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AEDES AEGYPTI (L.) (DIPTERA: CULICIDAE), A POTENTIAL VECTOR OF DENGUE VIRUSES IN SOUTH AFRICA: TAXONOMY, ECOLOGY AND VECTOR COMPETENCE.

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ABSTRACT

The potential of Aedes aegypti to act as a vector of dengue (DEN) viruses was assessed in the tropical and subtropical regions of South Africa. The prevalence in bamboo pot ovitraps, utilization of artificial larval habitats and anthropophilism of Ae. aegypti was measured in fifteen populations from coastal Natal and five from the Transvaal. Pot indices generally ranged from 40-100 per cent ($\bar{x}\pm s_{\bar{x}}$ = 60,3±9,8), with a mean abundance index of 0,43±0,15 ($s_{\bar{x}}$) mosquitoes/pot/day. Artificial container indices ranged from 11-83 per cent ($\bar{x}\pm s_{\bar{x}}$ = 56,8±5,6). Biting rates ranged from 0,2-29,0 per man-hour, in direct relationship to the level of urbanization. The feral Ndumu population was least anthropophilic, although pot and abundance indices were high.

To investigate the presence of subspp. aegypti and formosus, the pale scales on tergite-1 were counted in ten or more siblings from each of 196 families, representing eighteen populations. At least 118 of these families were heterogeneous, each containing some siblings with no pale scales and others with pale scales on tergite-1, thus invalidating this character for distinguishing between the subspp. Isozyme electrophoresis did not provide diagnostic electromorphs for distinguishing between different populations. Allelomorph frequencies and the number of pale scales on tergite-1 and tergite-2 differed significantly in individual populations but not between anthropophilic and non-anthropophilic populations. As there was no correlation between tergal morphology, isozymes and

anthropophilism, the populations could not be resolved into the two subspp. Based on the only morphological character that appears to be reliable, viz. the blackness of the background scales, all of the populations are probably Ae. aegypti formosus.

The vector competence of five populations for DEN-1 and DEN-2 viruses was tested in the laboratory after allowing mosquitoes to feed on an infective blood-virus mixture. Viral antigen was detected by indirect fluorescent antibody test. Head-squash infection rates (HSIRs) ranged from 11-54 per cent for DEN-1 and from 19-46 per cent for DEN-2. Transmission rates (TRs) were determined by in vitro capillary method and ranged from 67-100 per cent for DEN-1 and from 71-86 per cent for DEN-2. Mean vector competence indices (calculated from HSIRs and TRs) were 0,13-0,47 for DEN-1 and 0,18-0,34 for DEN-2. It is concluded that, should DEN be reintroduced to South Africa via the shipping or tourist industries, Ae. aegypti would be an efficient urban vector. The Durban population is of particular epidemiological importance because it was highly anthropophilic and was the most vectorially competent of the South African populations.

DECLARATION

I declare that this dissertation is my own, unaided work as performed in my capacity as entomologist in the Arbovirus Unit of the National Institute for Virology, except for the assistance acknowledged in the Preface. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg and has not been submitted before for any degree or examination in any other University.

29th day of June, 1993

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PREFACE

Dengue has become the world's most important arboviral disease, causing considerable human suffering and placing unexpected burdens on the health services (and economies) of countries that have been affected by epidemics. South Africa, too, is at risk, for several reasons:

i) several generations have passed since the last dengue epidemic of 1926/1927 in the Durban area, so that the human population is largely susceptible to infection with dengue virus;

ii) the mosquito responsible for transmitting dengue, Aedes aegypti, occurs within our borders and its population density is thought to have increased in the subtropical parts of Natal and the Transvaal due to increased urbanization;

iii) there have been real and suspected introductions of dengue through infected travellers returning from the endemic areas of Asia, a situation which can only escalate with the growth in air-links with these countries;

iv) between 1976 and 1984, dengue outbreaks have occurred in Mozambique and other East African countries where before they had only been recorded in West Africa, except for the Durban epidemic in 1926/1927.

In view of this, a study was initiated in the Arbovirus Unit of the National Institute for Virology (NIV), Sandringham to determine the potential for dengue epidemics in the tropical and subtropical regions of South Africa. The approach used was to investigate the potential mosquito vectors of dengue virus, of which

there are several, in these regions. The work submitted in this dissertat' concentrates on the ecology, taxonomy and vector competence of one of these mosquito species, viz. Aedes aegypti, forming a subset of the larger study.

I wish to thank Professor B.D. Schoub, Director of the NIV, for permission to submit work for this dissertation which was done in the course of my official duties as medical entomologist in the Arbovirus Unit. I am particularly grateful to Dr P.G. Jupp for initially providing me with the opportunity to work as his research assistant, and for his supervision and continual encouragement since that time. The ecological and vector competence work would have been impossible without his direct involvement and generous sharing of expertise. I also need to acknowledge Dr Jupp for generating the data in the morphological study of Aedes aegypti.

I am indebted to other staff-members of the Arbovirus Unit: the Deputy-Director, Dr N.K. Blackburn for advice on the indirect immunofluorescent antibody technique; Mrs G.M. Meenehan for preparing mouse anti-dengue ascites; Messrs O. Matlala, M.E. Chauke, B.J. Mahlangu, D. Tegedi and S. Nhlapu for technical assistance in the field and insectary.

Mosquitoes were collected from areas under the jurisdiction of the KwaZulu Bureau of Natural Resources and the Natal and National Parks Boards, and from farms belonging to Messrs J. Henderson of Port Shepstone (Armadale), J. Hiemstra of Mica (Hope) and R.J. Laburn of Magaliesburg (Kloofwaters). For hospitality and field collaboration, I thank the following: the Department of Community Services, Richards Bay; the Durban City Health Department; Mr O. Human of "Kinderstrand", Glenmore

Beach; the Kruger National Park; Dr B.M. McIntosh of Oslo Beach; the National Institute for Tropical Diseases, Eshowe and Tzaneen; the Ndumu Game Reserve; and the Research Institute for Diseases in a Tropical Environment, Congella.

Dr M. Coetzee, medical entomologist at the South African Institute for Medical Research (SAIMR) provided training in the technique of polyacrylamide gel electrophoresis of mosquito enzymes for which I am most grateful. Dr Coetzee and her colleagues, Dr R.H. Hunt (Head of the Medical Entomology Unit, SAIMR) and Mr A.J. Cornel gave invaluable advice on the interpretion of banding patterns. Drs Hunt and Coetzee kindly read the chapter dealing with isozyme electrophoresis and provided much appreciated comment. Dr Jupp read and commented on the earlier drafts, and he and my wife, Debbie, checked the readability and English usage. I gratefully acknowledge their proof-reading skills and the generous giving of their time. However, any errors or ommissions in the final copy are my own.

The chapters dealing with ecology, morphology and vector competence of Aedes aegypti have been published in relevant research journals as parts of three papers: Kemp and Jupp (1991), Jupp et al. (1991) and Jupp and Kemp (1993), respectively.

Figures 1, 2 and 3 were produced with the help of the Photographic Illustration Unit of the University of the Witwatersrand.

LIST OF ABBREVIATIONS

Ae. ..... Aedes;
ANOVA ..... analysis of variance;
C ..... Celcius;
D ..... genetic distance index;
DEN ..... dengue;
DHF ..... dengue haemorrhagic fever;
DSS ..... dengue shock syndrome;
F_1, F_2, etc. ... first filial generation;
h_e, h_o ..... expected and observed population heterozygosities at a locus;
$\bar{H}_e$, $\bar{H}_o$ ..... expected and observed mean heterozygosities;
HSIR ..... head-squash infection rate;
IFAT ..... indirect (immuno-)fluorescent antibody test;
M ..... moles per cubic decimeter;
MID_{50} ..... 50 per cent end-point of the mosquito infectious dose;
NIV ..... National Institute for Virology, Johannesburg;
PAGE ..... polyacrylamide gel electrophoresis;
RH ..... relative humidity;
rpm ..... revolutions per minute;
s ..... sample standard deviation;
$s_{\bar{x}}$ ..... standard error (standard deviation of the mean);
SAIMR ..... South African Institute for Medical Research, Johannesburg;
sp. ..... species (plural spp.);
subsp. ..... subspecies (plural subspp.);
T_1, T_2 ..... first tergite, second tergite;

TR ..... transmission rate;
var. ..... variety;
VCI ..... vector competence index;
w/v ..... weight/volume;
$\bar{x}$ ..... mean.

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CHAPTER 1. INTRODUCTION

1.1. The dengue viruses

The dengue (DEN) arboviruses (arthropod-borne viruses) are comprised of four antigenically related serotypes, designated DEN-1 to DEN-4, in the genus Flavivirus of the family Flaviviridae (Westaway et al. 1985). DEN viruses are small, with a diameter of approximately forty-five nm. They have a single strand of positive-sense RNA with a molecular weight of 4 X 10^6, a nonglycosylated capsid, a small polypeptide, and one envelope glycoprotein.

The disease manifestations of DEN infection are similar for all four serotypes. The mild type of DEN illness has symptoms which resemble those of chikungunya in the genus Alphavirus (Togaviridae), with which DEN has sometimes been confused (Carey 1971). The clinical symptoms can include sudden onset of fever lasting for three to seven days, frontal and retro-ocular pain, myalgia, rash, joint pain, nausea and vomiting (Gubler 1988). Normally, DEN causes either clinically inapparent infection or mild fever but may progress to the severe illness termed dengue haemorrhagic fever (DHF). The predisposing factors for DHF are not well understood (Rosen 1989), but are believed to include the virulence and serotype of the DEN virus and an abnormal immune response in the human host following sequential infection with a different serotype (Halstead 1988). In extreme cases of DHF, patients may present with circulatory failure and hypovolemic shock. This has been termed the DEN shock syndrome (DSS) and is often fatal.

DEN fever has displaced yellow fever as the most important arboviral disease to affect man, recently reaching pandemic proportions in China (Qiu and Zhao 1988), Cuba (Kourí et al. 1983) and Brazil (Figueiredo et al. 1990). The causative viruses share a world-wide distribution with their most important mosquito vector, Aedes (Stegomyia) aegypti (Linnaeus), throughout the tropics. The recognized natural hosts are primates and culicine vectors in the genus Aedes Meigen.

In the Afrotropical region, DEN virus is endemic or has been involved in epizootics in Burkina Faso and the Ivory Coast (Cordellier et al. 1983, Roche et al. 1983, Hervy et al. 1984), and in Senegal (Cornet et al. 1984, Zeller et al. 1992). Recent DEN epidemics and smaller outbreaks in human populations have occurred in the Seychelles in 1976-1977 (Metselaar et al. 1980), Burkina Faso in 1982 (Gonzalez et al. 1985), Kenya in 1982 (Johnson et al. 1982), Mozambique in 1984 (Gubler et al. 1986), Somalia and Sudan in 1984 (Saleh et al. 1985), and Senegal in 1990 (Zeller et al. 1992). Except for two isolates of DEN-4 in Senegal in 1983 (Saluzzo et al. 1986), DEN-1 and -2 were the only two serotypes isolated in the East and West African outbreaks, with DEN-2 becoming more predominant recently. The 1984 Mozambican outbreak provided the first isolations of DEN-3 in Africa. The threat of DEN epidemics and endemism in the other tropical regions of Africa where the mosquito vectors are present must therefore be considered to be very real.

DEN-1 occurred in Durban, Natal, and surrounding areas in 1926-1927 (Edington 1927) in an epidemic conservatively estimated to have affected over forty thousand people. Since then, there has been one isolation of DEN-1 from a traveller returning to South Africa from

India in 1985, and two suspected recent infections in a married couple returning from a visit to Saudi Arabia and Pakistan in the same year (Blackburn and Rawat 1987).

1.2. The arthropod vector, Aedes aegypti

Aedes aegypti, the suspected vector in the Durban DEN epidemic, is endemic in much of South Africa. The southern limit of the distribution of Ae. aegypti in Africa is approximately 33°40'S latitude, with the sp. being recorded from Grahamstown (Edwards 1941) and Port Alfred (Mattingly 1953) in the eastern Cape Province. Aedes aegypti is found throughout the Transvaal, and on the coastal plane of Natal, Transkei, Ciskei and the eastern Cape. Its distribution extends inland to the north-western Cape: de Meillon (1943) reported it from Upington, and Muspratt (1956) from Graaf Reinet, Kimberley and Prieska. The present study concentrated mainly on populations of Ae. aegypti from the tropical and subtropical climatic regions of South Africa, which are located in the Natal coastal plain and north-eastern Transvaal, because these are the regions where imported DEN is likely to infect the local vector population.

Current opinion holds that there are at least two behavioural forms of Ae. aegypti in Africa, the one associated with human dwellings where the mosquito oviposits in artificial containers and bites man, and the other associated with forests where it oviposits in rot-holes in trees (tree-holes) and does not appear to bite man (Christophers 1960). Mattingly (1957) classified Ae. aegypti into "type form", subsp. formosus (Walker) and var. queenslandensis (Theobald) based on morphology, behaviour and distribution. Only the first two forms are believed to occur in South Africa, primarily on evidence of anthropophilic and non-anthropophilic populations. For

convenience, I will use the term "zoophilic" to describe non-anthropophilic populations.

The type form of Mattingly (1957) is variously referred to as subsp. typicus and subsp. aegypti. I will use the latter to conform to the practice in the recent literature on the taxonomy of Ae. aegypti.

1.3. Scope of the study

The larval habitats (i.e. preferred sites for oviposition) and anthropophilic biting-preference of various geographically isolated populations of Ae. aegypti were studied in this dissertation. The occurrence of the two subspp., aegypti and formosus, was investigated on a morphological basis in these populations using Mattingly's (1957) taxonomic distinctions. Isozyme electrophoresis was employed, initially to find unique electromorphs (electrophoretic bands) that could distinguish the South African populations and, eventually to determine allele frequencies at polymorphic gene loci that coded for isozymes in two allopatric populations, representing anthropophilic and zoophilic Ae. aegypti. Finally, the vector competence of Ae. aegypti to DEN-1 and -2 was determined for populations from five localities in Natal and the eastern Transvaal. The ecological and vectorial attributes of Ae. aegypti were used to assess the abilities of the different populations to contribute to viral transmission in the event of DEN outbreaks in South Africa.

CHAPTER 2. ECOLOGY: DISTRIBUTION AND BEHAVIOUR OF *Aedes aegypti*

The aedine subgenus *Stegomyia* Theobald, contains several natural vectors of DEN virus, including *Ae. aegypti*, the principal urban epidemic vector. *Aedes aegypti* oviposits above the water-line in natural rain-filled receptacles such as tree-holes, rock-holes and the leaf-axils of cultivated bananas, *Mus* spp. (Musaceae). When the eggs are inundated by more rain, they hatch and the larvae feed on the aquatic microfauna (bacteria and protozoa) growing on the sides of the receptacle. In urban areas, the domestic (private properties) and peridomestic sites (business premises, motorcar scrapyards, rubbish dumps, etc.) contain artificial receptacles which are filled with water, either naturally by rain or artificially by man. Suitable larval habitats are created when the wind deposits leaf-litter in the water-filled container, and the process of decay produces organic molecules that act as ovipositional attractants for the adult mosquito, and provides nutrients for the microfauna that the larvae feed on.

Zoophilic populations of *Ae. aegypti* are associated with the forests (coastal scrub, riverine and montaine) and wooded savanna (bushveld) of eastern South Africa (Muspratt 1956, McIntosh *et al.* 1972, Jupp and McIntosh 1990), while anthropophilic populations breed in close proximity to man in the urban areas (Muspratt 1956). A survey of several localities in Natal and the Transvaal and one in the eastern Cape was made by setting bamboo pot ovitraps, investigating artificial containers and collecting biting mosquitoes with human bait. The

presence and abundance of Ae. aegypti in these larval habitats, and the diurnal biting rates were measured to assess the impact the sp. may have in the event of a dengue epidemic. Certain, more remote localities were also selected to provide further samples of Ae. aegypti for the morphological and biochemical studies (Chs. 3 and 4).

2.1. Materials and Methods

Collections and observations were made at the twenty-two localities mapped in Fig. 1. The three farms, Armadale, Hope and Kloofwaters, are situated near Port Shepstone in Natal, near Mica in the north-eastern Transvaal and near Magaliesburg in the Magaliesberg mountains, respectively. I will refer to the latter two localities as Mica and Magaliesberg, respectively. St. Lucia represents the town St. Lucia Estuary. Tzaneen represents two sites: Jaffray's farm and the neighbourhood of the National Institute for Tropical Diseases.

2.1.1. Ovitraps in trees

Ovitraps consisted of bamboo segments which were cut to form receptacles or "pots". The bamboo pots were filled with water and a few dried poplar leaves were added to each one. They were hung in trees at heights of betweeen one and four meters above ground level at several localities in Natal and the Transvaal (Fig. 1). Eggs, larvae and pupae were collected over periods of 38-153 days from 7 November 1989 to 17 May 1990.

A second type of ovitrap was used to supplement the bamboo pots at two of the localities in the Transvaal,

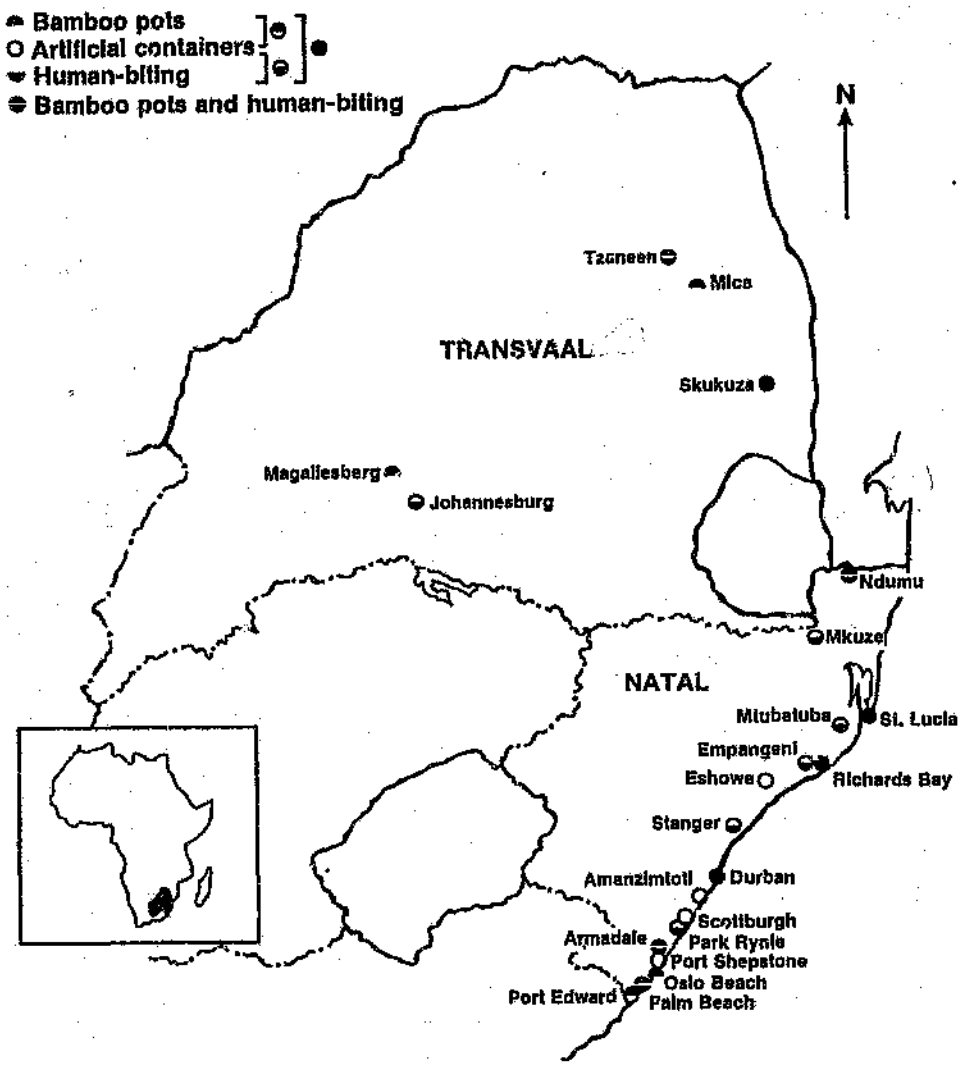


Figure 1. South African localities where Aedes aegypti was sampled, showing collection method.

viz. Magaliesberg and Mica. Eggs were collected on wooden tongue-depressors, which were inserted in water-filled, black plastic bottles suspended in trees. For the purposes of this study, no distinction was drawn between bamboo pots and the bottles.

Ovitraps containing eggs were returned to the laboratory along with any larvae and pupae (immature

stages) that were present. Eggs were flooded with tap-water and left for two days to hatch. All immature stages were reared to adults. The pot index is defined as the percentage of pots found to contain Ae. aegypti. The abundance index is the number of Ae. aegypti at each locality divided by the product of the number of pots and the number of days that they were exposed.

2.1.2. Leaf-axils

Samples of immature mosquitoes were taken from small collections of rain-water found in the leaf-axils of the Natal strelitzia or "wild banana", Strelitzia nicolai Regel and Koern (Strelitziaceae), at Palm Beach and of the Natal dracaena or "dragon plant", Dracaena hookeriana Koch (Agavaceae), at Oslo Beach.

2.1.3. Artificial containers

Immature mosquitoes were sampled from artificial containers in peridomestic sites such as motorcar scrapyards and municipal rubbish dumps at various localities (Fig. 1) from 5 November 1988 to 18 April 1989. Artificial containers consisted of discarded tyre-casings, floor-pans and tyre-wells in scrapped cars, metal drums, tins and beverage cartons. Where possible, the entire contents of containers were emptied into inspection trays. Containers holding large amounts of water, such as drums, and tyre-casings were sampled as follows: as much as possible of the floating leaf-litter was removed with forceps and a ladle was dipped repeatedly into the water until at least fifty larvae and/or pupae had been obtained, or at least three times until it was evident that there were no immature mosquitoes present. Larvae and pupae were reared to adults in the insectary and identified. Container indices

were recorded as the percentage of artificial containers colonized by Ae. aegypti at a particular site and were averaged for each locality.

2.1.4. Human-baited collections

Adult female mosquitoes were collected over the months of January to April 1989, November 1989 to February 1990, January to February 1991 and November to December 1991. The collection sites coincided with the artificial container sites, except for Palm Beach, Armadale, Ndumu and Tzaneen, where they coincided with the pot sites (Fig. 1). Collecting generally took place on days when the prevailing climatic conditions were warm, humid, partly cloudy and calm or slightly windy. Two to ten volunteers collected mosquitoes in the open during the daylight hours of 05h30 to 09h30 and 15h00 to 19h00, in view of the recorded diurnal activity-pattern of Ae. aegypti. Catchers used glass test tubes to trap mosquitoes that were probing on their legs and feet. At least two collections were made at each locality on different days and biting rates were based on the total number of mosquitoes collected in a locality, divided by the number of man-hours invested (the collection effort). Limited collections were also made from 10h00 to 15h00 in Durban and Palm Beach with the Durban collection being spaced one month apart in early 1991. Observations were made on various aspects of feeding behaviour.

2.1.5. Insectary conditions

Adult mosquitoes were held in cages measuring 160 X 160 X 160 mm^3 in an insectary maintained at 25-26 °C and 75-80 per cent RH. The daily light cycle consisted of a day period of twelve hours, a night period of ten hours, and two transitional periods of one hour each, consisting

of either decreasing or increasing light intensities to simulate dusk and dawn, respectively.

2.2 Results

2.2.1. Ovitraps in trees

Data from the pot survey of tree-hole breeding mosquitoes at eight different localities are given in Table 1. Aedes aegypti was not collected at Armadale and only 9 per cent of the pots at St. Lucia were utilized. The low pot index at St. Lucia was related to a low abundance index. Pot indices of 70 per cent and more consistently accompanied high abundance indices. The mean pot index was 60,3±9,8 ($\bar{x}\pm s_{\bar{x}}$) per cent and mean abundance index was 0,43±0,15 ($\bar{x}\pm s_{\bar{x}}$) Ae. aegypti/pot/day.

2.2.2. Leaf-axils

Although culicine larvae and pupae were found in the leaf-axils of Strelitzia nicolai and Dracaena hookeriana plants, these did not include the sp. Ae. aegypti.

Table 1. Survey for Aedes aegypti using pots at various localities in Natal and the Transvaal.

Locality	No. of pots	Exposure period(d)	Pot index[a]	Abundance index[b]
NATAL				
Port Edward	5	57	40	0,10
Palm Beach	10	57	100	1,17
Oslo Beach	15	57	93	0,85
Armadale	6	57	0	
Durban	15	57	53	0,14
Richards Bay	14	57	50	0,07
St. Lucia	23	56	9	<0,01
Ndumu	44	64	73	0,35
TRANSVAAL				
Magaliesberg	22	112	45	0,01
Skukuza	23	153	100	0,65
Mica	26	31	67	--
Tzaneen	15	38	93	1,39

a - $\frac{\text{No. of pots containing mosquitoes X 100}}{\text{Total No. of pots}}$

b - No. of Ae. aegypti/No. pots/No. days exposed.

2.2.3. Artificial containers

Table 2 lists the container indices for Ae. aegypti in artificial containers on the ground. Nine of the fifteen localities which were sampled had container indices greater than 50 per cent, with the three highest occurring at Port Shepstone, Empangeni and Durban.

Table 2. Immature Aedes aegypti in artificial containers and adults in human-baited collections at various localities in Natal and the Transvaal.

	Larvae		Adults	
Locality	No. of containers	Container index[a]	Man-hours	Biting rate[b]
NATAL				
Port Edward	17	47	--	--
Oslo Beach	--	--	2,0	0
Palm Beach	NF[c]	--	31,2	11,2
Port Shepstone	18	83	--	--
Park Rynie	37	54	4,0	18,0
Scottburgh	19	11	--	--
Durban	45	76	9,0	29,0
Stanger	13	54	5,0	12,4
Empangeni	18	78	4,5	18,0
Richards Bay	53	62	23,5	1,5
Mtubatuba	21	33	5,5	2,9
St. Lucia	9	56	8,0	0,8
Mkuze	10	70	5,5	3,5
Ndumu	NF	--	5,0	0,2
TRANSVAAL				
Johannesburg	33	45	2,0	13,0
Skukuza	10	70	13,8	4,8
Tzaneen	--	--	37,0	2,9

a - $\frac{\text{No. of containers with mosquitoes X 100}}{\text{Total No. of containers}}$

b - No. of biting females per man-hour

c - None Found

2.2.4. Human-baited collections

Aedes aegypti was collected at significantly high biting rates (11-29 Ae. aegypti per man-hour) at six of

the fourteen localities sampled (Table 2) including Johannesburg. Biting rates were low at localities in Natal north of Empangeni, correlating with generally low pot abundances (Table 1). In the paired adult collections made in Durban in early 1991, the first biting rate was 15 per man-hour and the biting rate one month later was 89 per man-hour. The rainfall totals for the thirty-day periods prior to each collection were 43 mm and 263 mm, respectively. The St Lucia and Ndumu populations were collected at biting rates lower than 1 per man-hour. Biting rates were also low in the north-eastern Transvaal* (Table 2), in spite of high abundances in the ovitraps (Table 1).

During the human-baited collections, male mosquitoes were observed to form small swarms, which rarely contained more than ten insects, about the catcher's exposed leg. These mosquitoes occasionally alighted on the "bait" or surrounding vegetation and could be caught with test tubes. They were identified as male Ae. aegypti. Usually within minutes of the formation of the swarm, the females arrived at the bait and would immediately be mated while still on the wing. Stationary males resting on the bait also became active in the presence of females. Copulating mosquitoes would either remain in flight or land on either the ground or the bait. After copulation, the females would approach the bait to feed. The females of Ae. aegypti were often slow to alight on the bait, particularly when a slight breeze was blowing. Females were also seen arriving at

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* Biting rates for Skukuza and Tzaneen differ from those published in Kemp and Jupp (1991) because of the addition of collection data acquired since that publication.

the bait to feed without the presence of the males.

At Palm Beach, where the only larval habitats that could be found were water-retaining leaf-axils of Strelitzia nicolai, adult Ae. aegypti were only caught in small numbers at any one collection site. In order to obtain a representative sample for this particular locality, human-baited collections were made at regular intervals during a roving search.

2.3. Discussion

The climate of the east coast of southern Africa is strongly influenced by the warm, tropical waters of the Mozambique and South Equatorial currents in the Indian Ocean, which become confluent just south of Madagascar to form the Agulhas current. The Agulhas flows southwards along the continental shelf off the Natal and eastern Cape coasts, and evaporation of the warm water from its surface provides good rainfall when carried onshore by the prevailing winds. The rain is responsible for the relatively luxuriant vegetation at the coast, inland as far as the escarpment and along the large rivers that derive from the climate and geology of the east-coast. The natural vegetation of the eastern coastal plane and Transvaal lowveld is similar and consists of bushveld and sour grassveld, interspersed with relict scrub and montaine forest (Acocks 1975). The lowveld rainfall is highly seasonal and less well dispersed than in Natal. Urban and resort development is particularly high on the Natal coast south of Durban, while northern Natal and the Transvaal lowveld are largely rural, farming areas interspersed with extensive game farms and nature reserves. As a result, these regions attract local and international tourism throughout the year.

Aedes aegypti on the south coast appears to be confined to the urban areas. The Palm Beach collections were made at a holiday resort consisting of large caravans (mobile homes) in permanent berths with attached storerooms and bathrooms. The vegetation of Palm Beach is typical for the region and includes dense stands of Natal strelitzias and dracaenas amidst the coastal scrub flora. Biting rates for Ae. aegypti were high. However, no discarded containers could be found in the resort and the only available breeding sites appeared to be roof-gutters, tree-holes and leaf-axils. Aedes aegypti could have been breeding in discarded containers just outside the resort but this was not investigated. The vegetation on the primary dunes at Oslo Beach was similar to that of Palm Beach, and was littered with discarded beverage cartons and cans. Artificial container indices and biting rates were only measured on the adjoining, urbanized, secondary dunes of Oslo Beach but Ae. aegypti was present in large numbers in the ovitraps on the primary dunes. Palm Beach and Oslo Beach lie about 40 km and 20 km, respectively, south of Armadale, near Port Shepstone. Armadale, however, is an uninhabited nature conservancy area established on several adjoining farms, and consists of near-virgin coastal forest with high populations of Natal strelitzias and dracaenas. Even though high densities of other phytotelmatous mosquitoes were present (Kemp and Jupp 1991), no Ae. aegypti were collected in the ovitraps or from human bait, except for a single specimen taken biting in late February 1991. It is possible that this specimen could have been transported in our vehicle from Palm Beach, which had been visited earlier that day.

In Durban, paired human-baited collections of Ae. aegypti were made one month apart where the second collection was fortuitously preceded by a six-fold

increase of monthly rainfall compared to the previous month. The biting rates increased dramatically from 15 to 89 per man-hour, reflecting a rapid increase in the density of the population with the improved breeding-conditions. Averaged over several collections, the Durban biting rates were the highest recorded for any of the localities.

The Ae. aegypti populations were highly anthropophilic in the urbanized areas as far north as Empangeni. Further north in Natal, however, the highest biting rate was only 3,5 per man-hour (Mkuze, Table 2), even though container indices were generally high because of the discarded tyres present at most of the chosen sites (Table 2). The Ae. aegypti populations at Ndumu are very poorly anthropophilic. Ndumu is a game reserve on the border between South Africa and Mozambique and attracts only small numbers of tourists because of its isolated position. Most of the ovitraps were set in riverine gallery forest and on the shores of a permanent pan in the reserve, where the dominant sp. is the sycamore fig, Ficus sycamorus Linnaeus (Moraceae). Even though the bamboo pots were heavily utilized by Ae. aegypti, only a single female was caught biting man. These results confirm the absence of biting Ae. aegypti during the extensive work undertaken by the staff of the Arbovirus Unit in the reserve in the 1960s (Dr Jupp, personal communication).

The Transvaal collections included anthropophilic populations of Ae. aegypti from Johannesburg in the south and Tzaneen in the north. The Johannesburg site was a small tyre-reprocessing business to the north of the city where shredded rubber was converted into commercial goods such as belts and sandals. The mosquitoes are believed to have been introduced from Durban via eggs in the used

tyres. As a result, it seems that there are temporary annual extensions of the range of Ae. aegypti from the tropics and subtropics to the densely populated, temperate Highveld region, where the sp. may only survive during the warmer seasons.

Skukuza was sampled for Ae. aegypti because it is the main tourist camp in the Kruger National Park and, therefore, is epidemiologically important to the dengue study. Skukuza supports a large permanent community, as it is also the administrational centre for the park, and comprises a staff village, light industry, and conservation and administration offices. The local population of Ae. aegypti was common in the ovitrap collections, as indicated by the high bamboo pot and abundance indices, and the moderate biting rates suggested that it was anthropophilic.

The remaining two Transvaal localities were selected to collect populations of Ae. aegypti that had not been exposed to humans and that were zoophilic. Aedes aegypti was not collected biting in the limited human-baited collections at Magaliesberg and was not very abundant in the ovitraps. No human-baited collections were made at Mica because the local population of Ae. aegypti was known to be zoophilic from an earlier study (Jupp and McIntosh 1990). These populations were needed for the taxonomic study on the subspp. of Ae. aegypti.

Most of the human-baited collections were made early in the morning or late in the afternoon, as these are the reputed times when Ae. aegypti is most likely to bite (Reed et al. 1901, Trpis et al. 1973). Teesdale (1955) also found a strong biting peak in the hour before midday and Lumsden (1957) reported biting peaks during the crepuscular periods. Among the South African populations,

both Durban and Palm Beach Ae. aegypti were collected at very high biting rates from 10h00 to 15h00 in human-baited collections. Christophers (1960) reviews several reports of Ae. aegypti feeding at night. Although this was not specifically investigated in the present study, Ae. aegypti was caught biting in artificial light indoors at Palm Beach. In general, the sp. is perhaps best described as a diurnal biter, especially as the mosquito is believed to rely primarily on visual stimuli to identify a potential blood-source at a distance (Christophers 1960). The behaviours of both male and female Ae. aegypti during the human-baited collections were similar to those reported by Hartberg (1971).

The highest biting rates in the South African populations of Ae. aegypti exceed the average rates recorded for East African populations. In twenty-four-hour human-baited collections during the 1952-1953 epidemic of chikungunya fever in Southern Province, Tanzania, biting rates for Ae. aegypti were 6,6 per man-hour (Lumsden 1957), while landing rates (possibly an over-estimation of biting rates) were 9,9 per man-hour in an automobile dump in Dar-es-Salaam, Tanzania (Trpis et al. 1973). Mukwaya (1974) collected Ae. aegypti in Kampala, Uganda from human bait and obtained biting rates over ten periods of twelve hours each of 0,01 per man-hour inside houses and 1,05 per man-hour outside.

The Ndumu and Skukuza localities are both close to international borders with Mozambique, a country that has recent experience of DEN. However, the introduction of DEN to South Africa through tourists from the endemic African and Asian regions is perhaps more likely than the co-incidental cross-border migrations of either human host or vector during an epidemic in a neighbouring country. The populations of Ae. aegypti in the major

tourist areas are clearly well adapted to coexist with humans and are highly anthropophilic, especially in Durban which is a densely populated city and international harbour. Therefore, there is a real danger that they may again become involved in human-to-human transmission of DEN virus, as happened before in Durban (Edington 1927).

CHAPTER 3. MORPHOLOGICAL VARIATION IN DIFFERENT POPULATIONS OF *Aedes aegypti*

According to Mattingly (1957), the absence of pale scales in the dorso-median area of the first tergite (first dorsal segment of the abdomen) distinguishes *Ae. aegypti* subsp. *formosus* from *Ae. aegypti* subsp. *aegypti* and *Ae. aegypti* var. *queenslandensis*. Subsp. *aegypti* is generally reported to possess a distinctly paler or browner body than subsp. *formosus* but this character was only considered by Mattingly to be taxonomically useful in the female. The value of the criterion of tergal ornamentation has remained unclear since McClelland (1960, 1974) disputed its taxonomical usefulness. Mattingly (1957) acknowledged that the presence of scales on the first tergite was "not, unfortunately, completely constant".

Several other studies besides McClelland's (1960, 1974) have also documented just how inconstant tergal ornamentation is in various African and Asian populations of *Ae. aegypti*. Summers Connal (1927) described morphological variation in "*Ae. argenteus* Poiret" (synonym of *Ae. aegypti*) from Nigeria. Based on the progeny from seven different matings, she found the entire range of tergal scale patterns, subsequently used by Mattingly to describe the three forms of *Ae. aegypti*, and concluded that the sp. was polymorphic rather than polytypic. In a World Health Organization mimeograph, Hartberg (1969) reported polymorphism in tergal ornamentation in siblings of wild-caught female *Ae. aegypti* from Dar-es-Salaam, Tanzania, using mosquitoes that were all dark in cuticle and "ground" colour (the

colour of the darker, background scales, which range from pale brown to black). Hartberg et al. (1986) studied the inheritance of the tergal scale pattern in two experimental strains of Ae. aegypti and found that tergal scale pattern was controlled by three independently assorting gene loci, and several mutant genes which could act as modifiers. They concluded that tergal scale pattern was not sufficient in itself to distinguish between mosquitoes from different populations. Mogi et al. (1984) found tergal scale patterns to vary considerably within populations from the Philippines, but failed to correlate scale pattern with any of the following: indoor vs. outdoor breeding, city vs. town breeding or occurrence of dry season. Mogi et al. (1989) also tested for correlation of tergal scale pattern with breeding habitat or season in northern Thailand and could find none, as before.

Perhaps the simplest way to conclusively demonstrate the veracity of a taxonomic character is to examine the offspring of individual females caught in the field. Female mosquitoes collected on human bait will have been fertilized under natural conditions where assortative mating can occur. Sibs of several South African populations of Ae. aegypti were examined to identify the two subspp. using Mattingly's (1957) criterion of white scales on the first tergite. The pattern of white scales on the dorsal area of the second tergite was also investigated in the sibling groups or families, as they will be referred to here. The term "family" should not be confused with the taxonomic category of the same name.

3.1. Materials and Methods

A series of populations of Ae. aegypti was sampled from eighteen localities, approximately covering the

distribution of the sp. in South Africa: one in the eastern Cape, twelve in Natal and five in the Transvaal (Fig. 1; Table 3). Mosquitoes were collected as eggs from bamboo pots, larvae and pupae from artificial containers, and adult females in human-baited collections (see Ch. 2). The anthropophilic nature of the mosquitoes in the egg and immature collections was deduced from the

Table 3. Populations of Aedes aegypti used in the morphological study.

Locality	Stages	Host-choice
CAPE		
Grahamstown	Larvae, pupae	Unknown[a]
NATAL		
Port Edward	Larvae, pupae	Unknown[a]
Palm Beach	Adults	Anthropophilic
Port Shepstone	Larvae, pupae	Unknown[a]
Park Rynie	Adults	Anthropophilic
Durban	Adults	Anthropophilic
Stanger	Adults	Anthropophilic
Empangeni	Adults	Anthropophilic
Richards Bay	Adults	Anthropophilic
Mtubatuba	Adults	Anthropophilic
St Lucia	Larvae	Anthropophilic
Mkuze	Larvae	Anthropophilic
Ndumu	Eggs	Zoophilic
TRANSVAAL		
Johannesburg	Adults	Anthropophilic
Magaliesberg	Eggs	Unknown[a]
Skukuza	Larvae, pupae	Anthropophilic
Mica	Eggs, larvae, pupae	Zoophilic
Tzaneen	Adults	Anthropophilic

a - Probably anthropophilic.

results of human-baited collections made previously at the same localities.

The F_1 progeny were obtained in separate families from samples of Ae. aegypti females collected in the field or reared from egg and larval collections in the laboratory. The gravid parental females (mothers) were placed in separate glass vials (50 mm length, 25 mm diameter), each containing a base of cotton-wool and a strip of filter-paper against the side. Vials were capped with fine netting. The mothers were released after depositing their eggs on the moistened cotton-wool and filter-paper substrates, and the vials were kept in the insectary until the substrates had dried. Eggs were hatched by filling the vial with a weak, filtered poplar-leaf infusion of tap-water (prepared at least four days before) and left overnight. Immature mosquitoes were reared through to adults and ten or more female sibs from each family were pinned.

Specimens were examined with a binocular dissecting microscope at 60-80 times magnification in blue-filtered, incident light. The silvery-white scales on the dorsal surfaces of the first tergite, T_1 and the second tergite, T_2 (forming basal bands when present on T_2) were counted. Where a mosquito specimen lacked pale scales on T_1, it was classified as the formosus form, otherwise it was classified as the "type" form, according to Mattingly's (1957) criteria. Samples of sibs representing the families were classified as "homogeneous" if all specimens in the sample were either formosus or type form, and "heterogeneous" if the sample contained both forms. Ranges, means, standard errors and 95 per cent confidence intervals were calculated for the two tergal scale counts in each population. The means and variances of the two tergal scale counts for the differ t

populations were subjected to one-way analysis of variance, Bartlett's test for homogeneity of variance and the non-parametric Kruskal-Wallis test (equivalent to the Mann-Whitney U test for two unpaired samples) using microcomputer software, Epi Info Version 5.1b (Dean et al. 1990) and Statgraphics Version 5 (Rockville, Maryland, USA: STSC, Incorporated).

3.2. Results

A selection of three of the families from Park Rynie in Natal demonstrates the range of tergal phenotypes on T_1 and T_2 (Fig. 2). The first two mothers were both of the formosus form and the third represented the type form. The formosus mothers gave rise to a wide range of T_1 phenotypes in their offspring, with sibs of both morphological forms of Ae. aegypti occurring. They also differed markedly in T_2 phenotype, with a basal band of white scales present in the one mother but absent from the other, yet the offspring of both possessed intermediate T_2 phenotypes ranging from presence to absence of basal bands. The type mother, however, produced only two sibs of the type form, the majority being typical of the formosus form. In this family, pale scales were also rare on T_2.

The F_1 families are classified in Table 4 according to the variation in T_1 phenotype. Out of 196 families, only three (1,5 per cent) were homogeneously formosus and seventy-five (38,3 per cent) homogeneously type form; 60,2 per cent were heterogeneous. The formosus forms were from the anthropophilic population of Park Rynie and the probably zoophilic population from Magaliesberg. All eighteen populations included families composed homogeneously of type forms except for the one from Park Rynie.

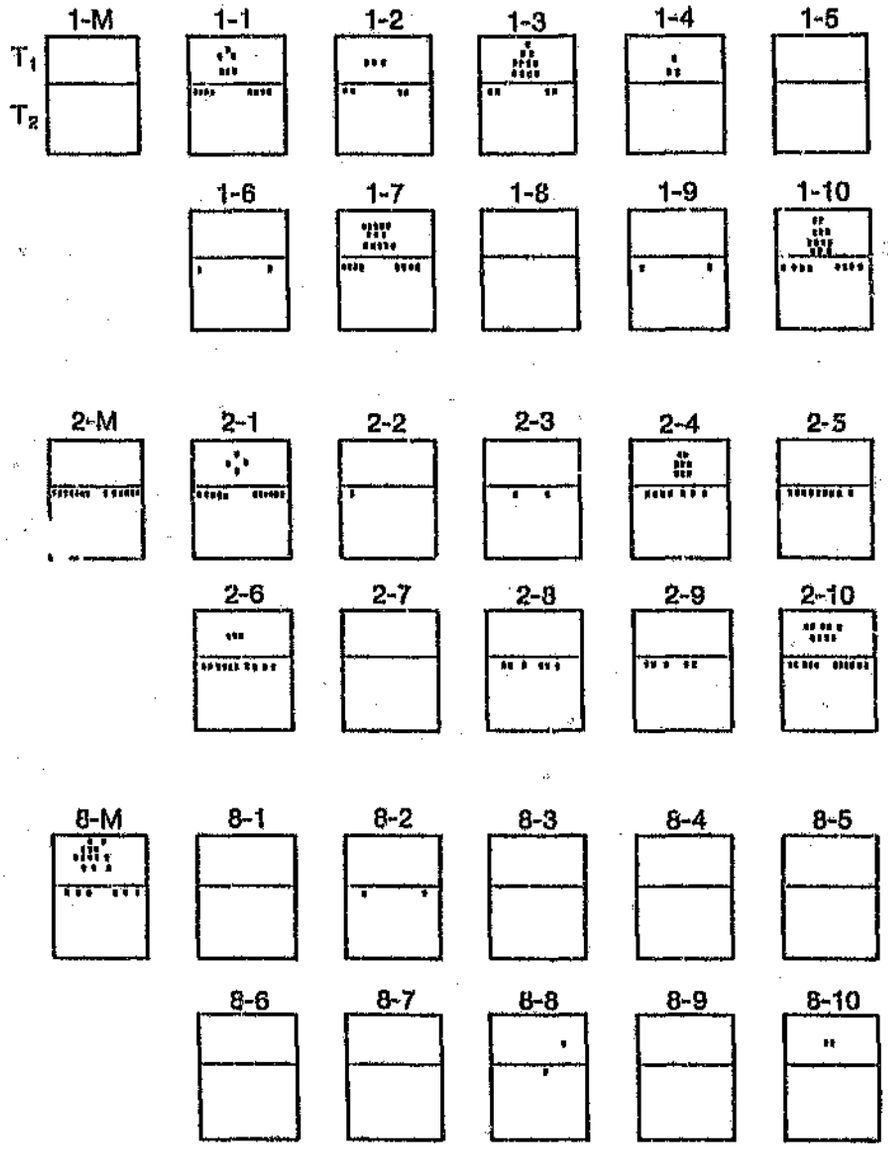


Figure 2. Distribution of white scales on the first (T_1) and second (T_2) tergites in three Aedes aegypti mothers (M) and samples of their respective daughters.

Table 4. Classification of F_1 families of Aedes aegypti from various localities according to the occurrence of formosus and type forms.

Locality	n°	Proportions (per cent) Homog.[a] formosus	Homog. type	Heterog.[b] both forms
Grahamstown	11	0	45	55
Port Edward	11	0	36	64
Palm Beach	12	0	25	75
Port Shepstone	12	0	33	66
Park Rynie	12	8	0	92
Durban	10	0	50	50
Stanger	10	0	40	60
Empangeni	9	0	44	56
Richards Bay	11	0	45	55
Mtubatuba	8	0	38	62
St Lucia	11	0	82	18
Mkuze	10	0	10	90
Ndumu	11	0	36	64
Johannesburg	11	0	36	64
Magaliesberg	15	13	20	67
Skukuza	11	0	73	27
Mica	13	0	31	69
Tzaneen	8	0	62	38

a - Homogeneous.

b - Heterogeneous.

c - Sample size (number of families).

In a similar treatment of tergal phenotype in respect of T_2, 55,6 per cent of the families were heterogeneous for the presence of the white scales in basal bands in the F_1 females.

Table 5 summarizes the scale counts of the two tergites for samples of Ae. aegypti from each of the various populations. A sample consisted of sibs in ten to thirteen families from each locality. For each of the eighteen samples, the range of values for T_1 and T_2 is given together with the respective mean and standard

Table 9. Number of white scales on the first (T_1) and second (T_2) tergites of F_1 female Aedes aegypti from various localities.

		T_1		T_2	
Locality	n[a]	Range	Mean[b]	Range	Mean
Grahamstown	104	0-45	7,6±0,9	4-35	16,5±0,6
Port Edward	112	0-40	5,6±0,7	0-18	7,5±0,4
Palm Beach	110	0-30	5,7±0,7	0-25	4,6±0,4
Port Shepstone	104	0-34	6,0±0,7	0-11	3,1±0,3
Park Rynie	110	0-24	2,0±0,4	0-15	4,2±0,4
Durban	102	0-35	8,8±1,0	0-22	5,8±0,5
Stanger	100	0-60	13,1±1,5	0-25	6,0±0,5
Empangeni	94	0-30	6,6±0,7	0-23	4,8±0,5
Richards Bay	109	0-40	7,2±0,7	0-16	5,4±0,4
Mtubatuba	81	0-24	5,8±0,6	0-17	7,1±0,5
St Lucia	100	0-40	11,9±0,9	0-19	3,9±0,4
Mkuze	96	0-15	2,8±0,3	0-21	4,4±0,4
Ndumu	125	0-19	4,5±0,4	0-27	9,7±0,5
Johannesburg	104	0-26	5,0±0,6	0-22	7,4±0,5
Magaliesberg	129	0-25	3,4±0,5	0-20	4,4±0,3
Skukuza	108	0-31	8,8±0,7	0-26	8,4±0,6
Mica	130	0-30	5,4±0,5	0-20	9,3±0,4
Tzaneen	63	0-30	8,1±1,1	0-16	5,7±0,6

a - Sample size (number of F_1 females).

b - $\bar{x}\pm s_{\bar{x}}$.

error. All the ranges of values for both T_1 and T_2 overlap, being similar for all populations except for differences at the upper end of the range. The ranges are generally larger for T_1 than for T_2, except in Mkuze and Ndumu.

The various 95 per cent confidence intervals of the mean scale counts on T_1 and T_2 have been plotted in Fig. 3. Contrary to the overlap in ranges, there are

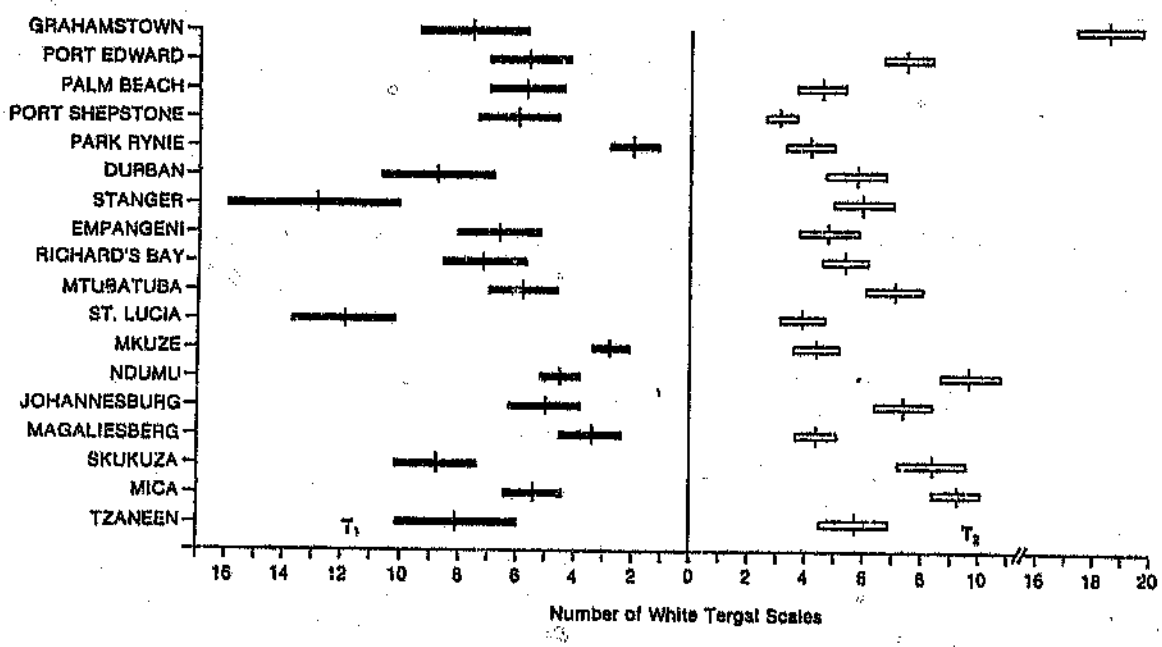


Figure 3. Sample means and 95 per cent confidence intervals for the number of white scales on T_1 and in the basal band on T_2 in various populations of Aedes aegypti.

noticeable differences in some of the confidence intervals. Bartlett's χ^2 test (Table 6) revealed a significant lack of homogeneity in the variances of the tergal scale counts. The Kruskal-Wallis H test revealed highly significant differences between localities in the sample means for both tergal scale counts (Table 6: data sets A and B). However, there was no significant difference between the mean scale counts on T_1 for the two behavioural forms, anthropophilic and zoophilic, of Ae. aegypti (Table 6: data set C).

Table 6. Statistical comparisons of the mean scale counts on T_1 and T_2 in the different populations and behavioural forms of Aedes aegypti.

Data[a]	Test	Statistic	df[b]	P-value
A:	Bartlett's χ^2	220,000	17	<0,001
	Kruskal-Wallis H	252,310	17	<0,001
B:	Bartlett's χ^2	56,097	17	<0,001
	Kruskal Wallis H	469,458	17	<0,001
C:	Bartlett's χ^2	94,948	1	<0,001
	Kruskal-Wallis H	0,748	1	0,387

a - A: Mean scale count for T_1 in the different populations.
B: Mean scale count for T_2 in the different populations.
C: Mean scale count for T_1 in populations grouped into anthropo- and zoophilic forms.

b - Degrees of freedom.

3.3. Discussion

The morphological heterogeneity within more than 60 per cent of the families studied, with silvery-white scales on T_1 present in some sibs and absent from others in the same family, clearly shows that this character is unsuitable for subspecific discrimination in Ae. aegypti. For this reason it must be emphasized that the references to formosus and type forms in Section 3.2. (Results) were conditional. This is consistent with the polymorphism found by Hartberg (1969), the only other work with Ae. aegypti where families reared from wild females caught in the field were studied, rather than the progeny of females mated selectively in the laboratory.

One of the three families that were homogeneously dark on T_1 originated from Park Rynie, a population that, paradoxically, is highly anthropophilic. The homogeneous presence of white scales on T_1 in the remaining families should not be unexpected. Machado-Allison (1971), as reported by VandeHey et al. (1978), described a gene termed "black tergite" (designated Bt) that is expressed phenotypically as absence of pale scales on T_1. Its allele Bt^+ controls the presence of white scales on T_1. Provided the parent mosquitoes are not homozygous for Bt and at least one parent is homozygous for Bt^+, simple dominance would ensure the expression of pale scales on T_1 in all of the sibs in the next generation.

The mean number of white scales on either tergite differed significantly in the various populations, but when the anthropophilic and zoophilic populations were grouped separately, the composite means for T_1 were not statistically different (Table 6). A more sophisticated statistical analysis (Jupp et al. 1991) of the same data, comparing each of the zoophilic or probably zoophilic

populations with each of the anthropophilic populations in turn, also failed to show significant differences (Waller-Duncan test, α=0,05). Jupp et al. (1991) also demonstrated by multivariate analysis that all eighteen populations differed significantly in respect of tergal ornamentation on T_1 and T_2. Gene flow is not likely to occur between geographically dispersed populations and a polymorphic character such as tergal scaling may be expected to vary considerably.

The earliest reference to dark and pale forms of Ae. aegypti is not from Africa but from the Townsville area of northern Queensland, Australia (Hill 1921), under the synonym, Stegomyia fasciata. Larvae of the dark form were collected from an artificial container in dense coastal scrub 550 m away from human dwellings, and adults were collected biting at the same site. This form was also found in a tree-hole. The pale form was collected within the precincts of a hospital and is described as larger than the dark form. In the laboratory, the pale form bred true, producing pale females for several generations, while the dark form produced both pale and dark phenotypes as well as intermediates. In both cases, the males were always dark. Mattingly (1957) interprets the description of the two forms as the type form and var. queenslandensis.

In sub-Saharan Africa, Mattingly's formosus is the feral form of Ae. aegypti, occurring widely throughout the region, and differs from the domestic form which breeds in stored-water containers inside huts in small, highly dispersed populations on the east coast of the continent. This description, however, is oversimplified. Haddow (1945) found Ae. aegypti to be largely sylvan and zoophilic in the Bwamba district of Uganda. Lumsden (1955) recorded predominantly anthropophilic Ae. aegypti

in Newala District and on the Makonde Plateau in southern Tanzania, describing the adults as commonly pale but with intermediates and dark forms also occurring. These Ae. aegypti were breeding in water storage pots inside villagers' huts as well as in tree-holes on the plateau, but it is not clear whether the tree-hole mosquitoes were different from the domestic forms. At Ganda, a village on the Kenyan coast, van Someren et al. (1958) and McClelland (1960) collected ae. aegypti larvae in abundance in villager's huts, and McClelland (1960) found that approximately 82 per cent of the Ae. aegypti biting in and around the huts were var. queenslandensis. However, McClelland (1959) collected anthropophilic Ae. aegypti in a plantation near Entebbe, Uganda, which were identified to be predominantly the dark form, formosus. Trpis and Hausermann (1978) demonstrated anthropophily in feral Ae. aegypti in the Rabai area of eastern Kenya by human-baited collections. Nasidi et al. (1989) reported on urban Ae. aegypti breeding well into the dry season in Nigeria during the 1987 epidemic in Ogbomosho, yet the population had a relatively low biting rate of 2,7 per man-hour. The Ogbomosho mosquitoes were shown to have isozyme profiles typical of West African Ae. aegypti (Miller et al. 1989), generally considered to be Mattingly's type form.

Mattingly (1957) notes several reports of feral Ae. aegypti (described as formosus) from Zaire, Nigeria and Zimbabwe that had become urbanized at inland localities, e.g., Liégeois (1944) who described highly urbanized populations from several localities in the then Belgian Congo, breeding preferentially within ten metres from houses. In contrast, Philip (1933) described Ae. aegypti breeding prolifically in bamboo pots set between thirty and ninety yards away from the nearest building in dense forest; these mosquitoes were treated as a continuation

of the domestic population near the compound of the West African Yellow Fever Commission near Lagos, Nigeria. These examples are intermediate between the two extreme forms, var. queenslandensis, which apparently has never been found in natural breeding-places, and the feral populations described by Haddow (1945) and Garnham et al. (1946).

In South Africa, Ae. aegypti is common along the east coast but is probably more dispersed elsewhere and absent from the west coast. Muspratt (1956) speculates that either there are two "strains", presumably the type and feral forms, or that the sp. is adaptable to both feral and peri-domestic habitats. Based on its adaptability and its inland, peridomestic distribution further north in Africa, it appears that Ae. aegypti in South Africa is exclusively Mattingly's subsp. formosus, as he had originally proposed (Mattingly 1957). The references to collections of "pale" forms from Durban and Ntambanana in Natal (Mattingly 1953), interpreted by Muspratt (1956) to be Ae. aegypti var. queenslandensis, may have represented limited introductions as there have been no subsequent recordings of pale forms from South Africa. In the present study, the blackness of the darker scales, which form the background to the white markings, lends further weight to this synopsis, and may also be more reliable taxonomically than the presence of pale scales on T_1 for distinguishing between the two subspp. However, the range of background colours given for Mattingly's type form could be problematic in the apparently limited cases where it occurs in sympatry with the classical, dark, sub-Saharan form.

CHAPTER 4. ELECTROPHORETIC ANALYSIS OF ISOZYMES IN *Aedes aegypti*

The separation of isozymes by polyacrylamide gel electrophoresis (PAGE) has been an important tool for distinguishing closely related spp. in, amongst others, the *Anopheles gambiae* complex, which include the principal mosquito vectors of malaria in Africa (Mahon *et al*. 1976, Miles 1979). Several studies have employed the technique to distinguish geographically isolated populations of *Ae. aegypti*, in particular the two putative subspp., *aegypti* and *formosus*.

Scott and McClelland (1975) investigated sympatric populations of "dark feral" and "pale domestic" forms of *Ae. aegypti* from the coastal area near Mombasa, Kenya, and found differences in isozyme allele frequencies between them. This was ascribed to "partial reproductive isolation" even though assortative mating could not be demonstrated from the tergal morphology of the progeny. Tabachnick *et al*. (1979) classed the two forms from the same area in Kenya as "sylvan" and "domestic", and showed that gene flow was restricted between populations but not that it was entirely absent. Tabachnick and Powell (1979) extended the earlier work into a world-wide study and related differences in allele frequencies in African and New World populations of *Ae. aegypti* with "light domestic" and "dark, often sylvan" forms. Wallis *et al*. (1983) also reported on allele frequency variation in the sp. on a "macro-geographic" scale.

A PAGE analysis of the isozymes of South African *Ae. aegypti* was undertaken to characterize different

geographically isolated populations and to identify the two putative subspp.

4.1. Materials and Methods

4.1.1. Mosquitoes

Female Ae. aegypti from the following localities were used for the PAGE study: Grahamstown, Palm Beach, Park Rynie, Durban, Mkuze, Richards Bay, Ndumu, Johannesburg, Magaliesberg (Kloofwaters farm) and Mica (Hope farm) (see Fig. 1). A long-established colony from the Rockefeller Institute (now the Rockefeller University) was included as a standard reference with some of the PAGE runs. F_1 and F_2 material obtained from field mosquitoes and reared in the laboratory were used in the electrophoretic work, except for the Rockefeller colony (generation unknown) and some of the work with the Richards Bay colony (F_9 and F_{10}). Mosquitoes were killed by freezing at -20 °C and stored in liquid nitrogen until required for PAGE.

4.1.2. Isozymes

The PAGE methods were those of Coetzee et al. (1993) and were adapted for use at the NIV.

A. Polyacrylamide gels

Polyacrylamide gels measuring 10 X 10 cm^2 were cast to a thickness of 1,6 mm on supporting plates of thin glass, with gel strength, T = 7,0 per cent. Each gel was composed of 7,0 ml of 25 per cent (w/v) acrylamide/N,N'-methylene-bis-acrylamide (MBA); 5 ml of 25 per cent (w/v) sucrose; 0,63 ml of 2 per cent (w/v) ammonium persulphate (initiator); 12,5 µl tetramethyl-ethylenediamine (accelerator); and 12,36 ml of the relevant gel buffer

solution. The proportion of MBA to acrylamide gave a cross-linking [illegible] (C) of 2,7 per cent. Before polymerization, [illegible] gel solution was degassed under slow agitation and vacuum for 15 minutes. Gels were usually layered two deep to allow two different enzyme systems with the same buffer solution requirements to be stained for one set of samples.

B. Sample preparation

Individual mosquitoes were thawed to room temperature and homogenized in a small droplet of distilled water on a frosted glass slide. A sample of the homogenate was absorbed by a small, 5 X 7 mm^2 piece of filter paper (Whatman No. 1).

C. Electrophoresis

Gels were run on a Bio-Phoresis Horizontal Electrophoresis System (Bio-Rad) with the gel cooled to 4 °C by refrigerant (distilled water mixed 3:1 with car radiator anti-freeze additive) circulating through the gel support. A Bio-Rad Model 500/200 power supply provided a constant potential difference of 250 V and maximum available current, ranging from 10-50 mA, depending on the particular resistance-characteristics of the various gel and electrode buffer systems. The buffer system recipes* were those of Steiner and Joslyn (1979) for most of the enzyme systems excepting malic enzyme and superoxide dismutase/ocatanol dehydrogenase (Barlow and Ridgway 1971). All gels were pre-electrophoresed at

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* "Recipe" is a common term for the chemical formulation of an isozyme stain or electrophoretic buffer solution.

250 V for 30 minutes. PAGE run-times depended on the time for a band of tracking dye, bromophenol-blue, to traverse the length of the gel.

After pre-electrophoresis, the gel was cut into two parts to give a strip 10 mm wide at one end (the cathodal end) and the two parts were left in contact with each other on the supporting glass plate. The sample was inserted between the two sections of the gel as soon as the filter paper had dried. Strips of cotton cloth or "wicks" were used to connect the anodal and cathodal ends of the gel to the respective electrode buffers to complete the circuit.

Included with every gel was a standard reference, consisting of a sample of haemolyzed blood (1:1 mixture of whole human blood and de-ionized water) stained with 0,1 mM bromophenol-blue in ethanol.

D. Enzymes and stains

The twelve enzymes that were studied, with the respective numbers of loci coding for isozymes in each enzyme system, are given in Table 7. The stain recipes of Steiner and Joslyn (1979) were used (occasionaly with modifications) for all enzyme systems except for Gdh (Harris and Hopkinson 1977), Pgm (Harris and Hopkinson 1977, Urbanelli and Bullini 1988), Me (Coetzee, SAIMR, personal communication) and the combined stain, Sod/Odh (Mahon et al. 1976). The recipes are presented in Appendix A.

Table 7. List of enzymes used in the PAGE study of isozymes of Aedes aegypti.

Enzyme system	E.C. No.[a]	No. of loci
Adenylate kinase (Ak)	2.7.4.3	2
Esterase (Es)	3.1.1.1	3
Glucose dehydrogenase (Gdh)	1.1.1.47	1
Glucose phosphate isomerase (Gpi)	5.3.1.9	1
Glyceraldehyde phosphate dehydrogenase (Gapdh)	1.2.1.12	1
Hexokinase (Hk)	2.7.1.1	4
Isocitrate dehydrogenase (Idh)	1.1.1.42	2
Malate dehydrogenase (Mdh)	1.1.1.37	2
Malic enzyme (Me)	1.1.1.40	1
Octanol dehydrogenase (Odh)	1.1.1.73	1
Phosphoglucomutase (Pgm)	2.7.5.1	1
Superoxide dismutase (Sod)	1.15.1.1	1

a - Enzyme classification numbers according to the Commission on Biological Nomenclature (1973).

4.1.3. Isozyme analysis

A. Diagnostic markers

Individual mosquitoes from the different populations were run on the same gel to find unique electromorphs that could serve as diagnostic markers for distinguishing the populations. One mosquito from the Rockefeller reference colony was included on each gel.

B. Allele frequencies

The allele frequencies of eight gene loci coding for

isozymes in three enzyme systems (Ak, Hk and Idh) were determined for the Durban and Ndumu populations of Ae. aegypti, representing anthropophilic and zoophilic behavioural forms, respectively. Gels were scored by measuring the distance of migration of the allelomorphs (allelic electromorphs) from the origin in millimeters. Firstly, the groups of electromorphs representing the different isozyme loci in a particular enzyme system were identified, numbering them in the order of progressive migration from the origin (Ayala et al. 1972, Munstermann 1979, Munstermann and Craig Jr 1979). Secondly, the band or bands representing the paired allelomorphs of each locus were identified for each genome. The allelomorphs were scored by dividing their distances of migration with that of the most commonly occurring allelomorph amongst the genomes on the gel, and grouped in classes of 10 per cent on either side of the most common allelomorph (having a value of 100 per cent and written, e.g., Ak-1^{100}). Where no allelomorph could be designated "100" on a gel, R_f values were determined relative to the haemoglobin electromorph on this gel, as well as one that did have a 100 per cent allelomorph, and the first gel was scored accordingly. These classes were classified alphabetically from the slowest, nearest to the origin, to the fastest, nearest to the anodal end of the gel, for the entire range of allelomorphs in both populations.

In a separate exercise, gels were also scored qualitatively by assigning alleles to genomes on a single gel according to the visually apparent banding patterns, without actually measuring the distances of migration. Alleles were assigned approximate R_f values relative to the most common allelomorph.

Each individual genome could be characterized by a couplet of alphabetic letters representing an allele

pair. Genomic data in this form may be converted into allele frequencies by the FORTRAN software, BIOSYS-1 Release 1.7 (Swofford and Selander 1981), implemented on an IBM-compatible personal computer. BIOSYS-1 provides statistical comparisons of allele frequency distributions against expected Hardy-Weinberg equilibria, and generates observed and expected heterozygosities as well as genetic distances and identities.

The observed heterozygosities are derived from direct counts of the heterozygous loci in a population and the mean, $\bar{H}_o$, is simply the average or arithmetic mean of these values. The mean expected heterozygosity, $\bar{H}_e$, is the average of the unbiased estimates of heterozygosity for each locus in a population (Nei 1978). The Student's t-tests used in the comparisons of the heterozygosities were generated using Statgraphics Version 5. When dealing with samples where the variances were shown to be equal using the variance-ratio (F) distribution for the two-tailed test, the probabilities produced by the computer programme (associated with 2n-2 degrees of freedom) were accepted. However, where samples had unequal variances, the more conservative probability associated with only n-1 degrees of freedom was used (Zar 1984).

BIOSYS-1 produces genetic distance (D) indices, including Nei's standard D and Rogers' D. Nei's D estimates the mean number of codon differences per locus between two populations that have accumulated since the populations diverged from their common ancestor (Nei 1972). As for this study, the codon differences are usually based on a sample of nucleotide substitutions that produce electrophoretically detectable, mutant alleles. Rogers' D differs from Nei's D by being a purely geometrical calculation of the mean distance between allelle frequency vectors per locus for the two

populations, using the Pythagorean theorem (Rogers 1972). The theoretical range of Nei's D is from zero to infinity and of Rogers' D is from zero to unity.

4.2. Results

All of the electromorphs in the twelve enzyme systems migrated anodally on the gel. All of the isozymes but one had banding patterns consistent with monomeric or dimeric quaternary structures, ranging from single bands (homozygotes) to two bands (monomeric heterozygotes) to three bands (dimeric heterozygotes).

4.2.1. Comparison of electromorphs in different populations

Members of the ten populations of Ae. aegypti from South Africa could not be conclusively distinguished in any of the twelve enzyme systems based on a comparison of electromorphs, although certain electromorphs in some systems occurred in only a proportion of the mosquitoes within a population (e.g., see "4.2.2. Allele frequencies of anthropophilic and zoophilic Aedes aegypti"). The South African mosquitoes also did not differ consistently from the Rockefeller reference colony with respect to electromorphs.

4.2.2. Allele frequencies of anthropophilic and zoophilic Aedes aegypti

The sample gel in Fig. 4 shows the Idh enzyme system for Ae. aegypti females from Ndumu and Durban. The slower group of electromorphs has been termed Idh-1 and the faster group Idh-2. Both isozymes are composed of dimers in the heterozygous genotype. Lanes 3, 8 and 9 were

heterozygous for Idh-1, while lanes 3 and 8 were composed of one type of heterozygote and lane 5 was composed of another for Idh-2. The most common allelomorph (seen in the homozygous genotypes in Fig. 4) often corresponds to one of the heterozygotic allelomorphs.

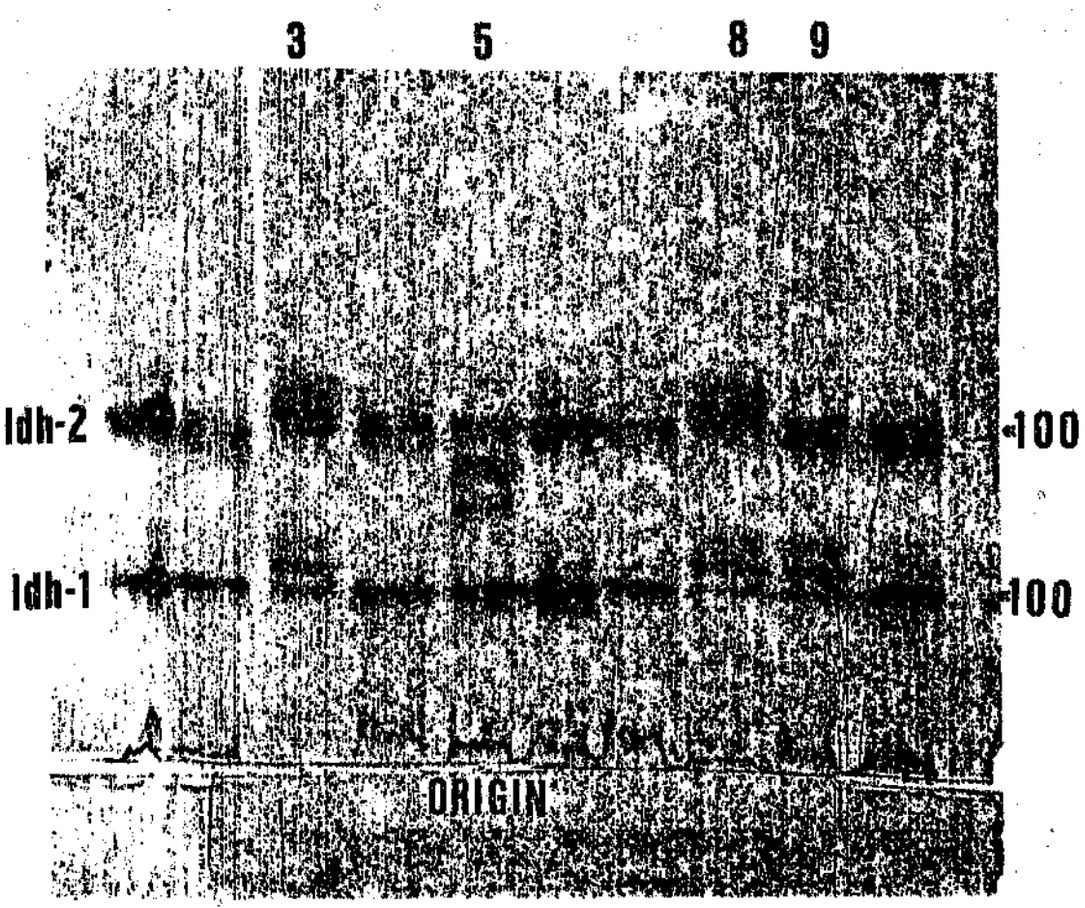


Figure 4. Sample electrophoretic gel of the isocitrate dehydrogenase isozymes of Aedes aegypti.

The allele frequencies for samples of the anthropophilic and zoophilic behavioural forms of Ae. aegypti are given in Table 8. Only one of the eight loci, Hk-3, was monomorphic* for both populations. Both Idh-1 and -2 were essentially monomorphic in the zoophilic population but polymorphic to varying degrees for the anthropophilic population. The degree of polymorphism at the other loci was similar for the two populations. The Hk-1^{90} allele was present at the same frequency as the Hk-1^{100} allele in the zoophilic population.

The allele frequency distributions differed significantly from Hardy-Weinberg expected frequencies at all loci, except for Ak-2 in the Durban population and Idh-2 in the Ndumu population.

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* A monomorphic locus is one where the most common allele has a frequency greater than 95 per cent (Ayala et al. 1972).

Table 8. Allele frequencies for isozymes in anthropophilic and zoophilic populations of Aedes aegypti from Durban and Ndumu, respectively.

Isozymes	Alleles	Durban	Ndumu
Ak-1	n[a]	57	57
	80	0,009	
	90	0,123	0,096
	100	0,728	0,588
	110	0,123	0,132
	120	0,018	0,114
	Other		0,070
AK-2	n	57	57
	80	0,105	0,035
	90	0,070	0,079
	100	0,754	0,833
	Other	0,061	0,053
Hk-1	n	24	25
	80	0,104	0,140
	90	0,438	0,200
	100	0,438	0,620
	Other	0,021	0,040
Hk-2	n	27	27
	80	0,019	
	90	0,037	0,037
	100	0,944	0,852
	110		0,111
Hk-3	n	27	27
	100	1,000	1,000

Table 8 continued.

Isozymes	Alleles	Durban	Ndumu
Hk-4	n	27	27
	90	0,037	
	100	0,926	0,926
	110	0,037	0,074
Idh-1	n	56	57
	90	0,027	
	100	0,902	1,000
	110	0,018	
	Other	0,054	
Idh-2	n	57	57
	80	0,026	0,009
	100	0,772	0,991
	110	0,202	

a - Genome sample size.

In the Ndumu mosquitoes, 62,5 per cent and, in Durban, 87,5 per cent of the loci were polymorphic. The genetic variability in the two populations are presented in Table 9. In both populations of Ae. aegypti, observed and expected levels of heterozygosity were clearly different for two loci, Ak-1 and Hk-1, while expected heterozygosities were generally high for Ak-1, Ak-2 and Hk-1. The Ndumu population did not differ significantly from the Durban population with respect to either of the two mean heterozygosities, $\overline{H}_o$ ($t = 1,5009$; $P = 0,1771$) or $\overline{H}_e$ ($t = 0,4083$; $P = 0,6893$). The observed and expected mean heterozygosities did not differ statistically for either Durban ($t = -1,9091$; $P = 0,0774$) or Ndumu ($t = -2.3030$; $P = 0,0547$).

Table 9. Genetic heterozygosity at eight isozyme loci in anthropophilic and zoophilic populations of Aedes aegypti from Durban and Ndumu, respectively.

Isozyme	Durban		Ndumu	
	h_o[a]	h_e[b]	h_o	h_e
Ak-1	0,018	0,443	0,105	0,618
Ak-2	0,421	0,415	0,105	0,298
Hk-1	0,083	0,619	0,040	0,566
Hk-2	0,037	0,108	0	0,266
Hk-3	0	0	0	0
Hk-4	0	0,143	0	0,140
Idh-1	0,143	0,185	0	0
Idh-2	0,211	0,366	0,018	0,018
Mean, $\overline{H}$	0,114[c]	0,285[d]	0,034	0,238
$s_{\bar{x}}$	0,051	0,073	0,016	0,087
s^2	0,021	0,043	0,002	0,061

a - Observed heterozygosity (estimate by direct count).
b - Expected heterozygosity (Nei's unbiased estimate assuming Hardy-Weinberg equilibrium).
c - $\overline{H}_o$, observed mean heterozygosity.
d - $\overline{H}_e$, expected mean heterozygosity.

The estimate of genetic distance between the two behavioural forms of Ae. aegypti from Durban and Ndumu given by Nei's D was 0,022 and by Rogers' D was 0,109.

The visual, qualitative scoring method did not provide significant differences in allele frequencies, heterozygosities or genetic distances from the qualitative method. In fact, none of the seven loci (Hk-3 is excluded because it was monomorphic in both

populations) had allele frequency distributions in Hardy-Weinberg equilibrium using the qualitative method.

4.3. Discussion

Atypical electromorphs were occasionally encountered in this study. Very infrequently, the Mdh-2 region of a gel consisted of a cluster of five bands instead of three bands or a single band. This may have represented a mutant genotype consisting of a tetrameric heterozygote or else have been a combination of the typical Mdh-2 heterozygote and the monomeric heterozygote of a third, rare Mdh locus. Occasionally, an electromorph would also develop in gels stained for Idh that migrated faster than Idh-1 and -2, possibly representing a rare locus, Idh-3. These atypical electromorphs were excluded from the isozyme analysis.

Behaviourally different forms of An. gambiae were shown to form a sp. complex and could be successfully differentiated by polytene chromosome mapping and characteristic isozyme electromorphs (Mahon et al. 1976, Miles 1979). Unfortunately, there are no comparable karyotypic or electrophoretic markers for characterizing the two behavioural forms of Ae. aegypti (Tabachnick and Powell 1979). Paterson et al. (1976) interpreted the allele frequency differences, reported by Scott and McClelland (1975) for ecologically separated subpopulations from a particular locality in Kenya, as evidence for assortative mating. However, Trpis and Hausermann (1975, 1978) suggested that mosquitoes from this locality hybridized naturally, based on the presence of a peridomestic form of Ae. aegypti, behaviourally and morphologically intermediate between the feral and domestic forms.

Nei's D (Nei 1972) and Rogers' D (Rogers 1972) are the two measures of genetic distance most commonly used in the electrophoretic literature. The estimates of these indices suggest very little phylogenetic divergence between the anthropophilic and zoophilic populations of Ae. aegypti. Ideally, a large number of loci should be included to obtain a reliable estimate of genetic distance, as this reduces the sampling error (standard error) of the mean heterozygosity. However, the sampling error can also be reduced to some extent by studying a large number of genotypes, even though the number of loci used may be small (Nei 1978). Indeed, Nei's D between the two behavioural forms from South Africa was almost identical to the average D of 0,018 between the "outdoor forms" (subsp. formosus) from West and East Africa, and roughly half the value of 0,049 calculated between the two putative subspp. from East Africa by Tabachnick and Powell (1979).

Grant et al. (1988) modified Thorpe's (1982) graph of the frequency distributions of published values of Nei's standard genetic identity for different categories of taxa to reflect values of Nei's D instead. D ranges from zero to 0,25 for the taxonomic category of "conspecific populations", and from approximately 0,10 to greater than 2 for the category of "congeneric species". By this definition, the two South African populations and the populations studied by Tabachnick and Powell (1979) and Wallis et al. (1983) all fall into a single sp.

Estimates of mean values of D between populations of Ae. aegypti from different regions and continents range from 0,017 to 0,055 (Wallis et al. 1983) and from 0,014 to 0,049 (Tabachnick and Powell 1979). The mean D between sympatric sylvan and domestic forms from coastal Kenya was 0,042 ± 0,004 ($s_{\bar{x}}$) (Tabachnick et al. 1979). These

values of D are significantly smaller than those found for subspp. of the fruitfly, Drosophila, by Ayala et al. (1974). Perhaps more aptly, they are comparable to those between populations of the culicine, Culex pipiens sensu stricto, from Italy and populations of the behaviourally different Culex pipiens var. molestus from Italy, Israel and Egypt, with D ranging from 0,023 to 0,067 (Urbanelli et al. 1985). These data would suggest that there is no evidence for subspp. of Ae. aegypti at the isozyme level.

The heterozygosities of the two populations of Ae. aegypti from South Africa (Table 9) are high when compared to other reported heterozygosities. Cianchi et al. (1985) listed $\bar{H}_e$ values of 0,07 to 0,21 for several different aedine species. Tabachnick et al. (1979) reported values of 0,141 for a "domestic" population and 0,163 for a "feral" population of Ae. aegypti but, by comparing only the h_e values for loci that correspond to six of the eight used in this study, viz. Ak-2, Hk-2 to -4 and IDH-1 and -2, a value of 0,269 is calculated for $\bar{H}_e$ for the "domestic" population. Tabachnick and Powell (1979) measured $\bar{H}_e$ values of between 0,096 and 0,155 for 19 loci in populations of Ae. aegypti representing Asia, the Americas and the two subspp. from Africa; however, the mean of the "average expected heterozygosities" for four highly differentiated loci (Idh-1, Pgm, Mdh and G-6-pdh) was 0,264. Differences between h_o and h_e are rarely provided in the extant literature, although differences in h_e for different gene-pools or geographic populations have been described, e.g., Tabachnick and Powell (1979). Unfortunately, Tabachnick and Powell (1979) calculated standard errors for the $\bar{H}_e$ values of the various populations without taking into account the inherent error in the estimate of h_e at each locus. The total error cannot be smaller than the largest error in any one constituent heterozygosity estimate. Therefore, the

standard error of $\bar{H}_o$ for the East African formosus population must be at least 0,046 and not 0,009 as reported. A confidence level of 95 per cent in the estimate of $\bar{H}_o$ requires the doubling of the standard error, giving 0,155 ± 0,092 so that this value is not significantly different from any of the other $\bar{H}_o$ values, contrary to the authors' interpretation of the data. The eight loci employed in the present study are clearly insufficient for determining representative mean heterozygosities for the two South African populations, even though their heterozygosities are comparable at the level of the locus.

The lack of agreement of allele frequency distributions with Hardy-Weinberg equilibria is notable, considering that these loci are believed to segregate in Mendelian, codominant fashion, particularly in the Hk and Idh systems (Tabachnick 1978, Munstermann and Craig Jr 1979). This suggests that gene flow between components of the mosquitoes sampled in either locality was restricted, possibly reflecting the temporal genotypic variation in a population sampled over several generations. The superficial deviation of $\bar{H}_o$ from $\bar{H}_e$ is seen to be statistically insignificant (P = 0,05) and reflects instead the inherent error due to the sampling methods used. PAGE is believed to detect as little as 30 or 40 per cent (Nei 1971) of the total isozyme polymorphisms present, largely because it is estimated that only 30 per cent of all possible nucleotide substitutions code for amino acids with different charges (Shaw 1970). If a proportion of the isozyme alleles go undetected, one would expect deviations from Hardy-Weinberg equilibrium to be common. Munstermann (1979) reported that twenty-seven out of 134 enzyme loci-Ae. aegypti population combinations deviated from Hardy-Weinberg equilibrium at P = 0,05.

Fairbairn and Roff (1980) tested the power of the χ^2 test for comparing observed allozyme frequencies with the expected frequencies of alleles segregating in Hardy-Weinberg equilibrium in the case of a dialelic locus, against three alternative hypotheses: random variation, variation due to post-translational modification of protein structure, and variation due to the segregation of an undetected ("null" or "silent") allele. They conclude that the χ^2 test has little power in all three cases when sample sizes are smaller than two-hundred, and when the sample is made up of several smaller collections rather than a single, large, homogeneous collection. Fairbairn and Roff (1980) note that Ak isozymes lose positively charged amide groups from glutamine or asparagine with sample storage, producing an increase in electromorphs towards the anode. Tabachnick (1978) and Tabachnick and Powell (1979) report on null alleles in Idh-2, Hk-2 and Hk-4. Interestingly, the one monomorphic locus in both the Ndumu and Durban populations, Hk-3 has been recorded as not having null alleles in other studies (Tabachnick 1978, Wallis et al. 1983).

The recent development of DNA- and RNA-based tests for characterizing different populations of Ae. aegypti (Ballinger-Crabtree et al. 1992, Wattam and Christensen 1992) may cast new light on the taxonomic status of the behaviourally different forms. Ballinger-Crabtree et al. (1992) demonstrated genetic variation in Ae. aegypti by amplifying randomly selected fragments of DNA in its genome by polymerase chain reaction (random-amplified polymorphic DNA polymerase chain reaction or RAPD-PCR). Using discriminant analysis of the frequencies of RAPD fragments, they could distinguish between populations of Ae. aegypti from central Africa and populations from other continents (described as subspp. formosus and aegypti, respectively), and calculated a relatively large

value of 0,3 for Nei's unbiased D (Nei 1978). Unfortunately, several of the Nigerian localities from which the "formosus" populations originated were urban (Ballinger-Crabtree et al. 1992), casting doubt on their true taxonomic status.

The scoring of gels is, to some extent, an arbitrary process due to several factors, including distortion in the gel which causes spurious banding patterns with equivalent electromorphs lying in a smooth curve relative to each other. This makes a strictly quantitative scoring method difficult to apply. Even in the absence of such an obvious irregularity in the gel, electromorphs can assume a large array of different mobilities, differing by less than 1 per cent from each other. One way to make sense of the data would be to determine the mean mobility and standard error for each individual isozyme in a large sample of mosquito genotypes. As this could be a laborious process, the data may be simplified by ordering the quantitative mobility data into suitable classes. The data may also be simplified by qualitatively allocating alleles according to the judgment of the experimenter. Both short-cut methods allow the experimenter to compensate for spurious mobility differences due to gross distortion of the gel, but loss of information is unavoidable. This loss may be a major contributor to gene frequency distributions not being in Hardy-Weinberg equilibrium, besides the problems caused by hidden heterogeneity and insufficient sample size.

In summary, the lack of Hardy-Weinberg equilibrium in the gene frequency distributions at six of the eight isozyme loci studied cannot be easily resolved. The accuracies of the genetic distance indices were therefore undermined. Mean heterozygosities were not statistically different but were high in comparison to published

figures, mainly because the number of loci sampled was too small to be representative of the mosquito genome. Despite these obvious shortcomings in the present study, the results at the very least indicate a high level of isozyme polymorphism in both the anthropophilic and zoophilic Ae. aegypti populations in South Africa.

CHAPTER 5. VECTOR COMPETENCE OF POPULATIONS OF *Aedes aegypti*

The natural vectors of DEN only include aedine species in the subgenera *Diceromyia*, *Finlaya* and *Stegomyia*. Forest canopy-dwelling mosquitoes from which DEN virus has been isolated in the field include the *Ae.* (*Finlaya*) *niveus* group in Asia (World Health Organization 1976, Rudnick 1978) and the *Ae.* (*Diceromyia*) *furcifer* group in Africa (Cordellier *et al.* 1983, Roche *et al.* 1983, Cornet *et al.* 1984). These studies demonstrated the involvement of mosquitoes and monkeys in forest cycles of DEN in similar fashion to the classic enzootic cycles of yellow fever virus. Hervy *et al.* (1984) refer to a single isolation of DEN-2 from a pool of *Ae.* (*Aedimorphus*) *cumminsii* (Theobald) but this is the only such record for this subgenus.

The vectors in the forest cycles also include several *Stegomyia* spp. that are believed to transmit DEN to humans in the rural and urban environments of Asia and Africa (Simmons *et al.* 1931, Mackerras 1946, Rosen *et al.* 1954, Rudnick and Chan 1965, Cordellier *et al.* 1983, Roche *et al.* 1983, Cornet *et al.* 1984, Hervy *et al.* 1984). Circumstantial evidence suggests a similar role for *Ae.* (*Gymnometopa*) *mediovittatus* (Coquillett) in the Caribbean (Gubler *et al.* 1985). This sp. and *Ae.* (*Protomacleaya*) *triseriatus* (Say) have been incriminated as DEN vectors in the laboratory (Freier and Grimstad 1983, Gubler *et al.* 1985), as have Japanese *Ochlerotatus* (Eshita *et al.* 1982, quoted in Gubler *et al.* 1985). Limited susceptibility has also been demonstrated experimentally with *Ae.* (*Aedimorphus*) *vittatus* (Bigot)

(Mavale et al. 1992). Jupp and Kemp (1993) demonstrated the vector potential of South African Stegomyia and Diceromyia in the laboratory for DEN-1 and DEN-2.

No natural DEN vectors have been found in other genera, except for reports of isolations from Culex (Culex) quinquefasciatus Say (Chen et al. 1982, Chen et al. 1986) during epidemics involving DEN-2 and DEN-3 in China. These findings are supported by laboratory evidence for the vector competence of Culex quinquefasciatus in China (Liu and Zhao 1985, Tong et al. 1987, quoted in Huang et al. 1985; Xue-dong and Gui-fang 1985, quoted in Gubler 1988). However, other recent studies have failed to show infection after intrathoracic inoculation of virus in populations of the sp. from Taiwan, the Philippines and Puerto Rico (Rosen et al. 1985, Huang et al. 1992). Rosen et al. (1985) found various spp. in the genera Armigeres, Anopheles, Culex, Heizmannia, Topomyia and Tripteroides to be refractory to infection by the oral route with DEN viruses. Eretmapodites quinquevittatus Theobald is similarly refractory to DEN-1 and DEN-2 in the laboratory (Jupp and Kemp 1993).

Aedes aegypti has long been recognized internationally as the urban vector of DEN (Siler et al. 1926, Smith 1956, Rao 1964), and knowledge of its vector competence is of great consequence. A certain degree of variation in susceptibility has been recorded for different populations of Ae. aegypti throughout its distribution (Gubler et al. 1979, Tardieux et al. 1990). The present study was concerned with the implications of variations in infection and transmission rates in South African populations.

The two serotypes, DEN-1 and -2, were selected for

the following reasons: DEN-1 virus is the only serotype to have been incriminated or isolated in South Africa (Kokernot et al. 1956, Blackburn and Rawat 1987), and recently DEN-3 virus has been the most active of the four serotypes in the Afrotropical region. The likelihood of DEN-2 being imported from south-east Asia is greater than from other parts of Africa. South Africa has strong trading ties with the Far East and the use of a DEN-2 strain from Taipei in this study seems a particularly apt choice.

5.1. Materials and Methods

5.1.1. Mosquitoes

Aedes aegypti populations from five localities (Fig. 1) were selected for the vector competence study. Mosquitoes originated from human-baited collections at Durban, Palm Beach and Skukuza; artificial-container collections at Richards Bay; and ovitrap collections at Durban, Ndumu and Skukuza (Tables 10 and 11). Several of the tests were done with Ae. aegypti females taken directly from field populations in the human-baited collections but most were done with F_1 and F_2 females, plus one test with the F_3 (Table 10). The ages of these field-caught adults were not determined but none of the adult mosquitoes reared in the laboratory were older than twenty-one days.

A reference colony of Ae. aegypti from the Rockefeller Institute was employed in the vector competence tests with DEN-1 to act as a standard against which to compare the local populations.

5.1.2. Amplification, preparation and detection of virus

DEN-1 (Cassim strain) was isolated at the NIV from the acute-phase serum of a patient from Durban in October 1985 (Blackburn and Rawat 1987) and had been passaged three times in suckling mouse brain and freeze-dried. DEN-2 (BC 5007 strain) was isolated from a febrile patient from Taipei, Thailand in November 1989 and had been passaged twice in Aedes albopictus tissue culture cells (C6-36) at the Centers for Disease Control, USA, and freeze-dried prior to being sent to the NIV.

Rehydrated DEN-1 and DEN-2 seed viruses were amplified by serial inoculation (see "5.1.3. Inoculation of mosquitoes with virus") of samples of adult Ae. aegypti females. The Cassim strain (DEN-1) was amplified by three or four passages and the BC 5007 strain (DEN-2) was amplified by two passages in Ae. aegypti. The stock-viruses were stored at -70 °C as whole, infected mosquitoes.

The viruses used in the experiments were based on units of thirty virus-stock mosquitoes per 2 ml of BPA incorporating 10 per cent heat-treated foetal calf serum (FCS), 20 µg/ml gentamicin, 500 units/ml penicillin G, 500 µg/ml streptomycin sulphate and 0,5 µg/ml amphotericin B. BPA is an abbreviation for 0,75 per cent (w/v) of bovine serum albumin in phosphate-buffered saline pH 7,2. One such unit of infective material was sufficient for inoculation into a group of mosquitoes to determine one infection rate while several units were required when a blood-virus mixture was made up to provide an infective blood-meal.

Virus-stock mosquitoes were homogenized in BPA and

centrifuged at 3000 rpm for thirty minutes in a refrigerated ultracentrifuge cooled to 4 °C. The supernatant was drawn off and passed through a 0,45 µm filter before being used to inoculate mosquitoes.

Mosquito infection was determined using an indirect immunofluorescent antibody test (IFAT), a variation of the method of Kuberski and Rosen (1977), to detect DEN viral antigen in mosquito tissues. Mosquitoes were decapitated by scalpel and the severed heads squashed under siliconized coverslips on glass slides. Head-squashes were washed in acetone to kill any virus present, then incubated in the presence of mouse anti-DEN hyperimmune ascitic fluid (HAF), rinsed, dried, and stained with a commercial anti-mouse fluorescent conjugate (conjugate of fluorescein isothiocyanate and anti-mouse immunoglobulins). Fluorescent antigen was visualized under ultra-violet light at 250 times magnification.

5.1.3. Inoculation of mosquitoes with virus

The intrathoracic method of inoculation as described by Rosen and Gubler (1974) was used to amplify, assay or titrate DEN virus in mosquitoes. Mosquitoes were anaesthetized with carbon dioxide and a small volume of inoculum ($\bar{x}\pm s$ = 0,34±0,02 µl; see "Appendix B") was injected intrathoracically into each mosquito. Inoculated mosquitoes were placed in suitable cages which were supplied with cotton-wool pads soaked in a 5 per cent sucrose solution and were covered with polythene bags. This provided both nourishment and a high humidity for the mosquitoes. The cages were kept in a security insectary at approximately 30 °C for eight days (see "Appendix C"). After the incubation period, mosquitoes were killed by freezing at -20 °C and stored at -70 °C

until required for either preparation of infective blood-meals or viral assay.

5.1.4. Titration of virus

Virus in the infective blood-meals were titrated at the start of each feed. Six ten-fold dilutions of a sample of the infective blood-meal were prepared and 0,34 µl aliquots were inoculated into batches of fifteen highly susceptible Ae. aegypti or Eretmapodites quinquevittatus mosquitoes per dilution. The mosquitoes were held in a security insectary for eight days at 30 °C (see "5.1.3. Inoculation of mosquitoes with virus"), after which they were killed by freezing and stored at -70 °C for later processing. The proportions of infected head-squashes were determined for ten mosquitoes from each assay batch by IFAT (see "5.1.2. Amplification, preparation and detection of virus"). The viral titres were calculated as the $\log_{10}$ MID_{50} per ml using the method of Reed and Muench (1938), representing the dilution at which fifty per cent of the inoculated mosquitoes became infected.

In two of the oral infection experiments, one with each DEN serotype, the infective blood-meal was titrated again three hours after the start of the experiment to measure the drop in titre during the feeding period.

5.1.5. Infective feeds and mosquito susceptibility

Samples of seventy-five to a hundred female Ae. aegypti per locality were put into each of two cages with dimensions of 160 X 160 X 160 mm^3. The mosquitoes were starved under standard insectary conditions (see "2.1.5. Insectary conditions") for eighteen to twenty-four hours before being moved to the security insectary for their

infective feed. Here, the cages were enclosed in polystyrene boxes which were provided with water-soaked pads of cotton-wool to provide a high humidity.

An infective blood-meal was prepared from one part of virus filtrate (see "5.1.2. Amplification, preparation and detection of virus") mixed with two parts of fresh, defibrinated, human blood donated just prior to the experiment. Using the "pledget method", a discardable petri-dish containing a cotton-wool pad soaked with approximately 5 ml of the infective blood-meal was placed in the bottom of each cage. (Two feeding pads required ninety virus-stock mosquitoes ground in six ml of BPA, giving approximately 5,5 ml of virus filtrate.) Due care was taken not to produce aerosols that may have infected the experimenter. After at least 35 mosquitoes had engorged on the blood-virus mixture, the pad was removed from the cage and disposed of safely. The mosquitoes were tubed singly in test tubes plugged with cotton-wool, and studied under a hand-lens of 10 times magnification to identify the engorged ones. The feeding rate was determined and well-engorged mosquitoes were transferred to a new cage. The unfed mosquitoes were destroyed.

Another <u>in vitro</u> feeding method was employed to expose mosquitoes to an infective blood-meal in the earlier experiments with DEN-1, i.e. the "droplet method" of Gubler and Rosen (1976). Single mosquitoes in vials closed with nylon mesh were presented with an infective blood-meal in the form of a small droplet on top of the mesh. This method was compared with the pledget method for the Rockefeller colony of <u>Ae. aegypti</u> to discover differences in feeding rates.

The cage of engorged mosquitoes was provided with a large cotton-wool pad soaked in 5 per cent sucrose

solution and was enclosed in a plastic cage-cover. The temperature in the security insectary was adjusted to approximately 30 °C and was monitored on a daily basis over an incubation period of fourteen to twenty days, whereafter infection rates were determined and transmission experiments done.

Infection was demonstrated by IFAT on head-squashes (see "5.1.2. Amplification, preparation and detection of virus"). Two head-squash infection rates (HSIRs), one for each DEN serotype, were calculated for each population of Ae. aegypti. The HSIR is defined as the proportion of a sample of mosquitoes which had engorged on an infective blood-meal, and that had developed disseminated infections that could be detected by IFAT on head-squashes.

The effect of varying the incubation period from fourteen to twenty days on the HSIR was investigated through regression analysis.

5.1.6. Experimental transmission

The in vitro feeding technique described by Aitken (1977) was employed to demonstrate the secretion of DEN virus in the mosquito's saliva, as an approximation of viral transmission. A sample of at least twenty mosquitoes, which had engorged on an infective blood-meal, were selected for each "transmission feeding" experiment. Mosquitoes were starved for eighteen to twenty-four hours prior to the transmission feed. Capillary feeding tubes (with one end drawn to about half the diameter of the tube) were filled with a 20 mm column of feeding solution consisting of equal parts of 10 per cent sucrose solution and FCS, equivalent to a volume of 6,8 µl. The wings and legs of the mosquitoes were removed

under carbon dioxide-anaesthesia. The proboscis of the mosquito was inserted into the feeding fluid contained in the capillary tube (the "capillary fluid") and the mosquito left to hang from its proboscis with the capillary tube supported vertically in a suitable stand. The mosquito was allowed to feed until the level of capillary fluid had dropped by at least 2 mm, whereupon it was removed and stored at -70 °C until the test for infection of the head-squash could be carried out. These mosquitoes will be referred to as "secreting mosquitoes".

The partially consumed capillary fluid was ejected from the capillary into 25 µl of BPA and the capillary flushed at least twice in the BPA to eject as much of the mosquito saliva as possible. The salivary solution was inoculated intrathoracically into six highly susceptible (assay) mosquitoes of the spp. Ae. aegypti or Eretmapodites quinquevittatus per capillary tube. The inoculated assay mosquitoes were placed into small, 100 mm high, cylindrical cages made from PVC piping with a diameter of 110 mm and enclosed with fine netting at each end. These were labelled according to the transmitting mosquito. The cages were provided with pads of cotton-wool soaked in 5 per cent sucrose solution and covered with polythene bags. After an incubation period of eight days in a security insectary at 30 °C (see under "5.1.3. Inoculation of mosquitoes with virus"), the assay mosquitoes were killed by freezing and stored at -70 °C for later processing.

In order to determine transmission rates with the two DEN serotypes for the different populations of Ae. aegypti, four of the six assay mosquitoes were tested by IFAT on head-squashes (see "5.1.2. Amplification, preparation and detection of virus"). Transmission was considered to have occurred if at least one of these four

assay mosquitoes had a positive head-squash infection. The transmission rate (TR) is thus defined as the proportion of a sample of infected mosquitoes which secreted virus to the capillary fluids. An alternative transmission rate was also calculated as the proportion of the original sample of engorged mosquitoes (regardless whether infection could be demonstrated in their head-squashes) which secreted virus into the capillary fluids.

Those engorged mosquitoes not needed for the transmission feeds were included in the determination of the HSIR for a population (see "5.1.5. Infective feeds and mosquito susceptibility"). The vector competence index (VCI) of each population of Ae. aegypti for DEN-1 and DEN-2 was calculated from the products of the decimal fractions of the relevant HSIRs and TRs. The maximum VCI of 1,0 represents 100 per cent HSIR and TR in a population.

5.2. Results

5.2.1. Incubation temperatures and viral titres

Incubation temperatures ($\bar{x}\pm s$) in the security insectary, over a long period and constituting a large number of observations (n = 286), ranged from a minimum of 28,8±1,4 °C to a maximum of 30,9±1,3 °C.

The origins and generations of the various samples of Ae. aegypti used in the infective feeds as well as the titres of virus in the blood-meals are shown in Tables 10 (DEN-1) and 11 (DEN-2). On the two occasions when blood-meals were titrated before and after the feeding period, the paired titres were $10^{-7,3}$ and $10^{-7,0}$ MID_{50}/ml for DEN-1, and $10^{-7,1}$ and $10^{-6,7}$ MID_{50}/ml for DEN-2. The drop-off in titre during the feed was usually lower than this as most of

the mosquitoes were observed to be engorged within the first hour.

Table 10. Vector competence experiments with DEN-1: *Aedes aegypti* populations and viral titres in infective blood-meals.

Population	Generation	Source	Viral titre (MID_{50}/ml)
Palm Beach	F_3	Biting	$10^{-6,1}$
	P[a]	Biting	$10^{-7,1}$
Durban	F_2	Biting	$10^{-6,3}$
	P	Biting	$10^{-7,1}$
Richards Bay	F_2	Tyres	$10^{-6,1}$
	F_1	Tyres	$10^{-7,1}$
Ndumu	F_1	Ovitraps	$10^{-6,3}$
	F_2	Ovitraps	$10^{-7,9}$
Skukuza	P	Ovitraps	$10^{-8,4}$
	P	Ovitraps	$10^{-6,9}$
Rockefeller[b]	-	Lab colony	$10^{-7,2}$
	-	Lab colony	$10^{-7,9}$

a - Parental generation.

b - Rockefeller Institute colony of unknown generation.

Table 11. Vector competence experiments with DEN-2: *Aedes aegypti* populations and viral titres in infective blood-meals.

Population	Generation	Source	Viral titre (MID_{50}/ml)
Palm Beach	F_1	Biting	$10^{-7,2}$
	F_1	Biting	$10^{-7,2}$
	F_2	Biting	$10^{-7,9}$
Durban	F_1	Biting	$10^{-7,5}$
	F_1	Ovitraps	$10^{-7,0}$
Richards Bay	F_1	Tyres	$10^{-7,5}$
	F_2	Tyres	$10^{-7,2}$
Ndumu	F_2	Ovitraps	$10^{-7,1}$
	F_1	Ovitraps	$10^{-7,1}$
	F_2	Ovitraps	$10^{-7,1}$
Skukuza	F_1	Biting	$10^{-7,9}$
	F_2	Biting	$10^{-7,0}$

5.2.2. Presentation of infective blood-meal

The pledget feeding method gave higher feeding rates than the droplet method in two of the three mosquito samples shown in Table 12, and it consistently provided a sufficiently large number of engorged mosquitoes in all three. Droplet feeding is a more labour intensive method because it requires the individual tubing of large numbers of mosquitoes. The pledget feeding method was therefore more convenient and more efficient than the droplet feeding method and was the method of choice in all subsequent experiments.

Table 12. A comparison of the effects of two *in vitro* feeding methods on feeding rate in *Aedes aegypti*.

Feeding method	Population		Colony
	Durban	Ndumu	Rockefeller
Droplet	38/132[a]	78/150	9/150
Pledget	58/95	48/150	37/150

a - $\frac{\text{No. of engorged mosquitoes}}{\text{No. of mosquitoes in feeding cage}}$

5.2.3. Vector competence

Susceptibility and transmission data for the replicate experiments with the different samples of *Ae. aegypti* are given in Tables 13 (DEN-1) and 14* (DEN-2). Transmission rate is either based on infected mosquitoes only (TR_I) or on engorged mosquitoes whether infected or not (TR_{II}). Replicate results for a particular population differ noticeably, e.g., with DEN-2 virus the HSIRs for four samples from Durban differ by as much as 38,9 per cent and the two TR_Is for Skukuza by 66,7 per cent (Table 14). This discrepancy between replicate results extends to TR_{II} as well, e.g., 46,7 per cent in the Durban population with DEN-1 (Table 13).

*HSIRs for Durban differ slightly from the published values in Jupp and Kemp (1993) due to the addition of new data.

Table 13. Head-squash infection rates (HSIRs) and transmission rates (TRs) in five populations and a reference colony of Aedes aegypti given infective feeds with DEN-1 virus.

Population	Incubation period, days	HSIR	TR_I[a]	TR_{II}[b]
Palm Beach	17,19	6/40	3/3	3/20
	16,17	9/32	3/6	3/20
Durban	17,19	15/22	12/13	12/15
	13,15	13/30	8/11	8/24
Richards Bay	18	19/40	5/9	5/20
	17,18,19	10/36	5/6	5/28
Ndumu	18,19	14/43	3/6	3/16
	18,19	18/45	3/3	3/20
Skukuza	14	3/16	3/3	3/16
	17,20	2/31	1/1	1/20
Rockefeller	18-20	16/30	10/10	10/20
	15,18	2/44	1/2	1/20

a - $\frac{\text{No. of mosquitoes that transmit virus}}{\text{No. of infected mosquitoes in the sample}}$

b - $\frac{\text{No. of mosquitoes that transmit virus}}{\text{No. of engorged mosquitoes}}$

Table 14. Head-squash infection rates (HSIRs) and transmission rates (TRs) in five populations of Aedes aegypti given infective feeds with DEN-2 virus.

Population	Incubation period, days	HSIR	TR_I[a]	TR_{II}[b]
Palm Beach	17,18	5/35	1/1	1/22
	17,18	6/36	0/3	0/24
	15	9/36	5/6	5/30
Durban	17,18	1/6	-----ND[c]-----	
	14,15	10/18	6/8	6/13
	14	2/7	-----ND-----	
	15	13/36	9/12	9/30
Richards Bay	15,18,20	15/36	9/11	9/28
	14,17,18	6/36	2/3	2/25
Ndumu	17,18	18/36	9/14	9/30
	14	7/11	-----ND-----	
	13,14	4/35	3/3	3/30
Skukuza	15,16	7/35	2/6	2/29
	19,20	13/36	12/12	12/30

a - $\frac{\text{No. of mosquitoes that transmit virus}}{\text{No. of infected mosquitoes in the sample}}$

b - $\frac{\text{No. of mosquitoes that transmit virus}}{\text{No. of engorged mosquitoes}}$

c - Not done.

Because of the discrepancies between them, the replicate values were treated as belonging to a single large sample and new HSIR and TR_I values, $HSIR_P$ and TR_P, were calculated for each population of Ae. aegypti with each of the DEN serotypes (Table 15). All five populations of Ae. aegypti were susceptible to DEN-1 and DEN-2 viruses. The population from Palm Beach was weakly

susceptible to both DEN serotypes while that from Skukuza was least susceptible of all the populations to DEN-1. The remaining populations were all more susceptible to DEN-1 than the Rockefeller colony of Ae. aegypti and the Durban population developed the highest $HSIR_p$s with both serotypes.

Values for TR_p were all significantly higher than 50 per cent but lower than the 91,7 per cent of the Rockefeller colony with DEN-1, except for the Skukuza population (Table 15). The Durban population was ranked as the most competent vector group for both serotypes based on VCI. The populations from Richards Bay and Ndumu had similar VCIs to the Rockefeller colony for DEN-1 while Palm Beach and Skukuza populations were

Table 15. The vector competence of five populations and a reference colony of Aedes aegypti given infective feeds with DEN-1 and DEN-2.

Population	DEN-1			DEN-2		
	$HSIR_p$[a]	TR_p[b]	VCI[c]	$HSIR_p$	TR_p	VCI
Palm Beach	20,8	66,7	0,14	18,7	60,0	0,11
Durban	53,8	83,3	0,45	38,8	75,0	0,29
Richards Bay	38,2	66,7	0,25	29,2	78,6	0,23
Ndumu	36,4	66,7	0,24	35,4	70,6	0,25
Skukuza	10,6	100,0	0,11	28,2	77,8	0,22
Rockefeller	24,3	91,7	0,22	------ND[d]------		

a - Population head-squash infection rate (per cent).

b - Population transmission rate (per cent) based on infected mosquitoes only.

c - Vector competence index = $HSIR_p/100 \times TR_p/100$.

d - Not done.

both poorer vectors. There was little difference in VCI between the populations for DEN-2, except for the one from Palm Beach.

A statistical evaluation of the significance of the differences in $HSIR_p$ and VCI between the populations of Ae. aegypti is given in Table 16 (χ^2 values and probabilities are tabulated in Appendix D). The Palm Beach and Skukuza populations both differ significantly from the other populations in $HSIR_p$ for DEN-1 but not from each other. Palm Beach differs significantly from Durban and Ndumu in $HSIR_p$ for DEN-2; there are no such differences between the other populations. With regard to VCI for DEN-1, all of the populations differ significantly from each other except for Richards Bay and Ndumu. For DEN-2, only Palm Beach differs from Durban, Richards Bay and Ndumu, there being no differences in VCI between the other populations. The TR_p values were compared by Fisher exact test because of the small sample sizes. No significant differences were found between the populations for this measurement.

Table 16. Statistical[a] significance of the differences in $HSIR_p$[b] and VCI[c] between five populations of Aedes aegypti for DEN-1 (below the diagonal) and DEN-2 viruses (above the diagonal). NS = not significant; S = significant.

Locality	Palm Beach	Durban	Richards Bay	Ndumu	Skukuza
$HSIR_p$					
Palm Beach		S	NS	S	NS
Durban	S		NS	NS	NS
Richards Bay	S	NS		NS	NS
Ndumu	S	NS	NS		NS
Skukuza	NS	S	S	S	
VCI					
Palm Beach		S	S	S	NS
Durban	S		NS	NS	NS
Richards Bay	S	S		NS	NS
Ndumu	S	S	NS		NS
Skukuza	S	S	S	S	

a - χ^2 test of independence with Yates continuity correction at the 5 per cent level of significance.

b - Population head-squash infection rate.

c - Vector competence index.

The incubation periods and HSIRs of mosquitoes infected with DEN are plotted in Fig. 5 and there were no discernable linear or curvilinear relationships between the two variables. Long incubation periods produced low and high HSIRs, as did the shortest incubation periods. A regression analysis of incubation periods on HSIRs gave a correlation coefficient, r of -0,15 and therefore the

probability of a linear relationship between them, r^2 was only 0,02. Logarithmic, power and inverse transformations of these variables did not improve the regression, with r^2 ranging from 0,01 to 0,03 regardless of the transformation.

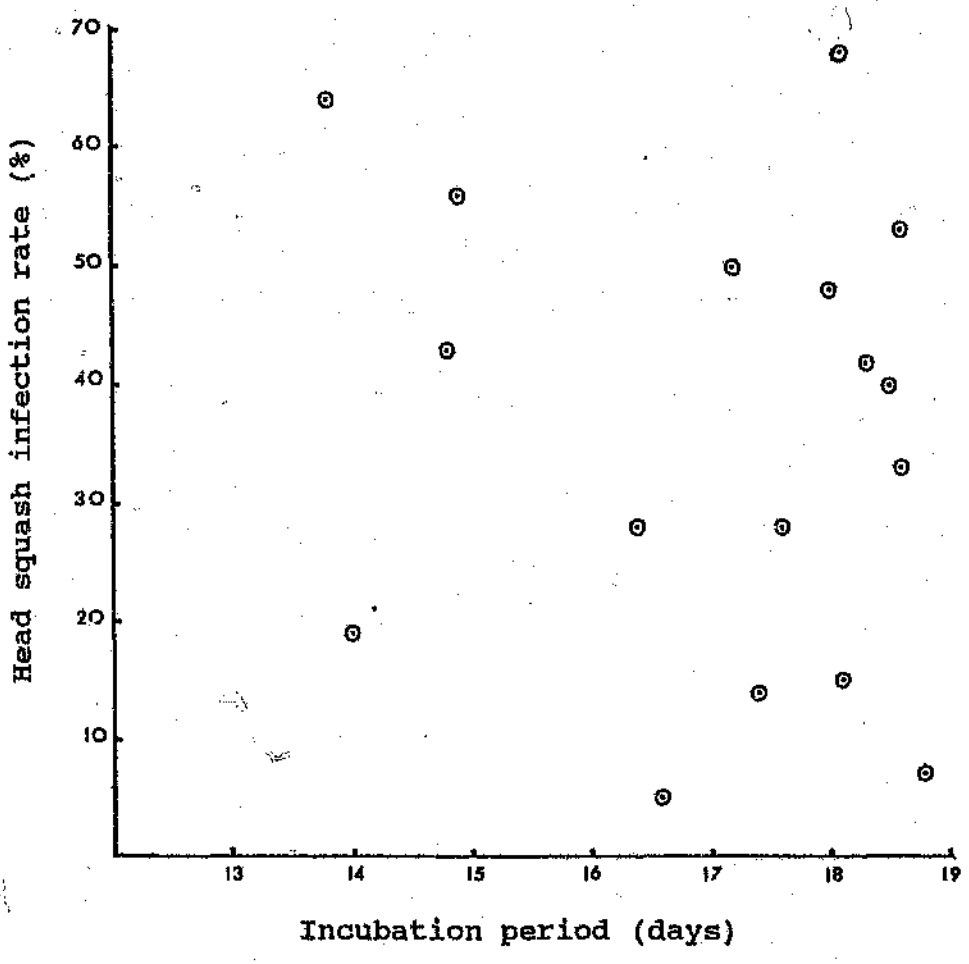


Figure 5. Scatter-diagram of HSIR against extrinsic incubation period for DEN virus in Aedes aegypti.

Discussion

In some of the vector competence experiments, only a small sample of infected mosquitoes survived through to the transmission feed, as varying levels of mortality occurred during the incubation period. Such experiments were repeated until an experiment could be replicated successfully with a suitable sample of infected and transmitting mosquitoes. This provided some basis for determining mean HSIR and TR rates but the significance of the differences between replicates could not be determined. At least seven or eight rate measurements would normally be required in statistical tests of confidence, but this is impractical to achieve because the experiments are so time-consuming. Large sample sizes for determining HSIRs were used to try and redress these shortcomings, yet TRs often depended on relatively small numbers of infected mosquitoes.

The HSIR is not directly comparable to all of the infection rates given in the literature. In the past, infection rates were often determined by testing the whole mosquito for virus, usually by inoculation of mosquito homogenate into suckling mice. Such an infection rate may include mosquitoes where the arboviral infection has not progressed beyond the midgut epithelium, e.g., the DEN-1 viral escape barriers in the midguts of Ae. (Protomacleaya) hendersoni (Freier and Grimstad 1983) and Ae. albopictus (Boromisa et al. 1987). Even worse, one may be detecting virus in the residues of the last bloodmeal in the lumen of the mosquito's gut. Therefore, it is preferable to measure infection rates based on disseminated infections in order to determine susceptibility more accurately. The head-squash technique tests for the pesence of virus in the central nervous system, the haemolymph and the head musculature of the

vector. There is a good correlation between head-squash IR and salivary gland IR for Ae. aegypti (Chen et al. 1990), with headsquashes being slightly less sensitive, resulting in more conservative estimates of IR. Although not determined in the present study, further intrinsic barriers to infection or transmission may exist at the level of the salivary glands, as demonstrated for Ae. albopictus with DEN virus (Boromisa et al. 1987) and Ae. hendersoni with La Crosse virus (Grimstad et al. 1985).

HSIRs were relatively low and TRs were relatively high in most of the populations. However, a low HSIR was not always correlated with a lower TR, e.g., Skukuza (DEN-1 and -2) and the Rockefeller colony (DEN-1). In order to rank the populations of Ae. aegypti according to the infection and transmission components of their vector competence, HSIR and TR were combined into one value, the VCI. This provides a convenient index for discriminating between populations and avoids the complexity of describing their abilities to act as vectors in terms of two characters.

...ler et al. (1979) generally found that HSIRs for DEN-2 were higher than for DEN-1 in Ae. aegypti. This was not the case in the South African populations and perhaps reflect the advantages of working with low-generation laboratory colonies. Gubler et al. (1979) and Rosen et al. (1985) obtained maximum DEN-1 HSIRs of 25 per cent, lower than any of the South African populations, except Palm Beach and Skukuza. Skukuza is unusual in our populations, with an extremely low HSIR for DEN-1 compared to DEN-2. DEN-2 HSIRs were generally comparable to or somewhat lower than those of Gubler et al. (1979), Rosen et al. (1985) and Tardieux et al. (1990).

Transmission data are not readily available in the literature, except for two. Gubler et al. (1979) report HSIRs and TRs for Ae. aegypti from Jakarta, Indonesia and Shimba Hills, Kenya, with DEN-2, determined using the droplet method, which may be converted into VCIs of 0,19 and 0,07, respectively. Boromisa et al. (1987) reports on the vector competence of a Houston, Texas population of Ae. aegypti with DEN-1 and the VCI is only 0,05. These values are low when compared to the South African populations, except for Palm Beach. The only other work giving TRs are for Ae. albopictus. Comparing DEN-1, VCIs calculated from IR and TR data are 0,88 (Mitchell et al. 1987), from 0,04 to 0,40 (Boromisa et al. 1987) and 0,37 (Miller and Ballinger 1988). For DEN-2, derived VCIs are 0,68 (Mitchell et al. 1987) and 0,27 (Miller and Ballinger 1988). The South African Ae. aegypti thus appear to be at least as competent as Ae. albopictus in the laboratory, considering IR and TR together, despite the reports of the higher susceptibility of Ae. albopictus (Rosen et al. 1985).

Among South African spp., Ae. aegypti was found to be the most competent vector (Jupp and Kemp 1993). Due to its prevalence in the urban, peridomestic environment, it is expected to play the principal role in the event of an urban epidemic.

CHAPTER 6. CONCLUSIONS

The two dichotomies, pale/dark morphology and anthropophilic/zoophilic host preference, underpin the whole Ae. aegypti controversy. However, the correlations between dark, feral and zoophilic traits are not inviolate (see "3.3 Discussion"). The relationship between background colour and domesticity could conceivably be compared to the classic l study on industrial melanism by Hasebroek (1934) in several lepidopteran spp., where the generally pale, greyish moths were gradually being replaced by dark forms in the industrial districts of England and western Europe. Kettlewell (1961) demonstrated that avian predators were being assisted by the air-borne pollution which was destroying the lichens that grew on tree-trunks. The birds could recognize the pale moths more readily because they stood out against the dark bark of the tree and thus provided a strong selection pressure for the dark mutation. Perhaps man is likewise driving selection in urban Ae. aegypti in the inverse of melanism. The process may be described as the genetical response of a sp. to an artificially altered (man-made) environment, in this case the pale interior walls of huts and houses. Pale larval colour (Summers Connal 1926; Trpis and Hausermann 1975, 1978) may also be selectively advantageous to indoor populations of Ae. aegypti.

McClelland (1974) suggested that the spatial "discontinuity between populations represents at least incipient speciation". If the presence of speciation is indicated by extensive genetic variation in several biological characters, then a degree of correlation

should exist between the characters. This is definitely not the case in terms of the feral or peridomestic habitat preference, host preference, tergal morphology, isozyme profile and vector competence of Ae. aegypti. The apparently haphazard relationships between the various spectra of characteristics in Ae. aegypti could indicate strong evidence for a morphologically, physiologically and behaviourally plastic and variable sp. The lack of Hardy-Weinberg equilibrium in allele frequencies at several isozyme-coding loci suggests the absence of gene flow between components of the same geographical population (i.e. assortative mating) but this is based on only six out of eight loci in samples smaller than the 200 required by Fairbairn and Roff (1980). The genetic distance between the two behavioural forms of Ae. aegypti in South Africa appears to be too small to postulate the presence of two spp. based on Nei's D. For these reasons, the South African populations of Ae. aegypti appear to belong to a single sp. displaying morphological and behavioural plasticity.

Mayr (1963) defined the subsp. as "an aggregate of local populations of a species inhabiting a geographic subdivision of the range of the species, and differing taxonomically from other populations of the species". This definition holds true for the subspp. aegypti and formosus in most of Africa, even though the taxonomic distinctions between them are not that clear. It only breaks down in the limited geographical region of Rabai on coastal Kenya where Trpis and Hausermann (1975, 1978) found the two forms to be sympatric. When two subspp. come into contact in a limited region of sympatry, they will, by definition, be able to hybridize naturally. The taxonomical evidence for subspp. elsewhere in Africa appears to consist of a morphological gradation between the overall pale and dark background colour characters,

behaviour and isozyme phenotype, as shown by the gene frequency differences between the two behavioural forms. Since the two different behavioural forms of Ae. aegypti in South Africa are not that easily differentiated, and from a comparison with the estimate of Nei's D by Tabachnick and Powell (1979), it is suggested that the South African populations all belong to Mattingly's (1957) subsp. formosus, involving feral forms that have become adapted to the urban, peridomestic environment.

Although all five populations of Ae. aegypti were capable of harbouring and transmitting DEN-1 and -2, it is particularly noteworthy that the most competent vector population was from Durban. Durban was also the most anthropophilic of the populations (Kemp and Jupp 1991), and was abundant in artificial containers at peridomestic sites in the city bounds. Not surprisingly, considering the long absence of DEN from the country, the human population is highly susceptible to infection. A serological survey in the high risk, eastern areas of South Africa conclusively identified only one of 1951 human sera with antibodies indicating recent DEN infection (Blackburn et al. 1987). How DEN-1 arrived in Durban to cause the 1926/1927 epidemic is not known. However, the establishment of new air-links with countries in south-east Asia and the increased trade by air and sea improves the likelihood of DEN being reintroduced to South Africa. These are all important considerations in the potential epidemiology of a South African DEN epidemic.

Seemingly, the main difference between climatic conditions in Durban today and those of 1926 and 1927 when the epidemic of DEN-1 occurred, is the uncharacteristically heavy rains that fell in those two years, with a fivefold increase in 1926 and an eightfold

increase in 1927 over the normal mean annual rainfall. This must have led to extremely high population densities of the vectors, which would presumably have been responsible for the extensive spread of the epidemic. Ambient temperatures were actually depressed below the annual means due to the intense precipitation, so that conditions in Durban were not ideal for the survival of this tropical virus. One may, however, speculate that the effects of adverse temperatures on the virus were successfully countered by the existence of large numbers of vectors which could rapidly distribute the virus in the human host population.

Modern public health practices may have provided improved control of artificial larval habitats compared to the situation in South Africa at the start of the century but there are limitations to what can be achieved by public health authorities. These authorities tend to be understaffed during periods between epidemics and are expected to provide other services than vector mosquito control. It is important to note that increasing the domestic and peridomestic areas, either by development of rural areas or by enlarging existing cities such as Durban, may lead to an increase in the number of artificial larval habitats available to Ae. aegypti. This could again create a fertile environment for DEN to gain a foothold in South Africa, should it be reintroduced via the shipping or tourist industries.

APPENDIX A

RECIPES FOR PAGE BUFFER SOLUTIONS AND ENZYME STAINS

i. Buffer recipes for the various enzyme systems adapted from Steiner and Joslyn (1972); all quantities have been trebled to give stain volumes of around 105 ml; solutions were made in distilled water.

Enzyme	Gel and electrode buffer system	Stain
Ak	CA-8 (8,45; 8,1)[a]	105 ml 0,05M Tris/HCl pH 8[b] 600 mg α-D-glucose 60 mg ADP 30 mg NADP 300 mg $MgCl_2.6H_2O$ 30 mg MTT 10 mg PMS 50 units glucose-6-phosphate dehydrogenase 80 units hexokinase
Es	LiOH (8,45; 8,25)	50 ml 0,2M NaH_2PO_4] [c] 10 ml 0,2M Na_2HPO_4] pH 6,4 40 ml H_2O 80 mg α-naphthyl acetate 50 mg Fast Blue RR salt

i. Buffer recipes continued.

Enzyme	Gel and electrode buffer system	Stain
Gapdh	CA-8 (8,45; 8,1)	105 ml 0,1M Tris/HCl pH 7,5 240 mg fructose-1,6-diphosphate 60 mg NAD 300 mg $Na_2HAsO_4.7H_2O$ 30 mg MTT 10 mg PMS 105 units aldolase
Gpi	LiOH (8,45; 8,25)	105 ml 0,1M Tris/HCl pH 8 160 mg fructose-6-phosphate 15 mg NADP 200 mg $MgCl_2.6H_2O$ 30 mg MTT 10 mg PMS 30 units glucose-6-phosphate dehydrogenase
Hk	CA-7 (7,1; 6,9)	105 ml 0,1M Tris/HCl pH 8 150 mg α-D-glucose 120 mg ATP 30 mg NADP 60 mg $MgCl_2.6H_2O$ 30 mg MTT 10 mg PMS 60 units glucose-6-phosphate dehydrogenase

i. Buffer recipes continued.

Enzyme	Gel and electrode buffer system	Stain
Idh	CA-8 (8,45; 8,1)	105 ml 0,1M Tris/HCl pH 8 150 mg DL-isocitric acid 30 mg NADP 30 mg MTT 10 mg PMS
Mdh	CA-8 (8,45; 8,1)	105 ml 0,1M Tris/HCl pH 8 800 mg DL-malic acid 30 mg NAD 30 mg MTT 10 mg PMS
Odh	LiOH (8,45; 8,25)	105 ml 0,2M Tris/HCl pH 8 6 ml octanol (dissolved in 3 ml methanol:3 ml ethanol) 60 mg NAD 30 mg MTT 10 mg PMS

a - System (gel buffer pH; electrode buffer pH) after Steiner and Joslyn (1972).

b - Stain recipe modified from Steiner and Joslyn (1972) and Urbanelli and Bullini (1985).

c - Stain recipe from Urbanelli and Bullini (1985).

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMS, N-methylphenazonium methosulphate; Tris, tris-(hydroxymethyl)-aminomethane.

The enzyme system, Me was developed and stained for according to the methods of the Medical Entomology Unit of the SAIMR (Coetzee, personal communication), and Sod and Odh were stained on the same gel using the method of Mahon et al. (1976). The electrode buffer solution used for these three enzyme systems was that of Barlow and Ridgway (1971). The stock buffer solution is made up as follows: 1,0 l solution of 2,0M Tris + 1,0M boric acid + 0,1M EDTA in distilled water. The electrode buffer solution is obtained by diluting a 100 ml sample of the stock solution to 1,0 l with distilled water and adjusting the pH to 8,6 with a saturated solution of sodium hydroxide. Both stock and electrode buffers can be stored at 4 °C for later use. The gel buffer solution is prepared freshly for each batch of gels by diluting 75 ml of electrode buffer to 250 ml with distilled water and adjusting the pH to 8,2 with saturated boric acid. The stain recipes used are as follows:

A. Me

40 ml 0,1M Tris/HCl pH 7,0 + 200 mg L-malic acid (readjust pH to 7);
200 mg $MgCl_2.6H_2O$ in 5 ml H_2O;
10 mg NADP in 2 ml H_2O;
10 mg MTT in 2 ml H_2O;
1 mg PMS in 0,2 ml H_2O;
1 g agar-agar in 50 ml H_2O.

B. Sod/Odh

100 ml 0,1M Tris/HCl pH 8,5;
0,4 ml octanol;
50 mg NAD;
100 mg NBT (nitro-blue tetrazolium);
10 mg PMS.

The buffers and stains for Gdh are taken from Harris and Hopkinson (1977):

Electrode buffer:- 0,1M Tris/HCl pH 9.

Gel buffer:- 0,02M Tris/HCl pH 9.

Stain:- 50 ml 0,025M $NaH_2PO_4.2H_2O$]
50 ml 0,025M $Na_2HPO_4.2H_2O$] pH 7,5;
18 g α-D-glucose;
60 mg NAD;
15 mg MTT;
15 mg PMS.

For Pgm, the electrode and gel buffers are taken from Harris and Hopkinson (1977) and the stain recipe is modified from Urbanelli and Bullini (1985):

Electrode buffer:- 0,1M Tris + 0,1M maleic acid + 0,01M $MgCl_2.6H_2O$ + 0,01M EDTA; adjust pH to 7,4 with saturated NaOH solution.

Gel buffer:- 1:10 dilution of electrode buffer.

Stain:- 90 ml 0,05M Tris/HCl pH 8,0;
240 mg α-D-glucose-1-phosphate;
30 mg NADP;
60 mg $MgCl_2.6H_2O$;
30 mg MTT;
10 mg PMS;
60 units glucose-6-phosphate dehydrogenase.

APPENDIX B

VOLUME OF INOCULATION

In order to determine the average volume per inoculum, a sample of twelve of the capillary tubes used to make inoculation needles were calibrated. A standard 10 µl of distilled water at an approximate temperature of 25 °C (similar to that at which inoculations generally took place) was measured out by micropipette and immediately drawn up into a capillary. The heights of the columns of water in the capillaries were measured and the radius of each capillary obtained from the standard

i. Determination of mean radius of capillary tubes used to make inoculation needles and feeding capillaries by volumetric method.

Capillary tube	Height of water column in mm (Radius[a])		
	A	B	C
1	30,0 (0,33)	30,5 (0,32)	29,5 (0,33)
2	29,0 (0,33)	28,3 (0,34)	29,0 (0,33)
3	29,8 (0,33)	30,3 (0,32)	31,0 (0,32)
4	29,0 (0,33)	28,0 (0,34)	29,5 (0,33)
5	29,0 (0,33)	30,0 (0,33)	29,0 (0,33)
6	30,0 (0,33)	32,5 (0,31)	31,0 (0,32)
7	28,8 (0,33)	28,0 (0,34)	29,5 (0,33)
8	30,0 (0,33)	28,8 (0,33)	29,0 (0,33)
9	27,3 (0,34)	28,0 (0,34)	29,5 (0,33)
10	32,0 (0,32)	32,5 (0,31)	31,5 (0,32)
11	29,5 (0,33)	30,5 (0,32)	29,8 (0,33)
12	27,0 (0,34)	27,0 (0,34)	26,5 (0,35)

a - Radius of capillary (mm) in parentheses.

formula for the volume of a cylinder. Three sets of measurements were made with each capillary.

The volume of a 1 mm column was calculated for each capillary tube and the mean (±s) was found to be 0,34±0,02 µl. Subsequent inoculations were based on the equivalent of 1 mm of inoculum from a capillary needle.

ii. Calculation of mean volume of inoculum delivered from an inoculation needle calibrated in one mm graduations.

Capillary tube	Volume of inoculum (µl)		
	A	B	C
1	0,34	0,32	0,34
2	0,34	0,36	0,34
3	0,34	0,32	0,32
4	0,34	0,36	0,34
5	0,34	0,34	0,34
6	0,34	0,30	0,32
7	0,34	0,36	0,34
8	0,34	0,34	0,34
9	0,36	0,36	0,34
10	0,32	0,30	0,32
11	0,34	0,32	0,34
12	0,36	0,36	0,38
$\bar{x}$±s	0,34±0,02		

APPENDIX C

INCUBATION OF INOCULATED DEN VIRUS

In an initial test with DEN-1 and a sample of Ae. aegypti from Empangeni, inoculated mosquitoes were kept under security insectary conditions for varying periods of between seven and twelve days. The effective incubation period was defined as the minimum number of days required for at least 95 per cent of the inoculated mosquitoes to become infected.

Infection rates of 100 per cent were obtained from day seven onwards. Consequently, an incubation period of eight days was adopted in all parenteral infections under standard security insectary temperatures of approximately 30 °C.

APPENDIX D

2 X 2 CONTINGENCY TABLES OF VECTOR COMPETENCE MEASUREMENTS IN DIFFERENT POPULATIONS OF *Aedes aegypti* FOR DEN VIRUSES

i. Statistical comparisons of $HSIR_p$ in five populations and a reference colony of *Aedes aegypti* by χ^2 test of independence with Yates continuity correction (DEN-1 below the diagonal and DEN-2 above).

	PB	DB	RB	ND	SK
PB		7,57[a]	2,11	5,88	1,69
		(0,006)	(0,146)	(0,015)	(0,194)
DB	13,11		1,04	0,07	1,31
	(<0,001)		(0,307)	(0,793)	(0,253)
RB	4,51	2,47		0,42	0,003
	(0,034)	(0,116)		(0,517)	(0,958)
ND	3,89	3,40	0,01		0,60
	(0,049)	(0,065)	(0,940)		(0,437)
SK	1,45	18,84	9,66	8,94	
	(0,229)	(<0,001)	(0,002)	(0,003)	
RC	0,09	10,24	2,72	2,20	2,66
	(0,759)	(0,001)	(0,099)	(0,138)	(0,103)

a - χ^2 value with probability in parentheses below.

Abbreviations of column and row headings:

PB = Palm Beach; DB = Durban; RB = Richards Bay; ND = Ndumu; SK = Skukuza; RC = Rockefeller colony.

ii. Statistical comparisons of TR_p in five populations and a reference colony of Aedes aegypti by Fisher exact test of independence (DEN-1 below the diagonal and DEN-2 above).

	PB	DB	RB	ND	SK
PB		0,331[a]	0,296	0,439	0,284
DB	0,277		0,572	0,526	0,573
RB	0,675	0,208		0,466	0,649
ND	0,690	0,277	0,668		0,460
SK	0,294	0,519	0,258	0,294	
RC	0,189	0,451	0,139	0,189	0,750

a - One-tailed probability.

Abbreviations of column and row headings:
PB = Palm Beach; DB = Durban; RB = Richards Bay;
ND = Ndumu; SK = Skukuza; RC = Rockefeller colony.

iii. Statistical comparisons of VCI in five populations and a reference colony of Aedes aegypti by χ^2 test of independence with Yates continuity correction (DEN-1 below the diagonal and DEN-2 above).

	PB	DB	RB	ND	SK
PB		8,83[a]	4,16	5,57	3,51
		(0,003)	(0,041)	(0,018)	(0,061)
DB	21,64		0,65	0,23	0,95
	(<0,001)		(0,420)	(0,633)	(0,330)
RB	3,19	7,93		0,03	0
	(0,074)	(0,005)		(0,868)	(1)
ND	2,63	8,85	0		0,11
	(0,105)	(0,003)	(1)		(0,739)
SK	0,18	27,01	5,72	4,99	
	(0,669)	(<0,001)	(0,017)	(0,026)	
RC	1,66	10,86	0,11	0,03	3,63
	(0,198)	(<0,001)	(0,739)	(0,867)	(0,057)

a - χ^2 value with probability in parentheses below.

Abbreviations of column and row headings:

PB = Palm Beach; DB = Durban; RB = Richards Bay; ND = Ndumu; SK = Skukuza; RC = Rockefeller colony.

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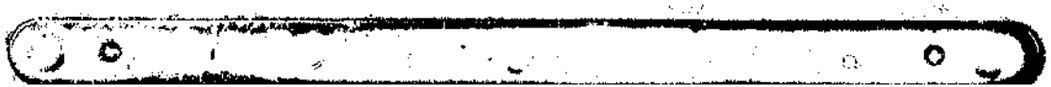
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