shown to fluctuate in salivary glands of Cx.tritaeniorhynchus mosquitoes(Takahashi,1976). Therefore,the fluctuations in transmission rate of WN virus could have been as a result of fluctuations in concentration of virus in the salivary glands of Cx.univittatus. Serial infectivity assays on salivary glands would,however,need to be done to confirm whether this also occurs with WN virus.

The graphs showed that the amount of virus transmitted followed the pattern of transmission rates. The incubation periods needed before maximum amounts of virus were transmitted
generally corresponded to those periods which produced the highest transmission rates.

Infection rates were,however,unaffected by temperature and remained well above 90% at all incubation temperatures used. Infection rates for WN virus in

Cx.univittatus were also shown by Jupp(1974) not to be affected by reduction in temperature and remained at 100% at both 18°C and 26°C in mosquitoes previously given a blood-meal with a titre of 4.1 to 4.6 $\log_{10}LD_{50}$ per 0.02ml of mouse inoculum.
Similarly,maximum titres of WN virus within mosquito carcasses were not affected by incubation temperature and reached similar average maximum titres at all temperatures. However,incubation periods required after the infective feed before maximum infective titres were reached were shortest at intermediate temperatures,slightly longer at 14°C,and interestingly longest at 30°C. The titre of WN virus within mosquito carcasses only showed initial decline at high temperatures,and such decline was totally absent at 18°C and 14°C. This is perhaps unusual as the opposite occurred with Ae.triseriatus infected with EEE virus,where initial decline in infective titres was most noticable at lower temperatures (Chamberlain and Sudia,1955). Perhaps,due to some physiological response of Cx.univittatus to high temperatures,it takes longer for WN virus to initially infect the mesenteronal cells of the midgut. If infectivity assays were done on the midgut of infected mosquitoes incubated at 30°C this could probably be confirmed.
Temperature also affected the survival of Cx.univittatus and the temperature tolerance of this mosquito,as measured by comparisons of mean life expectancies,was highest at fluctuating summer temperatures and at 26°C and lowest at 18°C and 30°C. Therefore,although viral multiplication was most rapid at 30°C,the longevity of Cx.univittatus was reduced at that temperature. Hence the chances of an infected mosquito living long enough to feed a second time(transmission feed) would be reduced,especially if environmental temperatures remained above 30°C for prolonged periods. Due to the opposing effects that high temperature (30°C) have on the longevity of the mosquito and transmission of virus,epidemics of WN

virus would most likely be expected in the summer,when temperature varies between daily maxima within the thirties and minima within the twenties.

At 14°C the exact opposite occurred. The multiplication of WN virus was substantially slower and this increased the period before large amounts of virus were transmitted and before high transmission rates occurred. However,the longevity of Cx.univittatus was so prolonged at 14°C that after 80 days the virus still had time to reach the salivary glands and develop high titres. At 14°C transmissior of WN virus would however,be reduced by the low feeding activity of Cx.univittatus. In spite of this,temperatures as low as 14°C may still be of epidemiological importance because at such temperatures Cx.univittatus could serve as an overwintering source of WN virus,particularly during mild winters.

A study was undertaken by Jupp(1969;1975) to investigate overwintering activities of highveld Culicine species including Cx.univittatus. Avian-baited traps showed intermittent presence of a few active females during the winter months in the highveld regions. Wilton and Smith(1985) however reported that host seeking activity and ovarian diapausing activity in mosquitoes of the Cx.pipiens complex in North America were two mutually exclusive phenomena and that hibernating(ovarian diapausing) females would not show any host seeking/blood feeding activity. Perhaps,as Jupp(1969) suggested,females caught with pigeon-baited traps during winter did not represent the entire mosquito population and diapausing individuals may have been overlooked. No true nulliparous Cx.univittatus have as yet been caught during winter in South Africa and those adults that were caught towards the end of winter were in a parous state(Jupp,1975). Some Cx.pipiens and Cx.theileri mosquitoes were collected in the highveld in June in a nulliparous state and at first glance it

appears that Cx.univittatus might have a different overwintering biology from these other two species. However,this would seem unlikely as all three species belong to the same subgenus and show a similar summer biology. The present vector competence study at 14°C with WN virus supports the idea that Cx.univittatus may to some degree overwinter in a parous state as some parous mosquitoes infected with WN virus lived for longer than 3 months. A proportion of such individuals in natural conditions could find microhabitats that remain relatively warm during winter. If these mosquitoes were infected they could provide for overwintering of virus and act as a source of virus the following spring when temperatures rise again. Further experiments would be required to confirm this possibility. Infected individuals would need to be held for long periods at temperatures lower than 14°C to test their feeding activities as well as their longevity at these low temperatures. It should also be borne in mind that the present experiments,involving the transfer of infected Cx.univittatus
from 14°C to 26°C,proved that this mosquito can survive lengthy periods of cool temperatures and transmit virus when temperatures rise again. The transfer of infected mosquitoes from cold to warm temperatures has been done with several other arboviruses and has similarly resulted in increased replication of virus (Bates and Roca-Garcia,1945; 1946;LaMotte,1963;Turrell et al,1985).
Subjecting infected Cx.univittatus mosquitoes virus to fluctuating summer temperatures, seemed to have the effect of a temperature intermediate between 30°C and 26°C. Hence past vector competence studies conducted at a constant temperature of 26°C in this laboratory have probably given a good indication of the vector competence of Cx.univittatus for WN virus (Jupp,1974;1976).

4.3.Temperature and competence of Cx.univittatus as a vector of SIN virus.

As with WN virus,infection rates were unaffected by incubation temperatures,and rates of 95% were obtained at all temperatures. Titres of SIN virus within mosquito carcasses were similarly not affected by temperature,except perhaps at 30°C which had slightly lower average titres. Incubation periods required before maximum infective titres of SIN virus were reached interestingly followed the same pattern as those obtained with WN virus,which were shortest at intermediate temperatures;slightly longer ar 14°C and longest at 30°C. Amounts of SIN virus within the mosquito carcasses were also not higher than the titres of WN virus at corresponding temperatures.

Titres of SIN virus transmitted did not follow a similar trend to the transmission rates and did not fluctuate nearly as much as the transmission rates. Highest amounts of SIN virus were transmitted shown by mosquitoes held at 26°C and 18°C and were higher than the amounts of WN virus transmitted by mosquitoes held at corresponding temperatures. Lowest virus titres were unexpectedly demonstrated in mosquitoes subjected to outside summer temperatures, and were lower than titres of WN virus in mosquitoes held outside.

Rates for transmission of SIN virus by Cx.univittatus held outside at fluctuating summer temperature rapidly reached a maximum of 100%,but thereafter decreased and dropped continuously until after 40 days very few mosquitoes were still able to transmit virus. A similar pattern of transmission was obtained for Ae.aegypti infected with another alphavirus,Semliki Forest virus (Mims and Marshall,1966),and the authors believed the decrease in transmission was caused by the destruction of salivary gland cells by the virus. Similar alteration of salivary glands of Cx.univittatus infected with SIN virus was in the

present study demonstrated by examining H and E sections. Furthermore,salivary glands of SIN virus-infected mosquitoes examined by immunofluorescence on day 12 PI had large areas of structural change (Fig.23b) which were not apparent in glands infected with WN virus(Fig.23c). In the light of the above observations,and because SIN virus-infected mosquitoes only lived 14-16 days at 14,5°C,there is good evidence to suggest that SIN virus causes changes to the salivary glands of Cx.univittatus which prevent normal functioning and secretion of saliva after a certain incubation period. At all constant temperatures,however, transmission rates fluctuated for the complete duration of the experiment. These fluctuations suggest that the salivary gland cells were periodically able to recover from the virus infection,subsequently allowing new virus to develop. In fact,fluctuations in transmission rates bec. ' larger at longer incubation periods suggesting that it took longer for the cells to recover after each subsequent cycle of replication of SIN virus.

SIN virus replicates and spreads more rapidly than WN virus within the mosquito,and as would be expected the EI periods were shorter for SIN virus at all corresponding temperatures. Despite this,SIN virus did not reach maximum titres within the mosquito body or saliva earlier than WN virus at corresponding temperatures. This may also be due to the cytopathic effect of SIN virus on mosquito cells.

As mentioned before,a temperature of 30°C reduced the lifespan of Cx.univittatus,but,coupled with this,an incubation temperature of 30°C was also detrimental to the replication of SIN virus within the mosquito. This becomes clear when the vector competence of Cx.univittatus with SIN virus at 30°C is compared to that at 26°C. Although the EI period was slightly shorter at 30°C,it took longer to reach a transmission rate of 100% at 30°C,viz.8 days at 26°C and greater

than 25 days at 30°C. Furthermore,average maximum titres of virus transmitted were 1.5 logs higher at 26°C and average maximum infective titres of mosquitoes were almost 1 log higher at 26°C. Therefore,temperatures higher than 30°C would be even more unfavourable for transmission of SIN virus than for WN virus,which possibly explains why more cases of WN virus occurred during an epidemic of both viruses in 1974 in the Karoo and Northern Cape when temperatures were often higher than 30°C (McIntosh et al,1976). Conversely,average summer temperatures in the Johannesburg-Pretoria region are lower and more favourable for transmission of SIN virus and thus could explain why more cases of SIN virus occurred in this region during the summer of 1982 and 1983 (Jupp et al,1986).

Cx.univittatus mosquitoes infected with SIN virus responded completely differently to those infected with WN virus at 14°C and died within 14-18 days. Here again,the death of the mosqutioes must have been due to the effects of SIN virus,which might possibly be even more extensive and damaging at lower temperatures. Hence,it seems that it is important to use infected mosquitoes for overwintering experiments if one wishes to demonstrate that adult mosquitoes could act as an overwintering source of virus. If further studies confirm that overwintering mosquitoes are not able to serve as reservoirs of SIN virus,then the seasonal introduction of virus by birds migrating from other regions where SIN virus is prevalent such as India (Shah et al,1960),might be the major seasonal source of SIN virus in South Africa.

4.4. Longevity of Cx.univittatus infected with WN and SIN viruses respectively.

Any models of the natural history of an arbovirus would naturally have to include a study of any measurable effects that the virus would have on the

vector. This would not only be important in terms of overwintering but also during summer months when epidemics occur.Arboviruses may cause definite pathogenesis thereby reducing the survival of the vector or they may simply affect certain functions within the vector due to increased energy requirements needed to support both the vector and the virus,or a combination of both (Turrell et al,1985). Few studies have been done to test this and most that have been done have shown no discernable difference between survival of infected and uninfected mosquitoes (Hayles et al,1972). Some evidence has,however,accumulated from specific studies. For instance,following oral exposure to RVF virus Cx.pipiens with a disseminated infection showed a 21% reduction in refeeding rates and a 20% reduction in the number of eggs laid as compared to uninfected individuals (Turrell et al,1985). Reduced ability to refeed was also demonstrated in Ae.triseriatus(Say) infected with LaCrosse virus (Grimstad et al,1980). One further study,already mentioned,was that of SF virus that seemed to cause destruction of salivary glands in Ae.aegypti. Both WN and SIN viruses reduced the mean life expectancies of Cx.univittatus from 40 days to 28 days at 26°C.In terms of WN virus these results seem to be somewhat contradictary,as,this virus did not seem to be detrimental to the survival of the mosquito at 14°C and did not appear to cause lesions of salivary gland cells. Destruction of salivary gland tissue by SF virus
appears to have been paralleled by SIN virus in Cx.univittatus and consequently reduces the lifespan of this mosquito. However,if SIN virus is indeed shown to cause lesions in mosquito salivary glands and possibly other organs,confirming the results obtained in this study,then this would be an unusual vector-virus relationship. It would be more unusual than that observed with SF virus and Ae.aegypti,since Ae.aegypti is not the natural vector of SF virus and

therefore the vector-virus relationship is not one that has had thousands of years to evolve. Perhaps,SIN virus might only have been introduced into South Africa recently so that the relationship between virus and mosquito has not had enough time to evolve into a true commensalistic relationship. SIN virus therefore does not seem as well adapted as WN virus to the South African climate and vector mosquito population. Cx.univitatus may therefore not be such an efficient vector of SIN virus as originally thought since it only transmits virus for short periods,and this only becomes apparent as mosquitoes are subjected to fluctuating ambient temperatures. Reduction in the number of days that Cx.univittatus is able to transmit virus is of particular epidemiological importance as overall transmision of virus would be reduced,possibly further explaining why SIN virus was less prevalent than WN virus,during the epidemic in 1974 in the Karoo and Northern Cape regions when climatic conditions were ostensibly perfect for transmission.

CHAPTER 5.

5.1.GENERAL CONCLUSIONS.

Subjecting infected mosquitoes to outside summer temperature conditions when evaluating the vector competence of mosquitoes with arboviruses approaches the natural situation more closely and therefore will produce more valid results than controlled-temperature studies. Very often it would be acceptable to assume that fluctuating temperatures would have intermediate effects on the vector competence of mosquitoes with arboviruses as was indeed the case with WN virus. However,this was quite obviously not the case with SIN virus. Therefore it is advocated that vector capability tests should be run at fluctuating temperatures simulating natural conditions or in cages exposed to outside ambient temperature.

The vector competence of Cx.univittatus was greatest for both WN and SIN viruses within a temperature range of 18°C to 26°C,particularly towards the higher end of this range. Temperatures higher than this were detrimental to the survival of Cx.univittatus and in the case of SIN virus actually reduced virus replication. Temperatures below 18°C reduced replication of both WN and SIN viruses and also adversely affected those mosquitoes infected with SIN virus.

Epidemics of WN and SIN viruses are most likely to occur when summer temperatures reach means in the vicinity of 26°C,and also when large numbers of vectors are available after heavy rainfall and when there is a large susceptible population of wild avian species and humans available.

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APPENDIX.

Appendix A.
Data for Figures 8 to 10.

Day	Temp °C	Titres										% Trans
		Capillary fluid					Mosquito					
		No.	Mean	SD	High	Low	No.	Mean	SD	High	Low	
2	30	5	0	0	0	0	5	4.8	1.3	6	1.75	0
3	30	5	0	0	0	0	5	4.6	0.7	5.5	3.75	0
4	30	5	0	0	0	0	5	4.5	0.4	4.75	2.75	0
5	30	5	4.4	0	4.4	4.4	5	4.8	1.1	6.5	2.75	20
6	30	5	0	0	0	0	5	5.3	0.9	6.5	4	0
7	30	5	5.9	0	5.85	5.85	5	5.2	0.6	5.75	4.5	20
9	30	5	4.4	0	4.4	4.4	5	5.2	0.7	6	4.5	20
11	30	5	5.2	0.8	6.1	4.6	5	5.6	0.8	6.25	4.5	40
13	30	5	6.9	1	7.6	5.9	5	5.6	0.3	6.75	6	80
15	30	5	6.8	0.8	7.6	5.6	5	6	0.4	6	5.5	100
17	30	5	6.6	1.2	8.1	5.35	5	6.2	0.6	6.75	5.5	100
20	30	5	6.3	1.2	7.85	5.35	5	6	0.3	6.5	5.75	100
24	30	5	7	1.2	8.1	4.85	5	6.4	0.7	7.25	5.5	100
2	26	5	0	0	0	0	5	4.9	0.1	5	4.75	0
3	26	5	0	0	0	0	5	4.2	0.9	4.75	2.5	0
4	26	5	0	0	0	0	5	4	0.6	4.75	3.5	0
5	26	5	5.6	0	5.6	5.6	5	4.75	1	6.5	3.75	20
6	26	5	5.8	0	5.8	5.8	5	4.9	0.4	5.25	4.5	20
7	26	5	5.5	0.9	6.1	4.85	5	5.6	0.6	6	4.5	40
9	26	5	4.4	0	4.4	4.4	5	5.6	1.3	6.75	3.5	40
11	26	4	6.1	0.8	6.85	5.35	4	6.7	0.4	7.25	6.25	75
13	26	5	6.2	1.2	6.75	5.75	5	6.2	0.5	6.75	5.75	80
13	26	23										79
15	26	5	6.2	0.9	7.35	5.35	5	7	0.2	7.25	6.75	100
17	26	5	6.2	1.3	8.1	4.85	5	6.35	0.3	6.75	6	100
20	26	5	6	1.2	7.1	4.4	5	5.6	0.6	6.25	5.5	100
24	26	5	6.5	0.8	7.6	5.85	5	6.4	0.3	6.75	6.25	100
31	26	5	6.4	0.6	7.1	5.6	5	6.1	0.7	6.75	5.25	80
1	a*	8	4.4	0	4.4	4.4	8	5.6	0.9	4.25	1.75	35
2	a	10	0	0	0	0	10	5.5	0.6	4.25	4.25	0
3	a	10	0	0	0	0	10	5.4	0.4	6	4.5	0
4	a	9	4.4	0	4.4	4.4	9	5.3	0.6	6	4.5	11
6	a	10	4.4	0	4.4	4.4	10	5	0.5	5.75	4	10
8	a	10	5.2	0.7	5.85	4.4	10	4.7	0.6	5.25	4	40
13	a	10	5.5	0.6	6.35	4.6	10	4.8	0.9	5.75	2.5	70
16	a	9	5.4	0.6	6.1	4.6	9	6.1	0.6	7	5.25	100
21	a	10	7	0.3	8.1	5.35	10	6.1	0.5	7	5	100
27	a	9	6.4	1.1	7.35	4.6	9	5.7	0.4	6.25	5.25	77
30	a	7	6.3	0.4	6.35	5.6	7	5.8	0.3	6.25	5.25	57
38	a	7	6.1	0.7	6.6	4.85	7	5.4	0.5	6.25	4.75	100

Appendix A.
Data for Figures 11 to 12.

Day	Temp °C	Titres										% Trans
		Capillary fluid					Mosquito					
		No.	Mean	SD	High	Low	No.	Mean	SD	High	Low	
1	18	10	0	0	0	0	5	2.7	0.5	3.5	2.5	0
2	18	10	0	0	0	0	5	4.6	0.4	5	4.25	0
3	18	10	0	0	0	0	5	4.7	0.3	5	4.25	0
5	18	10	0	0	0	0	5	4.7	1.3	6	2.5	0
7	18	10	0	0	0	0	5	5	0.4	5.5	4.75	0
8	18	9	0	0	0	0	4	4.18	0.3	4.5	4	0
10	18	10	0	0	0	0	5	4.25	1.4	5.5	4.25	0
14	18	10	4.9	1	6.1	4.4	5	4.9	1	6.5	3.5	35
17	18	10	5.3	0.8	6.6	4.6	5	5.9	0.7	6.75	5	60
22	18	8	5.3	0.7	6.6	4.4	4	6	0.5	6.5	5.5	100
24	18	10	5.4	0.9	7.1	4.4	5	6.3	0.4	6.75	6	90
29	18	9	6.3	1	7.6	5.5	4	6.3	0.9	7.5	5.5	100
36	18	10	7	1	8.1	5.35	5	5.5	1	6.25	3.75	90
42	18	10	6.9	1.2	7.85	4.85	5	6	0.6	6.75	5	100
49	18	5	6.6	0.8	7.6	5.6	5	6	0.5	6.5	5.25	80
58	18	3	6.6	1.9	7.85	4.4	3	5.75	0.3	6	5.5	100
1	14	10	0	0	0	0	5	3.5	0.5	4	3	0
2	14	10	0	0	0	0	5	3.5	0.6	4	3.4	0
3	14	10	0	0	0	0	5	0.5	3.75		3.4	0
5	14	10	0	0	0	0	5	3.9	0.9	5	3	0
7	14	10	0	0	0	0	5	3.7	0.6	4	3	0
8	14	10	4.4	0	4.4	4.4	5	3.7	0.9	5.25	3	10
10	14	10	0	0	0	0	5	4.3	0.4	4.5	3.75	0
14	14	9	0	0	0	0	4	4.2	0.3	4.5	3.75	0
17	14	10	4.7	0.2	4.85	4.6	5	3.5	0.8	4.25	2.25	20
22	14	10	4.4	0	4.4	4.4	5	4.3	0.6	5	5.5	10
24	14	9	4.6	0	4.6	4.6	4	4.7	0.2	4.75	4.5	11
29	14	10	5.4	0	5.4	5.4	5	4.8	0.3	4.8	0.3	10
36	14	10	5	0.7	6.1	4.4	5	4.5	2	6.5	4.5	60
43	14	10	5	0.4	5.6	4.6	5	4.6	0.5	5.25	4	80
49	14	5	5.2	1.2	6.1	4.4	5	5	1	6.25	3.5	40
49	14"	5	6.1	0	6.1	6.1						20
51	14"	5	8	1.4	7.6	4.85						80
56	14	4	5.2	1	6.35	4.4	4	4.3	0.9	5.25	3	75
56	14"	5	5.6	0.3	5.85	5.35	5	5.5	0.7	6.5	5	60
58	14	3	5.6	1.2	6.35	4.4	3	4.9	1.4	6.25	3.5	100
58	14+	3	7.2	2.2	8.1	4.4	3	6.4	0.8	7	5.5	100
65	14	5	5.4	0.4	5.85	5.1						60
65	14"	3	7	0.2	7.1	6.85						66
65	14+	3	4.9	0.7	5.35	4.4						66
79	14	4	5.9	0.9	7.1	4.85						100
90	14	5	6.6	1	7.85	5.35						100
99	14	3	6.1	0	6.1	6.1	3	5.3	1	6	4	33
114	14	4	5.2	0.5	5.85	4.6	4	6.4	0.3	6.75	6	100

"=Infected mosquitoes transferred to 26°C and not given a second blood meal on a pigeon.+=Infected mosquitoes transferred to 26°C and given a second blood meal on a pigeon.*=Ambient temperature.

Appendix B.
Data for Figures 13 to 15.

Day	Temp °C	Titres										% Trans
		Capillary fluid					Mosquito					
		No.	Mean	SD	High	Low	No.	Mean	SD	High	Low	
2	30	5	5.2	0.7	5.35	4.4	5	5.2	0.4	5.75	4.75	40
4	30	5	6	0.9	6.6	5.1	5	5.2	0.7	5.75	4	40
5	30	5	5.1	0.7	5.85	7.6	5	5	2	6.5	2.5	60
7	30	4	4.7	1.2	6.6	4.85	4	5	0.5	5.5	4.25	50
9	30	5	4.4	0	4.4	4.4	5	4.6	1.4	5.75	2.25	20
12	30	5	5.5	0.9	6.1	4.85	5	5	0.2	5.25	4.75	40
16	30	10	5.1	0.6	6.1	4.4	10	5.4	0.5	6.25	4.75	60
19	30	4	5.1	0	5.1	5.1	4	5.7	0.8	6.25	4.5	60
23	30	4	4.9	0.4	5.1	4.6	4	3.1	0.5	3.75	2.75	40
27	30	4	4.7	0.6	5.6	4.4	4	4.9	0.5	5.5	4.5	100
2	26	5	0	0	0	0	5	5.5	0.8	6.5	4.75	0
4	26	5	4.8	0.3	5.1	4.4	5	5.2	1.2	6.25	3.5	80
5	26	5	6	1.6	7.1	4.85	5	4.5	1.3	5.5	2.5	40
7	26	4	4.5	1.5	7.6	4.4	4	6.1	0.3	6.5	5.75	100
9	26	5	5	0.3	5.35	4.6	5	5.7	0.8	6.25	4.25	80
12	26	5	7.5	0.4	7.6	6.85	5	6.2	0.7	7.25	5.5	60
14	26	7	5.4	1.3	8.1	4.4						77
16	26	10	5.1	0.6	6.1	4.4	10	5.4	0.5	6.25	4.75	60
19	26	5	56	0.5	5.35	4.4	5	5.8	2.3	7	1.75	80
23	26	3	5.3	0.8	6.1	4.6	3	3.2	0.5	6.35	2.8	100
2	a*	10	4.4	0	4.4	4.4	10	5.6	0.3	6	5	10
4	a	10	4.9	0.6	6.1	4.4	10	5.6	0.8	7.5	3.75	90
6	a	9	5.3	0.6	6.6	4.6	9	5.2	0.2	6.25	6	100
8	a	10	5	0.6	5.85	4.4	10	6	0.4	7.55.5		90
10	a	10	5.6	0.7	7.35	4.4	10	5.9	0.5	6.75	5.25	90
14	a	10	5.4	0.7	6.35	4.4	10	5.9	0.5	6.75	5.25	90
18	a	10	4.9	0.4	5.35	4.4	10	5.3	0.9	7.5	4.25	80
22	a	9	5	0.5	6.6	4.6	9	5.7	0.5	6.5	4.75	55
28	a	9	4.4	0.1	4.6	4.4	9	5.1	0.5	6	4.5	33
44	a	9	4.4	0	4.4	4.4	9	5.1	0.6	6.25	4.5	11

Appendix B.
Data for Figures 16 and 17.

Day	Temp °C	Titres										% Trans
		Capillary fluid					Mosqutio					
		No.	Mean	SD	High	Low	No.	Mean	SD	High	Low	
1	18	10	0	0	0	0	5	5.7	0.5	6.25	5.25	0
2	18	10	4.6	0	4.6	4.6	5	5.2	0.5	5.75	4.5	10
4	18	10	4.9	0.6	6.1	4.4	5	5.8	0.9	6.5	4.5	60
6	18	10	4.9	0.6	6.1	4.45	5	4.8	2.2	7	4.25	100
8	18	9	6	1	8.1	4.85	5	6.4	0.4	6.5	5.75	88
9	18	10	5.9	0.4	6.35	5.35	5	5.8	1.4	7.5	3.75	80
12	18	9	5.5	0.6	6.35	4.6	4	5.3	0.2	6.5	6	88
16	18	5	5.5	0.4	5.85	5.1	5	5.3	0.5	6	4.75	100
23	18	5	4.4	0.3	4.85	4.4	5	5.5	0.6	6.25	4.5	60
26	18	5	4.5	0.1	4.6	4.4	5	5.5	0.8	6.5	4.75	40
44	18	5	4.7	0.5	5.1	4.4						40
55	18	5	7.5	1.2	8.1	5.35						100
1	14	10	0	0	0	0	5	5.2	1.2	6.25	2.5	0
2	14	10	0	0	0	0	5	5.7	0.4	6	5	0
4	14	10	6.1	0	6.1	6.1	5	5.6	0.7	6.75	4.75	10
5	14	10	4.9	0	4.9	4.9	5	5.3	0.5	5.75	4.5	10
6	14	10	4.6	0.5	5.6	4.4	5	5.9	1.1	6.75	3.75	60
8	14	10	4.5	0.2	4.6	4.4	5	5.9	1.3	7	4.25	40
9	14	10	5.5	1.3	7.85	4.4	5	5.3	1.4	6.75	3.75	60
12	14	10	4.9	0.6	6.1	4.4	5	6	1.2	7	4	80
16	14	4	5.9	0	5.9	5.9	4	4.1	1.4	6	2.75	25

*=Ambient Temperature.

Appendix C.
Calculations for the Student Newman Keuls test for longevity of uninfected mosquitoes at various temperatures.

	Temperature ranked by magnitude of means.			
	30°C	18°C	26°C	Ambient
Y	14days	19days	40days	51days
Median	13days	18days	41days	48days
n_i	588	502	611	418
SD	9	5.3	19	17
S.	81.1	28.1	369.8	309.2

The pooled variance=MS within

$$= \frac{\Sigma(n_i-1)s_i}{\Sigma(n_i-1)}$$

$$= \frac{(588-1)81.1+(502-1)28.1+(611-1)369.8+(418-1)309.2}{587+501+610+417}$$

$$= \frac{287256.79}{2115}$$

=135.8 the square root=11.65.

The differences are computed as row mean -column mean.

Rank		1	2	3	4
	Y	14	19	40	51
1	14	-			
2	19	5	-		
3	40	26	21	-	
4	51	37	32	11	-

For a difference in the above table to be significant at the α level,it must be equal to or greater than $LSR=Q_{\alpha}(K,v).MSwithin=\sqrt{\frac{n_i+n_2}{2n_in_2}}$

where K=1+difference in the ranks of two means.This equals the number of means in the set whose range we are testing.The degrees of freedom equal v=dfwithin(=2115).The sample sizes of the two means are n_i and n_2 and $Q_{\alpha}(K,v)$ is a value from the table of critical values of the Studentized range which gives the upper α points of the studentized range of K values when the estimate of the error variance has v=df at the 0.05 level of significance.The following is a list of the $Q_{.0.05}(K,.)$-values we shall need for the present example for K=2,3 and 4.

32.3 38.6 42,32

To test any particular range between two means we need only compare it with these quantities multiplied by $\sqrt{\frac{n_i+n_2}{2n_in_2}}$.

1.Test total range of the 4 means:k=4

$$LSR=Q_{\alpha}(K,v).MSwithin\sqrt{\frac{n_i+n_2}{2n_in_2}}$$

$$42.32\sqrt{\frac{588+418}{2*588*418}}=42.32\sqrt{\frac{1006}{491568}}$$

LSR=1.91.
Since the observed range(37) is greater than this it is significant at the 5% level,therefore they are significantly different.
2.Compare range of three means for K=3

$$=38.6\sqrt{\frac{611+588}{2*611*588}} \qquad =38.6\sqrt{\frac{1199}{718536}}$$

LSR=1.57.
Since the observed range(26) is greater than this it is significant at the 5% level,therefore they are significantly different.
3.Compare range of two means (26°C and Ambient)

$$32.3\sqrt{\frac{418+611}{2*418*611}} \qquad =32.3\sqrt{\frac{1029}{590352}}$$

LSR=1.5
The observed difference=11,therefore they are significantly at the 5% level.
4.Compare range of two means (18°C and 30°C)

$$LSR=32.3\sqrt{\frac{502+588}{2*502*588}} \qquad =\sqrt{\frac{1090}{510796}}$$

LSR=1,4
Observed difference=5,therefore they are significantly different at the 5% level.
5.18°C was not compared to 26°C as these would also be significantly different.

Appendix D.
Calculations for the Student Newman Keuls test on longevity of infected and uninfected mosqutoes at 26°C.

	WN	SIN	26°C
Y	28days	27days	40days
n_i	452	377	611
SD	12	17.36	17
S.	144	301.4	309.2

The pooled variance =MSwithin

$$=\frac{\Sigma(n_i-1)s_i}{\Sigma(n_i-1)}$$

$$=\frac{(451*144)+(376*301.4)+(610*309.2)}{451+376+610}$$

$$=\frac{64944+113326+188612}{1437}=\frac{366882}{1437}=255.31=\text{MSwithin}$$

square root=15.98.
Computed differences.

Rank		1	2	3
	Y	-	-	-
1	28	-	-	-
2	27	1	-	-
3	40	12	13	-

The following is a list of the Q0.05(K,v)-values we shall need for the present test for K=2 and 3.
2,772 3,314
When multiplied by .MSwithin =15.98,these values become 44,39 52,96
To test any paricular range between tho means we need only compare it with these quantities multiplied by $\Sigma(n_i+n_2)/2n_in_2$.
1.Test total range of three means.K=3,

$$=52.96*\sqrt{\frac{377+611}{2*377*611}}=52.96*\sqrt{\frac{988}{460694}}$$

=52.96*0.05=2.45
since the observed range (13) is greater than this it is significant at the 5% level,therefore they are significantly diff.
2.Test ranges of 2 means
WN and SIN mosquitoews at 26°C.

$$=44.39*\sqrt{\frac{452+377}{2*377*452}}=44.39*\sqrt{\frac{829}{340808}}$$

=44.39*4.449=2.189
Since the observed range (1) is less than this,longevity of mosquitoes infected with WN and SIN are not significantly different at the 5% level.

3.Test ranges of two means
WN and 26°C.

$$=44.39*\sqrt{\frac{452+611}{2*452*611}} \quad =44.39*\sqrt{\frac{1063}{552344}}$$

=44.39*Ø.Ø44=1.9

The observed range (12) is more thsn the LSR value,they are significantly different at the P=Ø.Ø5 or 5% level.

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