STUDIES ON FACTORS INFLUENCING THE ESTABLISHMENT AND DEVELOPMENT OF FILARIA IN MOSQUITOES

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To
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ABSTRACT

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Factors affecting the establishment and development of Brugia and Wuchereria microfilariae in various strains of C. quinquefasciatus, C.p. molestus, An. stephensi and Ae. aegypti SS were studied. The investigations concentrated mainly on the factors affecting exsheathment and migration of microfilariae from the midgut of refractory and susceptible mosquito species.

It was shown that the rickettsia Wolbachia pipientis is not a requirement for the successful infection of C. quinquefasciatus with W. bancrofti, and that the growth and development of W. bancrofti proceeded at the same rate and infective larvae attained the same size in aposymbiotic and normal strains.

Four geographical strains of C. quinquefasciatus and C.p. molestus were found to have low frequencies of the gene sb (filarial susceptibility to B. pahangi). These mosquitoes did not respond to selection for increased susceptibility or refractoriness to B. pahangi, indicating that control of susceptibility was polygenic. Inoculation of C. quinquefasciatus (DAR) and An. stephensi with sheathed and exsheathed B. pahangi, increased the number of larvae that develop to the infective stage.

Increasing the heparin concentration in the bloodmeal decreased the migration rate of B. pahangi in C. quinquefasciatus mosquitoes. Exsheathment and migration of B. pahangi occurred normally in Ae. aegypti SS, a susceptible mosquito. It was shown that natural exsheathment of microfilariae in refractory mosquitoes does not appreciably increase migration. Neither did experimental exsheathment of microfilariae prior to infection increase the migration rate. Feeding C. quinquefasciatus (DAR) on mixed infective bloodmeals of W. bancrofti and B. pahangi, increased the exsheathment and migration rate of B. pahangi in this mosquito.

Red blood cells played no role in inhibiting migration of B. pahangi in C. quinquefasciatus (DAR) and it was shown that systemic factors in the abdominal haemocoel of this mosquito did not inhibit migration. However, the microfilariae which reached the thorax did not develop further indicating a 'thoracic barrier'. The stimulus for migration was shown not to be located in the abdominal haemocoel.

Cations decreased the rate of blood digestion and larval development of B. pahangi in Ae. aegypti SS but had no effect on exsheathment and migration. In vitro studies on exsheathment of B. pahangi, showed the presence of factors which inhibit endopeptidase induced exsheathment of B. pahangi in the abdomen homogenates, but there was no evidence that the factor was located in the midgut. In vitro exsheathment of B. pahangi occurred in the presence of midgut and thoracic tissues as well as in mosquito cells in suspension and in monolayers. Motility of B. pahangi microfilariae in the incubating media was prolonged by the presence of midguts, particularly those of Ae. aegypti SS, and MK/VP12 + 20% FBS medium was shown to prolong motility of microfilariae, compared to other media.

CONTENTS

		Page
ABSTRACT		i
CHAPTER 1	INTRODUCTION	1
CHAPTER 2	LITERATURE REVIEW	6
	2.1 Important Vectors of *Brugia* and *Wuchereria* infections.	6
	2.2 Members of the *Culex pipiens* complex	9
	2.3 The genetic basis of susceptibility to parasites in mosquitoes.	10
	2.3.1 Susceptibility to Malaria	10
	2.3.2 Susceptibility to Arboviruses	14
	2.3.3 Susceptibility to Filariae	20
	2.4 The Migration of Microfilariae to the site of development in the vector.	29
	2.5 Factors affecting the Migration of Microfilariae to the site of development in the vector.	31
	2.5.1 The Cibarial and Pharyngeal armatures	31
	2.5.2 Coagulation of Bloodmeal	33
	2.5.3 Digestive enzymes	35
	2.5.4 Exsheathment of Microfilariae	37
	2.5.5 The Peritrophic Membrane	39
	2.5.6 Regulation by the gut wall	41
	2.6 Factors affecting the development of microfilariae in the vector.	43
	2.6.1 Age of mosquitoes	43
	2.6.2 Encapsulation	44
	2.6.3 Micro-organisms	46
	2.6.4 Temperature	48
	2.6.5 Chemical treatment of adult mosquitoes	49
	2.6.6 Chemical treatment of mosquito larvae	51
	2.6.7 Irradiation	52
	2.6.8 Enzymes	53

		Page
CHAPTER 3	MATERIALS AND METHODS	55
	3.1 The Mosquitoes	55
	3.2 Rearing and Maintenance of mosquitoes	55
	3.3 The Filarial parasites	58
	3.4 Mating of mosquitoes	58
	3.5 Thawing and Reconstitution	59
	3.6 Infection of mosquitoes	60
	3.7 The Estimation of microfilariae in the blood	62
	3.8 Dissections	63
CHAPTER 4	THE SUSCEPTIBILITY OF SOME MEMBERS OF THE CULEX PIPIENS COMPLEX TO WUCHERERIA AND BRUGIA FILARIAE.	64
	4.1 The development of W. bancrofti in normal and aposymbiotic strains of C. quinquefasciatus (DAR)	64
	4.1.1 Introduction	64
	4.1.2 Materials and Methods	65
	4.1.3 Results and Discussion	66
	4.2 The susceptibility of B. pahangi to geographical strains of C. quinquefasciatus (DAR)	69
	4.2.1 Introduction	69
	4.2.2 Materials and Methods	70
	4.2.3. Results and Discussion	70
	4.3 The susceptibility of C.p. molestus to B. pahangi.	75
	4.3.1 Introduction	75
	4.3.2 Materials and Methods	75
	4.3.3 Results and Discussion	76
CHAPTER 5	THE SELECTION OF REFRACTORY AND SUSCEPTIBLE STOCKS OF CULEX QUINQUEFASCIATUS (DAR) TO B. PAHANGI	81
	5.1 Introduction	81
	5.2 Materials and Methods	82
	5.3 Results and Discussion	83

		Page
CHAPTER 6	THE SELECTION OF BLOODFEEDING REFRACTORY AND SUSCEPTIBLE STOCKS OF C.P. MOLESTUS TO B. PAHANGI	89
	6.1 Introduction	89
	6.2 Materials and Methods	89
	6.3 Results	92
	6.4 Discussion	94
CHAPTER 7	THE SUSCEPTIBILITY OF SOME MEMBERS OF THE CULEX PIPIENS COMPLEX, AE AEGYPTI AND AN. STEPHENSI INOCULATED WITH EXSHEATHED AND SHEATHED B. PAHANGI.	100
	7.1 Introduction	101
	7.2 Materials and Methods	101
	7.3 Results and Discussion	104
CHAPTER 8	THE EFFECT OF DIFFERENT CONCENTRATIONS OF HEPARIN ON THE EARLY MIGRATION OF MICROFILARIAE B. PAHANGI FROM C. QUINQUEFASCIATUS BLOODMEALS.	118
	8.1 Introduction	118
	8.2 Materials and Methods	118
	8.3 Results	119
	8.4 Discussion	120
CHAPTER 9	THE EXSHEATHMENT AND MIGRATION OF B. PAHANGI IN VARIOUS MOSQUITO SPECIES.	125
	9.1 The Exsheathment and Migration of B. pahangi in C. quinquefasciatus (DAR) C.p. molestus, Ae. aegypti SS and An.s tephensi.	125
	9.1.1 Introduction	125
	9.1.2 Materials and Methods	125
	9.1.3 Results	126
	9.1.4 Discussion	127
	9.2 The migration of endopeptidase exsheathed B. pahangi in C. quinquefasciatus (DAR), Ae. aegypti SS and An. stephensi at various heparin concentrations.	131
	9.2.1 Introduction	131
	9.2.2 Materials and Methods	131
	9.2.3 Results	132
	9.2.4 Discussion	134

Page

9.3 The exsheathment and migration of B. pahangi and W. bancrofti in C. quinquefasciatus (DAR) and Ae. aegypti SS fed on mixed infective blood meals. 141

9.3.1 Introduction 141

9.3.2 Materials and Methods 142

9.3.3 Discussion 144

9.4 The Exsheathment and Migration of W. bancrofti in C. quinquefasciatus (DAR) and Ae. aegypti SS 158

9.4.1 Introduction 158

9.4.2 Materials and Methods 158

9.4.3 Results 158

9.4.4 Discussion 160

9.5 The Migration and development of exsheathed B. pahangi suspended in serum, fed to C. quinquefasciatus (DAR) and Ae. aegypti SS. 167

9.5.1 Introduction 167

9.5.2 Materials and Methods 167

9.5.3 Results 167

9.5.4 Discussion 168

9.6 The development of exsheathed B. pahangi inoculated into the abdominal haemocoel of C. quinquefasciatus (DAR) and Ae. aegypti SS. 173

9.6.1 Introduction 173

9.6.2 Materials and Methods 173

9.6.3 Results 174

9.6.4 Discussion 175

CHAPTER 10 THE EFFECT OF VARIOUS SUBSTANCES ON THE SUSCEPTIBILITY OF AE. AEGYPTI SS AND C. QUINQUEFASCIATUS (DAR) TO B. PAHANGI. 182

10.1 The effect of Cations on development of B. pahangi in Ae. aegypti SS. 182

10.1.1 Introduction 182

10.1.2 Materials and Methods 182

10.1.3 Results 183

10.1.4 Discussion 184

Page

10.2 The Effect of sulphadiazine, penicillin, ascorbic acid and whole body extracts on the susceptibility of Ae. aegypti SS and C. quinquefasciatus (DAR) to B. pahangi. 190

10.2.1 Introduction 190

10.2.2 Materials and Methods 190

10.2.3 Results 191

10.2.4 Discussion 192

CHAPTER 11 STUDIES ON THE IN VITRO EXSHEATHMENT OF B. PAHANGI 197

11.1 The effect of abdomen homogenate of Ae. aegypti SS, An. stephensi and C. quinquefasciatus (DAR) on endopeptidase induced in vitro exsheathment of B. pahangi. 197

11.1.1 Introduction 197

11.1.2 Materials and Methods 197

11.1.3 Results 200

11.1.4 Discussion 202

11.2 The effect of midgut homogenates of C. quinquefasciatus (DAR) An. stephensi and Ae. aegypti SS on endopeptidase and protease induced in vitro exsheathment of B. pahangi. 212

11.2.1 Introduction 212

11.2.2 Materials and Methods 212

11.2.3 Results 214

11.2.4 Discussion 215

11.3 The exsheathment, motility and migration of B. pahangi incubated in vitro with midguts of C. quinquefasciatus (DAR), Ae. aegypti SS and An. stephensi. 223

11.3.1 Introduction 223

11.3.2 Materials and Methods 223

11.3.3 Results 227

11.3.4 Discussion 229

11.4 The in vitro exsheathment of B. pahangi incubated in mosquito cells. 239

11.4.1 Introduction 239

11.4.2 Materials and Methods 239

11.4.3 Results 241

11.4.4 Discussion 241

		Page
CHAPTER 12	GENERAL DISCUSSION	246
CHAPTER 13	SUMMARY	258
CHAPTER 14	ACKNOWLEDGEMENTS	264
CHAPTER 15	REFERENCES	265

1. INTRODUCTION

Filarial nematodes of the super family Filarioidea are general parasites of all vertebrates, with the exception of fishes. The Filarioidea are characterized by a life cycle in which there are no free living stages and in which transmission is achieved by haematophagus arthropods. The adult worms inhabit the body cavities, blood or lymph vessels or connective tissues of the definitive host, and after fertitization, the female liberates numerous first stage larvae, or microfilariae, which circulate in the blood or in the skin of the host. In certain species of filarial worms, the microfilariae appear in the peripheral blood stream only during a certain period of time in the day or night, this phenomenon being referred to as periodicity.

Transmission is achieved when the microfilariae are ingested by the appropriate haematophagus arthropod, in which they undergo development to the third larval stage, which is infective to the definitive host. The relationship between the filarial parasite and the vector has been reviewed in some detail by several workers (Macdonald, 1967; Macdonald, 1976; Denham and McGreevy, 1977).

The known vectors of filarial nematodes include members of the Culicidae, the Ceratopogonidae, the Simuliidae, the Psychodidae, the Tabanidae, the Hippobosidae, the Siphonaptera, the Mallophaga and the Acarina (Macdonald, 1971). The geographical distribution of the filarial parasites infecting man is limited primarily by the geographical distribution of the vector.

It has been recognized that the eradication of filariasis can be achieved by control of the vectors and by chemotherapy of the infected population. In recent years, most of the research has concentrated on

the biological and genetical control of the vectors, especially with the development of insecticide resistance in mosquitoes.

Macdonald (1962a,b) demonstrated that filarial susceptibility in Ae. aegypti was controlled by a single sex-linked recessive gene f^m (filarial susceptibility to B. malayi) and Macdonald and Ramachandran (1965) later showed that the same gene controlled its susceptibility to B. pahangi and W. bancrofti, but not to D.immitis and D. repens in the Malphigian tubules of the mosquito. Thus the susceptibility gene seemed to be acting on the site of filarial development rather than on the parasite. His work resolved finally the genetic basis of filarial susceptibility that had been partly demonstrated by Kartman (1953) working on the development of strains of Ae. aegypti with altered levels of susceptibility to Dirofilaria immitis.

However, little or nothing is known of the underlying basis for susceptibility or its opposing character refractoriness. Much of the work on filarial parasites has been done with a mosquito species Ae. aegypti, which is not a vector, but a good laboratory host of various filarial parasites. Efforts to demonstrate the operation of a gene similar to the f^m gene, in C. quinquefasciatus, the principle vector of W. bancrofti, have not been successful. Thomas and Ramachandran (1970) failed to select a strain of C. quinquefasciatus highly susceptible to a rural strain of W. bancrofti and although Zielke and Kuhlow (1977) managed to produce a strain of C. quinquefasciatus refractory to infection by a Liberian strain of W. bancrofti, in 3 generations, this same strain was found to be susceptible to a Sri-Lankan strain of W. bancrofti, suggesting that different strains of filariae may vary in their infectivity to mosquito vectors. Obiamiwe and Macdonald (1973) showed that susceptibility of C. 'pipiens' to infection with B. pahangi

was controlled by a sex-linked recessive gene designated sb, which also controlled development of sub-periodic B. malayi, but had no influence on susceptibility of this mosquito to W. bancrofti.

Not many studies have been carried out to determine the physiological expressions of these genes and how this ultimately affects the susceptibility of the mosquito to filariae. Some physiological aspects of the vector-parasite relationship between Ae. aegypti and D. immitis were investigated experimentally by Kartman (1953). He noted that the bloodmeal in Ae. aegypti clotted much more rapidly than that of the highly susceptible Anopheles quadrimaculatus Say. A significant increase in the migration rate of microfilariae from the midgut to the tubules was obtained by feeding Ae. aegypti through a membrane on infectious blood containing anticoagulant. This indicated that the normal or natural course of events in the vector-parasite relationship could be experimentally altered. Obiamiwe (1970) succeeded in increasing the migration of B. pahangi from the midgut in homozygous refractory C. 'pipiens' to the level observed in C. 'pipiens homozygous for sb, by altering the heparin concentration. As a result of his investigations he proposed two types of barrier mechanisms to filarial infection in mosquitoes. The barriers depended on the genotype and species of mosquitoes and also on the strain and species of mosquito. In the first, the microfilariae were mostly destroyed in the gut in genotypically refractory mosquitoes, and in the second, there was migration from the bloodmeal to the thorax, but in genotypically refractory mosquitoes most of the microfilariae were destroyed.

C. quinquefasciatus, which has a wide distribution in the sub-tropics, is the principal vector of nocturnally periodic W. bancrofti.

In recent years, there has been an increase in the prevalence of Bancroftian filariasis, due to the rapid unplanned urbanization taking place in tropical developing countries coupled with poor sanitation and an increase in the available breeding site for larvae. The increase in numbers of C. quinquefasciatus has led to increased use of insecticides for control which has resulted in the development of insecticide resistance in some populations of C. quinquefasciatus. An alternative means of control has been attempted using knowledge of the cytoplasmic incompatibility phenomenon, which enables the replacement of populations of C. quinquefasciatus (Curtis et al. 1974; Curtis, 1976). However, due to the variability observed in wild and laboratory populations and their genetic analysis, further knowledge of the mechanism by which the symbiont Wolbachia causes incompatibility is required before successful population replacement can be achieved.

For these reasons, it becomes more evident why it is necessary to elucidate the mechanisms through which the action of genes controlling susceptibility to filariae is mediated. Due to the unavailability of a suitable laboratory model for W. bancrofti and the decreased viability of cryopreserved parasites, the filarial parasite used in these studies is B. pahangi, which is an animal filaria and is easily maintained in the laboratory. However, if it is possible to elucidate the basis of refractoriness to B. pahangi in C. quinquefasciatus, this information would be useful in developing strains of C. quinquefasciatus which are resistant to W. bancrofti and these could be used in an intergrated vector control programme.

The purpose of this study is to characterize some of the factors which are involved in the exsheathment, migration and development of

Brugia and Wuchereria in the mosquitoes Culex quinquefasciatus, Aedes aegypti and Anopholes stephensi. Most of the studies have concentrated on the gut barrier which seems to be the major factor inhibiting normal development of B. pahangi in C. quinquefasciatus. It is intended that the results of this study will throw more light on the mechanisms by which the genes controlling the susceptibility of mosquitoes to filariae, express themselves. This information may be useful for further studies on vector-parasite relationships.

2. LITERATURE REVIEW

2.1 Important Vectors of Brugia and Wuchereria Infections

The transmission of Brugia and Wuchereria by mosquitoes, in the field and laboratory has been reported from four main genera: Anopheles, Aedes, Culex and Mansonia.

Wilson (1961), Edeson and Wilson (1964), Omori (1966) and Sasa (1976) have reviewed the transmission of Brugia and Wuchereria by mosquitoes. Brugia malayi infections in man and animals are found exclusively in South East Asia. The nocturnally periodic and subperiodic strains of the parasite occur in man and the latter also in animals. Many of the endemic areas of the nocturnally periodic type of Brugia malayi in South and South East Asia have been found to be open swamp where Mansonia uniformis, M. indiana and M. annulifera breed abundantly as the main vectors (Wilson and Reid, 1951; Wharton, 1962; and Edeson and Wilson, 1964).

An. campestris, An. barbirostris and An. donaldi were also shown to be good vectors of nocturnally periodic B. malayi in Malaya (Reid, 1962; Wharton, 1962).

In China, An. sinensi was reported to be the main vector in the rice paddy areas (Edeson and Wilson, 1964).

Ae. togoi, a mosquito species which breeds in rock pools on the beach in East Asia, was incriminated as the vector of nocturnally periodic B. malayi in Japan (Sasa et al. 1952). The sub-periodic race of B. malayi is transmitted by night and day biting mosquitoes of swamp forests. Mansonia dives and M. bonneae are the main vectors in Malaya and in the Philippines (Wilson et al. 1958; Wharton, 1962; Cabrera and Rozeboom, 1964).

Brugia timori, which is endemic only in Timor and the Flores

Islands of the Indonesian archipelago is transmitted by An.barbirostris. Ae. togoi has been found to transmit it experimentally in the laboratory (Partono et al., 1977). Recently, Lee et al., (1983) have reported that A.subpictus is the main vector of bancroftian filariasis in Flores Island, A. vagus being a secondary vector.

B. pahangi which occurs in some Malayan domestic and wild animals is transmitted by M.dives (Wharton 1962). B.patei in East African domestic animals is transmitted by A.pembaensis (Heisch et al, 1959).

Culex quinquefasciatus have been shown to serve as an excellent intermediate host for the urban form of nocturnally periodic W.bancrofti. In North and Central America, West Indies, South America, urban areas in East and West Africa, South Asia, urban areas in South East Asia and East Africa. C.quinquefasciatus is the main vector of "urban" nocturnally periodic W.bancrofti (Davies 1935; Causey et al., 1945; Jordan and Goatly, 1962; Omori, 1966). In Japan, the transmission of nocturnally periodic W.bancrofti is by a member of the C.pipiens complex - C.p.pallens (Omori, 1966). Yeoli (1957) pointed out that C.p.molestus (Israel strain) was an excellent natural vector of nocturnally periodic W.bancrofti in Jewish donors who emigrated from India.

Although C.quinquefasciatus is an excellent intermediate host for the urban form of W.bancrofti, the species was demonstrated to be less effecient than Anopheles species for larval development of some rural races of W.bancrofti in West Africa (Maasch, 1973), in Malaya (Wharton, 1960) and the Pacific Islands. Wharton (1960), found that C.quinquefasciatus was an excellent host for the urban strain in Singapore but was a poor vector of a rural strain of the parasite in Pahang, Malaya. An.whartoni was found to be an excellent vector of the rural periodic W.bancrofti.

Various workers in West Africa have shown that An.gambiae s.l is the main vector of W. bancrofti (Taylor, 1930; Gordon et al., 1932; Kartman, 1946; Gelfand, 1955; Brengues et al., 1968; Subra et al., 1968).

An funestus and An.melas have also been demonstrated to be naturally infected with W.bancrofti in West Africa (Taylor 1930; Gordan et al., 1932; Gelfand, 1955; Brengues et al., 1968), An.welcomei was shown by Brengues et al., (1968) to be naturally infected with W.bancrofti.

In East Africa, W.bancrofti infections in mosquitoes were seen mainly in members of the An.gambiae complex, An.funestus and C.quinquefasciatus (Nelson et al., 1962; Smith, 1955; White, 1971; Wijers and Kilu, 1977; Bushrod, 1979, 1981).

In Sarawak and Brunei, An. leucosphyrus and An. barbirostris (Zulueta, 1957) were found to be vectors of W.bancrofti. In addition, there were areas in tropical South America where An.darlingi is as important as C.quinquefasciatus in transmitting the urban strain of nocturnally periodic W. bancrofti (Giglioli, 1948). Ae. poecilus is the principal vector of nocturnally periodic W.bancrofti in abaca growing areas in the Philippines (Rozeboom and Cabrera, 1956).

Two strains of W. bancrofti exist in the South Pacific. First, the nocturnally periodic strain which occurs in Micronesia and the northern and western area of Melanesia. In these areas C. quinquefasciatus is the principal vector. For instance. C.quinquefasciatus is an important vector in New Guinea (Van Dijk, 1965). In south eastern portions of Melanesia, the vectors are An. farauti, An. bancrofti, An. karwari, C. annulirostris, C. bitaeniorhynchus and M. uniformis. (Sasa, 1976)

Secondly there is the duirnally sub-periodic W. bancrofti found in the Polynesian Islands. The principal vectors are day biting

mosquitoes of the Ae. polynesiensis group. (Rosen, 1955). Ae. fijiensis have been shown to act as efficient vectors in certain Polynesian Islands. They breed in leaf axils of plants. In New Caledonia and the Loyalty Islands, where mosquitoes of the Aedes polynesiensis group are completely absent, Ae. vigilax, a day biting mosquito which breeds in open brackish marshes near the coast, is the major vector.

2.2 Members of the Culex pipiens Complex

Mattingly et al., (1951) recognized five entities in the Culex pipiens complex - C.p.pipiens Linnaeus, C.p.quinquefasciatus Wiedamann, C.p. var molestus Forskal, C.p. var pallens Coquillett and C.p. var comitatus Dyar and Knab. Since 1951 two further forms have been added to the Complex: C.p. australicus Dobrotsworsky and Drummond (1953) and C. globocoxitus Dobrotsworsky,(1953.)

C.p. pipiens is mainly found in the northern temperate region. C. quinquefasciatus has a tropical and subtropical distribution. C.p. molestus has a range of distribution from the most temperate region of C. quinquefasciatus and throughout the tropical range of C.p.pipiens. C.p. comitatus and C.p. pallens are found in North America and Japan respectively.

Overlapping and hybridization of various members of the complex over large areas in north America, Europe and Japan, have led to systematic controversy by taxonomists, but the older view of Mattingly (1951) is still maintained.

Members of the Culex pipiens complex are highly domestic and widely distributed throughout the world. The females are night biters, usually highly anthropophilic and excellent intermediate hosts of W. bancrofti. However, they are less adapted than certain anopheline species for the development of certain rural strains of W. bancrofti

and are less susceptible to the Pacific (diurnally sub-periodic) race of W. bancrofti (Maasch, 1973; Wharton, 1960; Brengues and Coz, 1971).

The larvae of this species usually breed in open ditches sewage pools and artificial containers of polluted water. (White, 1971).

Variation in the susceptibility of C. quinquefasciatus to different populations of Wuchereria bancrofti, has been reported between West African and Asian populations of this mosquito (Zielke and Kuhlow, 1977; Jayasekera et al., 1980).

Miles (1982) has discussed the genetic relationships among members of the Culex pipiens group of taxa, in which he points out the need to establish a common specific-male recognition system which would aid in assigning geographically - separated populations to specific taxon. This would also enable a clearer understanding of the genetic relationships within and between those taxa of practical interest.

2.3 The genetic basis of susceptibility to parasites in mosquitoes

Examples of the widespread variability of mosquitoes of different genera, species and strains, to act as vectors of different parasites, are numerous in literature. That this variation in genetically controlled has been demonstrated in some vector/parasite relationships, such as mosquitoes and malaria, mosquitoes and viruses, and mosquitoes and filariae.

So far, various workers have only managed to map the location of the genes controlling susceptibility, using various markers. The exact mechanisms by which these genes control susceptibility in vectors are still unknown.

2.3.1 Susceptibility to Malaria

Studies of the inheritance of susceptibility to malaria have been made in three genera of mosquitoes.

2.3.1.1 Culex

Huff (1927,1929, 1931, 1933) was the first to provide evidence for the inheritance of susceptibility of a mosquito to a parasite. He selected lines of C. pipiens susceptible and non susceptible to Plasmodium cathemerium by selection of progenies from infected and non-infected individuals. As a result of experiments with C. pipiens (1931) he concluded that susceptibility was controlled by a simple recessive Mendelian factor. The F_2 data gave a perfect 3:1 ratio of refractory to susceptible mosquitoes. However Kitzmiller (1953) and Macdonald (1967) noted that Huff's results from the back-cross progeny did not give a perfect 1:1 ratio as expected. Micks (1949) increased the susceptibility of C. pipiens to Plasmodium elongatum from 13% to nearly 49% in six generations of selection.

2.3.1.2 Anopheles

Boyd and Russell (1942) were the first to attempt to demonstrate the inheritance of susceptibility to malaria in anophelines, using An. quadrimaculatus and Plasmodium vivax. For six generations they attempted to mate several sisters with a single brother and to compare the susceptibility of the mother to the offspring. However, due to low mating success and oviposition, their data did not permit any conclusion on the inheritance of susceptibility.

In 1948, workers at the Rockefeller Foundation succeeded in increasing the susceptibility of An.quadrimaculatus to P. gallinaceum from 20% to 100% by selection over a few generations. However they did not succeed in selecting a totally refractory line. Further crosses were made between the susceptible stock and the parental stock. The offspring of such crosses were found to be intermediate in susceptibility (Rockefeller Foundation, 1950). They concluded that although there was

evidence of a genetic basis for susceptibiltiy, the factor could not be a simple gene but more likely multiple interacting genes.

Frizzi et al., (1975) studied the mechanisms influencing the susceptibility of An. stephensi to Plasmodium gallinaceum. They did this by examining data of crosses and back crosses between a susceptible and refractory stock. They used the presence or absence of oocysts to measure susceptibility. The F1 progeny contained some susceptible females, which should not have been the case if resistance behaves as a Mendelian dominant phenotype. Their back cross data also did not fit well the expected 1:1 ratio, but they thought this was due to different variables interfering with the expected Mendelian distribution. However data of segregation fitted well the 3:1 expected ratio and hence they concluded that resistance is controlled by a single dominant genetic locus.

Van der Kaay and Boorsma (1975, 1977) selected a fully susceptible strain of An. atroparvus to P. berghei after 18 generations. They used both number of positive mosquitoes and mean number of oocysts to measure susceptibility. However, they did not succeed in selecting for a completely refractory strain. Reciprocal matings between the susceptible and refractory strain individuals of F18 decreased the susceptibility to approximately 50%. Back crosses of F1 offspring with the susceptible parents increased susceptibility to 66%, whilst backcrosses to the refractory parents decreased susceptibility to 11%. It was suggested that multiple interacting genes probably control susceptibility.

During investigations on the susceptibility of An. gambiae to P. berghei berghei, Al Mashhdani et al., (1980), were able to select fully susceptible and refractory lines in nine generations. They

recorded the presence or absence of sporozoites in the salivary glands as well as number of oocysts and the number of females infected with oocysts. Results from reciprocal crosses of the susceptible and refractory stocks were found to be different. This difference could not be attributed to cytoplasmic inheritance, as they showed no closer resemblance to the female parent. From the back crosses and F2's, it was concluded that the control of susceptibility is polygenic.

2.3.1.3 Aedes

Trager (1942) using Ae. aegypti and Plasmodium lophurae, established a large colony of Ae. aegypti which for over a year proved to be at least twice as susceptible as the stock colony from which it was derived. The mode of inheritance was not determined. Jeffery (1944) failed to increase the susceptibility of Ae. albopictus to P. lophurae after several generations of selective inbreeding (quoted by Kilama and Craig, 1963). In 1947, Hovanitz attempted to select a susceptible line of Ae. aegypti to P. gallinaceum. He recorded the numbers of malarial oocysts on the stomach of the mosquito as a measure of susceptibility. After 6 generations of selection, he did not observe any genetic mechanism affecting susceptibility and no parent offspring correlations were found. Ward (1963) thought that this might be due to the presence of extreme inbreeding in his laboratory colonies. Workers at the Rockefeller Foundation (1948) using Ae. aegypti and P. gallinaceum attempted to select a resistant stock of Aedes, but were unsuccessful.

Ward (1963) working with Ae. aegypti and P. gallinaceum attempted to select for increased or decreased susceptibility and to present an interpretation of the inheritance of susceptibility. The number of malarial oocysts developing on the midgut of a mosquito was used to express susceptibility. Reciprocal crosses between a refractory and a

susceptible line gave F1 and F2 hybrids that were intermediate. Back cross data had a tendency toward bimodality. He concluded that a single pair of genes or a block of closely linked genes with incomplete dominance was responsible for observed differences. To explain his observations, he proposed a complicated model in which S_1S_1 designated his refractory line, while his susceptible line consisted of a mixture of S_2S_2 and S_1S_2. The phenotypes (numbers of oocysts) from these three genotypes, however showed considerable overlap. Kilama and Craig (1969) undertook a study to establish more complete information on the genetic aspects of susceptibility of Ae. aegypti to P. gallinaceum. They established two refractory strains within one generation of selection and by crosses with susceptible stocks established that the refractory condition was controlled by a simple autosomal recessive factor, plasmodium-susceptibility (pls) located on linkage group 2 between the markers. Silver-mesonotum (S_1) and dieldrin resistance (D1), at a distance of seventeen crossover units. Kilama (1973) found that the pls allele was absent from Ae. aegypti from Asia and America but present in all eight strains tested from Africa at frequencies of 0.27 to 1.0.

2.3.2 Susceptibility to Arbovirsuses

Tesh et al., (1976) reported variation among geographic strains of Aedes albopictus in susceptibility to infection with Chikungunya virus (CHIK). Up till then, the possibility that genetic factors may control susceptibility to arbovirus infection in mosquitoes had not been investigated. They investigated the susceptibility of 16 different strains of A. albopictus to two CHIK strains, the Ross-strain and the Barsi strain. Their studies showed that A. albopictus mosquito strains originating from different geographic localities vary not only in their

oral susceptibility to infection with CHIK virus, but also in their ability to replicate the virus once infected. Attempts to develop increasingly resistant or susceptible mosquito lines through genetic selection were unsuccessful. Infection rates did not change significantly despite 3 to 6 generations of selective breeding. However crosses between strains of high and low CHIK susceptibility yielded hybrid mosquitoes with infection rates and mean virus titers intermediate between those of the parent colonies. Their data suggested that at least one factor controlling the susceptibility of Ae. albopictus to CHIK infection was genetic. They also observed that two lines of Ae. albopictus with a marked difference in susceptibility to oral infection with dengue viruses were equally susceptible to oral infection with CHIK virus.

Gubler and Rosen (1976) investigated the comparative susceptibility for four prototype strains of dengue virus infection of 13 geographic strains of Ae. albopictus. They fed mosquitoes on virus-erythrocyte sugar suspensions. Significant variation in susceptibility for each of the four dengue serotypes was observed among the geographic strains. Mosquito strains which were more susceptible to infection with one dengue serotype were also more susceptible to the other dengue serotypes. There was a direct relationship between the amount of virus ingested and the infection rate in a given mosquito strain. Crossing experiments between susceptible and resistant mosquito strains produced hybrid progeny with intermediate susceptibility. The susceptibility to infection by dengue virus was decreased by selective inbreeding in one strain from 74% to 13% in two generations. They also found that susceptibility to dengue virus was stable and not significantly influenced by inbreeding. However from the data they collected during the investigation, they were

unable to determine the mode of inheritance of susceptibility to dengue virus. It is also of interest that susceptibility to CHIK and to dengue virus infection in the same Ae. albopictus strains did not appear to be related. Some geographic strains highly susceptible to dengue were relatively resistant to CHIK infection and vise-versa. Furthermore, the CHIK susceptibility to the Poona dengue resistant line and the Poona colony strain were similar, suggesting that the factors which control susceptibility to infection with these two arboviruses were distinct. They suggested that the barrier to infection was in the midgut, but once this had been overcome further virus growth is the same in all mosquito strains. In 1977, Aitken et al., compared the susceptibility of 3 geographical strains of Ae. aegypti to infection with low passage yellow fever virus, as well as for their ability to transmit virus by bite and varying extrinsic incubation periods. The Santo Domingo strain appeared the most competent and Kampala the least, when mosquitoes were exposed to low level virus infecting blood meal. At higher virus levels, a similar trend was noted but differences were less evident and not statistically significant. All three strains were infected and transmitted yellow fever virus. However they did not indicate the mode of inheritance of susceptibility of Ae. aegypti to yellow fever virus.

Grimstad et al., (1977) screened 20 strains of Ae. triseriatus for transmission efficiency of La Crosse virus. These mosquito strains were from endemic and non-endemic areas. Infection rates ranged from 40% to 93% and rate of transmission ranged from 20% to 90%. A control strain tested in seven trials showed no significant variation in susceptibility (71-77%) or transmission ability (54-68%). The susceptibility and transmission rates of strains from areas where

La Crosse virus is endemic were lower than that of strains from non-endemic regions, showing 71% vs 87% for susceptibility to infection, and 46% vs 74% for ability to transmit. They argued that these differences might be due to laboratory factors and not factors intrinsic to the individual strains, but if this was so, then the control mosquitoes, reared under the same conditions should also have been affected. There were no significant differences in either infection or susceptibility among the control groups. Consequently they concluded that the geographical differences among test strains were real and did not reflect field-induced phenomenon. These differences were thought to be due to genetic drift and a simple genetic difference governing the susceptibility of Ae. triseriatus to viral infection. However the absence of any correlation between infection and transmission in the small colony strains indicates that other factors affect the transmission of the virus by the mosquito.

In 1979, Gubler et al., reported the comparative susceptibility of 13 geographic strains of Ae. aegypti to oral infections with dengue viruses, by feeding the mosquitoes on a virus-erythrocyte sugar suspension. Significant variation in the susceptibility to four dengue serotypes were observed among the geographic strains tested. Mosquito strains which were more susceptible to one serotype were also more susceptible to other types. The amount of virus required to infect mosquitoes orally varied inversely with the susceptibility of the geographic strain. The thresholds of infection were not the same for dengue types 1,2, 3 and 4. Crossing experiments with four geographic strains resulted in hydrid progeny which had the same susceptibility as the resistant parent. No difference was observed between resistant and susceptible mosquito strains in the rate or the amount of viral

replication after infection by the parenteral route, or in their ability to transmit dengue 2 virus after infection by the oral route. As previously shown with Ae. albopictus, the barrier to infection appears to be in the midgut. Since the results of this study showed that the F1 progeny inherited the susceptibility of the resistant parent, unlike Ae. albopictus, the mode of inheritance of susceptibility to dengue viruses in these two mosquito species may therefore be different. The data in this study suggested that susceptibility is genetically controlled.

2.3.2.2 Culex

Hardy et al., (1976) evaluated four field populations and four laboratory colonies of Culex tarsalis from California to infection with western equine encephalitus (WEE) virus by intra thoracic inoculation and by feeding on pledgets soaked with a virus-blood-sucrose mixture. All mosquito strains were uniformly susceptible to infection by intrathoracic inoculation but there was alot of variation in field and colony strains infected by pledget feeding. It was also found that some field and laboratory strains of C. tarsalis were quite heterogenous for susceptibility. Refractoriness to infection appeared to be related to a gut barrier. These observations did not determine if genetic or non-genetic factors are responsible for variability observed in susceptibility of C. tarsalis to WEE virus.

In an extension of the above studies, Hardy et al., (1978) succeeded in selecting two hybrid strains of C. tarsalis that were highly resistant to infection following ingestion of WEE virus. Resistance was associated with a mesenteronal barrier since both refractory and parental strains were equally susceptible to infection by intrathoracic inoculation. Susceptibility was dominant, possibly incompletely

dominant over resistance. They speculated that inheritance was probably polyfactorial although this could not be determined with certainty. In follow-up studies with WEE virus and C. tarsalis, Kramer et al., 1981 reported a second barrier which prevents infection of the salivary glands. They designated this the "salivary gland infection" barrier which was related to the dose of virus ingested and whose effectiveness decreased with time.

In an attempt to select lines of C. tritaeniorhynchus for increased and decreased susceptibility to West Nile (WN) virus, Hayes and Baker (1980) got 2 lines with increased susceptibility but no lines showed a decrease in susceptibility. The F1 progeny of crosses between the susceptible line and naturally resistant strains, resembled the susceptible parent, suggesting susceptibility was dominant over resistance. However, it was found that although the parental susceptible line had approximately 10-fold virus than the resistant line, the F1 progeny had intermediate titres to the 2 parental stocks, suggesting that different genetic factors may be responsible for controlling initial susceptibility and subsequent viral replication in the mosquito.

Chamberlain et al., (1959), in their investigations on susceptibility of various strains of C.p. pipiens and C.p. quinquefasciatus from the United States, found that although there was little variation in susceptibility to St. Louis encephalitis (SLE) virus, the transmission rate was different. Thus, 100% of C.p.pipiens were able to transmit SLE virus after 12 day extrinsic incubation compared to only 18 to 29% for C. p. quinquefasciatus after a comparable incubation period. The latter species required 20 or more days to reach 100% transmission efficiency.

Similar findings were reported by Mitchell et al.,(1981) in their studies on the experimental transmission of Rocio virus by mosquitoes. They found that although C. tarsalis from Arizona and C.p.pipiens from Ilinois had infectivity rates of 92% and 71% respectively after 20 days incubation, only 36% of C.pipiens from Tennessee and 23% of C.p. quinquefasciatus from Argentina transmitted the virus.

The above review shows that the factors controlling susceptibility and transmission efficiency are different in various mosquitoes and for different viruses. Further investigations are required for each vector-virus system in order to use this information for controlling arthropod borne viral diseases.

2.3.3 Susceptibility to Filariae

The most comprehensive studies on the inheritance of mosquitoes to filarial nematodes have been carried out with Ae. aegypti. Although it is not a natural vector of human filariasis, the ease with which it has been colonized in the laboratory, has made it the most suitable mosquito species for use in studies of mosquito susceptibility to filariae. Natural vectors of bancroftian filariasis such as Culex quinquefasciatus and Ae. polynesiensis, and Malayan filariasis such as mosquitoes of the Mansonia group, have not yielded to analyses readily.

2.3.3.1 Aedes

Roubaud et al., (1936) and Roubaud (1937)were the first to note differences in susceptibility rates between colonies of Ae. aegypti from different geographic areas following exposure to the same strain of Dirofilaria immitis. Roubaud suggested that the variation in susceptibility was heritable. This suggestion was supported by Kartman (1953) and Raghavan et al., (1967) who showed that the

susceptibility rate of a colony of Ae. aegypti to infection with D. immitis could be increased or decreased by selecting progeny from infected and non-infected mothers.

Ramachandran et al. (1960) observed variation in susceptibility to infection with both periodic and sub-periodic forms of Brugia malayi in stocks of Ae. aegypti. Macdonald (1962a) reported selection of a West African strain of Ae. aegypti, to semi-periodic B. malayi, which was maintained in cats. By initial single pair sib matings and selection from individual mosquitoes, susceptibility measured by percentage of mosquitoes with mature larvae nine to eleven days post infection, rose from 17.1% to 93.8% in one generation. After 15 generations of selections, 85% of the mosquitoes dissected contained mature larvae. The inheritance of susceptibility was studied by crossing this strain with uniform refractory strains and testing the susceptibility of the F1, F2 and backcross offspring, Macdonald (1962b). Results of these tests suggested theat susceptibility was controlled by a recessive sex-linked factor. Macdonald designated the recessive susceptibility gene f^m and its dominant allele F. Since only 85% of his selected strain supported maturation of malayi larvae, Macdonald (1963a) tested seemingly refractory individuals and found that they were homozygous for f^m. This indicated that f^m is incompletely penetrant and suggested that there were modifying genes present. Macdonald (1963b) obtained a cross over value between f^m and the sex locus of 7.8%, but later Macdonald and Sheppard (1965) obtained a crossover distance of 3.4 ± 1.1. This heterogeneity in crossover frequencies was thought to be due to inversions on the sex chromosomes.

Macdonald and Ramachandran (1965) showed that Ae. aegypti homozygous for f^m, were susceptible not only to sub-periodic B. malayi but also to periodic B. malayi, B.pahangi, periodic and subperiodic W. bancrofti but not to forms which developed in the Malphigian tubules D. immitis and D. repens. McGreevy and McClelland (1973) found that f^m homozygotes were also susceptible to D.corynoides which develops in the fat body. The finding that f^m homozygotes are susceptible to all muscle parasitic filariae tested is surprising since natural vectors of filariasis do not show this property; they are usually susceptible to only a single kind of filarial worm. Barr (1975) suggests that selection has altered a basic defence mechanism of the mosquito so that it can no longer defend itself against these muscle-inhabiting worms.

Rodriguez and Craig (1973) tested 43 geographically diverse strains of Ae. aegypti for susceptibility to infection with B. pahangi, to obtain information on the distribution of the f^m gene. They found it was absent from thirty of the strains, including all six from Asia and all seven tested from West Africa. Most of the positive forms were from East Africa, but as Macdonald (1976) points out, many of the strains had been maintained in the laboratory for a number of years, and may have different frequencies of the gene from the field populations. The studies of Zielke (1973); McGreevy et al., (1974); Coluzzi and Cancrini (1974); Sulaiman (1979) and Sulaiman and Townson (1980), showed that the development of Dirofilaria species in Ae. aegypti, is controlled by different genes and not the f^m gene. Susceptibility to D. immitis is under the control of a single major sex-linked gene designated fi (=ft), susceptibility being recessive to refractoriness.

The gene has high penetrance and expressivity. Susceptibility to D. repens is also predominantly under the control of a sex-linked gene f^r but the results of Townson et al., 1981 suggest that modifier genes may also be involved.

Terwedow and Craig (1977) found that susceptibility of Ae. aegypti to the frog filaria Waltonella flexicauda was controlled by an incompletely recessive sex linked gene close to the f^m gene. The authors claim that the two genes are allelelic as the REFM stock, which is homozygous for f^m was also susceptible to W. flexicauda, and hybrids from the crosses between REFM and the stock selected for susceptibility to W. flexicauda were also susceptible. However, Curtis (1977) points out they do not have sufficient evidence for this, as REFM may be homozygous for both genes. An appropriate test for allelism was made by Townson et al., (1981) and Matthews (personal communication) from one stock susceptible to both D. immitis and B. pahangi and from another stock refractory to both. The F1 hydrid was crossed to the doubly susceptible homozygote and 7/1284 singly susceptible recombinants were obtained hence demonstrating that the genes f^m and f^i were at separate though closely linked loci.

As a result of evidence provided by Macdonald studies on the genetic basis of susceptibility to filarial nematodes in Ae. aegypti, several workers have extensively investigated the susceptibility of several populations and laboratory stocks of this mosquito species to filariae. Rodriquez (1973) investigated the susceptibility of 13 inbred laboratory stocks of Ae. aegypti to B. pahangi and found that nine out of 13 stocks were completely refractory.

Terwedow (1973) and Kilama (1976) observed a susceptibility rate ranging from 0-18% of W. flexicauda in 12 geographic stocks of

Ae. aegypti. Paige and Craig (1975) examined 29 stocks of Ae. aegypti from East Africa, for susceptibility to B. pahangi. They found susceptibility rates ranging from 0-59% in their stocks. The sylvan strains were more susceptible to B.pahangi than either the peri-domestic or domestic stock. In 1976, Kilama investigated the susceptibility of Ae. aegypti to the local urban strain of W. bancrofti in East African stocks. Susceptibility rates ranged from 0-30% in the 17 stocks tested. Again, he found sylvan stocks more susceptible, the peri-domestic stocks showed intermediate levels of susceptibility, while the domestic stocks were refractory. He suggested that the continuous exposure of domestic mosquitoes to the parasite could have exerted selection pressure, in faviour of refractoriness. Susceptible mosquitoes were found to have higher mortalility rates (Townson, 1971). Another interesting observation was that stocks which were susceptible to W. bancrofti had very similar susceptibility rates to those previously observed with B. pahangi (Rodriguez and Craig 1971; and Paige and Craig 1975). These observations tend to support the findings of Macdonald and Ramachandran (1965) that susceptibility to Brugia and Wuchereria were controlled by the same gene in Ae. aegypti.

Studies on the inheritance of filarial susceptibility in the Ae. scutellaris group are not as advanced as those of Ae. aegypti. Macdonald (1976) reported observations that hybrids of the cross between Ae. s. malayensis females and Ae. polynesiensis males were refractory to infection with B. pahangi, so that susceptibility maybe a recessive trait. When Ae.s. malayensis females were back crossed to the hybrid males all the offspring were refractory. Trpis et al. (1981) crossed the refractory species Ae. alcasidi and the susceptible species Ae. polynesiensis and Ae. cooki. They tested the parents, hybrids and

backcrosses for susceptibility to B. malayi. From the results of these tests, they suggested maternal or cytoplasmic inheritance, which has not been found in any other mosquitoes. Meek and Macdonald (1982) crossed female A. alcasidi and Ae. s. katherinensis with males of Ae.polynesiensis or Ae. cooki, both of which were susceptible to B. pahangi and are natural vectors of W. bancrofti. The female hybrids were back crossed to the parental susceptible stocks and the hybrids and backcross progeny were tested for filarial susceptibility. These results suggest that nuclear factors are involved in the inheritance of susceptibility to filarial infections, but there is no evidence of a single major gene.

2.3.3.2 Culex

Studies on the susceptibility of the Culex group of mosquitoes to filarial parasites have not been many in comparison to Aedes. Members of the Culex pipiens group are important vectors of bancroftian filariasis. Studies on the susceptibility of C. quinquefasciatus to B. pahangi have shown them to be either refractory to B. pahangi (Ogunba 1969) or to have relatively low susceptibility rates (Desowitz and Chellapah 1962; Ewert 1965). Obiamiwe (1977a) found five geographic stocks of C. quinquefasciatus to have susceptibility rates ranging from 5-20% to B. pahangi. The susceptibility to Brugia of C. p. molestus is usually higher than that of C. quinquefasciatus. Schacher and Khalil (1965) obtained 14.1% and 19.1% mature larvae rates in Lebanese C. p. molestus infected with B. pahangi; Ogunba (1969) reported a 36.6% rate in a London strain of the same mosquito. Obiamiwe and Macdonald (1973) reported that susceptibility to infection with B. pahangi in C. pipiens was controlled by a sex-linked gene designated sb. The gene lacks complete penetrance and has variable expressivity. A preliminary estimate of the crossover distance between sb and the sex locus in males was 12.8 $\pm$ 2 whilst two

point linkage data for females gave an average distance between sb and re of 42. As re is very close to the sex locus, these results indicate that further information is needed. In selecting a red-eye susceptible strain of C. pipiens, Obiamiwe (1976) crossed a strain of C. quinquefasciatus from Bobo-Dioulasso with females of C. p. molestus. Assuming that C. quinquefasciatus was refractory, such a cross would introduce refractory genes into the hybrid stocks hence slowing down selection. Obiamiwe (1977) found that the gene sb (filarial susceptibility to B. pahangi) also controls development of sub-periodic B. malayi, but has no influence on the development of periodic W. bancrofti (Ceylon strain). This is in contrast to Ae. aegypti, where control of susceptibility to Brugia and Wuchereria are by the same gene. Thomas and Ramachandran (1970) were the first to note a response to selection for the Malayan rural strain of W. bancrofti to five different Malaysian stocks of C. quinquefasciatus. They succeeded in increasing susceptibility, but due to the small numbers of mosquitoes dissected, they were unable to give conclusive results.

Several attempts have been made to select a strain of C. quinquefasciatus refractory to W. bancrofti and these have been reviewed and discussed by Curtis and Graves (1983). Partono and Oemijati (1970) using Indonesian C. quinquefasciatus and W. bancrofti, selected for refractoriness for three generations. A small increase in refractory mosquitoes was noted, but there was no evidence of a major gene for refractoriness. They suggested that they may have been selecting for a modifier gene. Singh and Curtis (1974) attempted to select a refractory strain of Delhi C. quinquefasciatus but after five generations of selection, they concluded that the initial population did not contain any genes for refractoriness to W. bancrofti. Similarly, Chakraverty et al. (1977) failed in their attempt to increase refractoriness after twenty

generations of selection. They suggested that their results show that susceptibility is polyfactorial. It is interesting to note that in their attempts to select for refractoriness to periodic W. bancrofti in C. quinquefasciatus, susceptible to diurnally sub-periodic W. bancrofti, they noticed that there was a decrease in susceptible mosquitoes. Zielke and Kuhlow (1977) also failed to select a refractory stock from Delhi C. quinquefasciatus. In 1977, Zielke and Kuhlow did succeed in selecting in five generations, for a refractory stock of a Liberian strain of C. quinquefasciatus from a stock originally showing 21.6% susceptibility for W. bancrofti. From the results of crossing experiments between the refractory Monrovia stock and the Delhi susceptibile stock, it appeared that susceptibility in the latter was dominating refractoriness of the former. The F1 hybrids were not as susceptible as the Delhi colony.

This would suggest incomplete dominance of susceptibility. The authors suggest that the results indicate that the genetic factors are sex-linked and that there are at least two different factors in C. quinquefasciatus which control the susceptibility for W. bancrofti. Jayasekera et al. (1980) reported that Liberian C. quinquefasciatus were as susceptible to Sri-Lankan W. bancrofti as were Sri-Lankan C. quinquefasciatus. Neither did the Liberian stock respond to selection for refractoriness. These results indicated that the two strains of W. bancrofti from Liberia and Sri-Lanka differ in their ability to infect specific mosquito strains. Such differences have been reported before between strains of W. bancrofti.

Kalra (1974) reported different strains from the Andaman Islands and the mainland of India in relation to Indian C. quinquefasciatus.

Several workers in Africa have observed that rural periodic W. bancrofti is transmitted by Anopheline mosquitoes (Gelfand, 1955; Hamon et al. 1967; White 1971; Crans, 1973; Maasch, 1973; Brengues et al. 1975: Brunhes, 1975; Zielke and Kuhlow, 1977). It is suggested that the West African C. quinquefasciatus have evolved a specific protective mechanism against its local filarial strain because of the harm which filarial infection can cause to mosquitoes (Townson, 1970). However changes in the ability of filarial strains can be brought about by selection (Laurence and Pester, 1967) and the Liberian strain of W. bancrofti would only have to resemble the Sri Lankan strain to extend its range of potential vector mosquitoes to the dense populations of C. quinquefasciatus now found in West African towns. Minjas (1981) observed initially relatively low susceptibility of the Monrovian and Thailand stocks to Tanzanian strain of W. bancrofti. This low susceptibility persisted for 2 years. Subsequently an increase in susceptibility to the Tanzanian strains of W. bancrofti was observed. Little information is available on the inheritance of susceptibility to infection with D. immitis by C. quinquefasciatus. Kartman (1953) and Zielke (1973) both attempted to select for increased and decreased susceptibility. Zielke did succeed, but did not study the mode of inheritance.

The above discussion indicates that information is accumulating on the modes of inheritance of susceptibility to parasites. Most research has concentrated on the genetics of susceptibility while relatively little research has been done on the physiological and morphological mechanisms which play a role in determining whether a mosquito species is an efficient vector or not. Further investigations are required on the mechanisms controlled by these genes, which determine the susceptibility or refractoriness of a mosquito species to a particular filarial strain or species. Such information would be useful for vector control since it would be possible to alter the susceptibility status of mosquito populations by genetic manipulations.

2.4 The Migration of Microfilariae to the site of development in the vector.

From the time of ingestion of microfilariae by a mosquito, various factors may inhibit the successful development of the parasite to its infective stage. These factors may be morphological, biochemical and physiological. These factors are probably all influenced by nuclear genes of the mosquito, which determine whether or not it is susceptible to certain filarial parasites. It has been demonstrated that taxonomic and morphologic features cannot be used to determine which species of mosquitoes are efficient vectors for the various parasites. Little consideration has been given to the characteristics of the infection in inefficient mosquito hosts. A knowledge of reliable and readily detectable features of filarial development which can be used to differentiate between efficient and inefficient mosquito hosts would be useful, especially if they are evident early in the infection as an indicator of vector potential.

Ewert (1965a) was the first to suggest that differentiation between a refractory and susceptible mosquito species might prove possible by an analysis of microfilarial migration. Laurence and Pester (1961a,1961b) found that 98% of B. patei microfilariae ingested by Mansonia uniformis reached the thorax within 24 hours after feeding. These authors also reported that in strains of An. gambiae (an inefficient vector) from East and West Africa, over 90% of the microfilariae of B. patei died in the stomach, while in M. uniformis 98% reached the thoracic muscles successfully. Ramachandran et al.(1961) found that 90% of B. pahangi microfilariae ingested by M. uniformis and 50% ingested by a Malayan strain of Ae. aegypti had migrated to the thorax 24 hours after feeding. Ramachandran and Zaini (1967) found that all the ingested microfilariae

in Ae. togoi reached the thorax within 24 hours of feeding and Ramachandran (1966) further reported that in the susceptible (Liverpool strain) of Ae. aegypti, 80% of the B. pahangi microfilariae ingested during feeding reached the thorax after 24 hours. Ewert (1965a) observed that 93% of B. pahangi microfilariae ingested by Anopheles quadrimaculatus, 93% by Ae. sollicitans, 61% by Ae. aegypti and 44% by Ae. albopictus reached the thorax 24 hours after feeding. Of these mosquitoes only An. quadrimaculatus was susceptible to infection with B. pahangi and in an attempt to obtain a more precise differentiation, he examined the migration percentages one hour after feeding. In An. quadrimaculatus he found that 71% had migrated after one hour, in Ae. sollicitans 34%, in Ae. albopictus 19%, in Ae. aegypti 14%, and in C. quinquefasciatus less than 1%, Of these, only An quadrimaculatus was a good host, the others being poor hosts.

Ewert (1965a) also noticed that although a reasonable percentage of microfilariae reached the thorax of the poor hosts, they did not develop to the infective stage. Hence he speculated that the extent of migration to the thorax as an indicator of a favourable host parasite relationship should be used with caution. He also noted the lack of development of microfilariae in the thorax having reached there, indicated that the failure to penetrate the gut wall was not the sole reason for refractoriness, but was one in a series of barriers that the microfilariae must successfully cope with in order to complete development in the mosquito. Some of these barriers include, the cibarial and pharyngeal armatures of the vectors, the coagulation of the bloodmeal, digestion by the vector, stimulation for exsheathment of sheathed microfilariae, the peritrophic membrane and the gut wall of the vector. The importance of thoracic factors (for Brugia and Wuchereria) and Malphigian tubules

factors (for Dirofilaria) is emphasized by the genetic studies reviewed above.

2.5 Factors affecting the Migration of Microfilariae to the site of development in the vector.

2.5.1 The Cibarial and Pharyngeal armatures.

The digestive tract of insects is often interrupted by a variety of sclerotinized teeth and spines that protrude from the gut wall into the lumen. In the foregut, these structures may be concentrated into rows or groups often referred to as armatures. Some species of mosquitoes, blackflies and sandflies are known to possess these armatures. Adler and Theodor (1926) were the first to note the diagnostic values of the morphology of the buccal cavity in the taxonomy of Phlebotomus. This suggestion was confirmed by Sinton (1927). Subsequently, Sinton and Covell (1927) examined the buccal cavity of 52 species of anophelines in India and found that it could be useful in the differentiation of species, sub-genera and groups of the anophelines. Barraud and Covell (1927) also examined the buccal cavity of anophelines and discussed its value as a feature for differentiating species of mosquito.

However it was Coluzzi and Trabucchi (1968) who were the first to suggest the role of these armatures as first lines of defence against filarial infection by inflicting lethal lacerations on microfilariae as they are ingested with an infective blood meal. They observed damaged microfilariae of D. repens only in the midguts of some anophelines and Culex species and accordingly, these mosquitoes were either not infected or had very few larvae in the Malphigian tubules.

Microfilariae introduced in the intestine through the anus were not found to be damaged and developed normally even in species previously considered completely resistant or almost so. Microfilariae were not

injured when incubated in intestinal and salivary gland extracts. It was concluded that microfilarial damage was inflicted by the teeth of the cibarial armature and was not the result of digestive enzymes or putative antifilarial toxins. They also suggested that the probability of microfilariae being injured was inversely related to the size of the pharyngeal pump and particularly to the amount of blood flowing through the anterior pharyngeal valve when it opened. An. pharoensis, which is an unusually large mosquito with an exceptionally large pharyngeal pump was found to have a low percentage of damaged microfilariae. They also suggested that the percentage of microfilarial damage is dependent on the length of the parasite. They observed that the microfilariae of Eufilaria sergenti, being only half the length of D. repens, suffered very little damage.

Shawcross (1971) found that Anopheles and Culex possess cibarial armatures which varied between sub-genera. Damage of microfilariae of B. pahangi was found to be most extensive in An. (Nyssorhynchus) albimanus. McGreevy et al. (1978) studied the lethal effects of cibarial and pharyngeal armatures of Anopheles. C. quinquefasciatus and Aedes mosquitoes on the microfilariae of B. pahangi and W. bancrofti. They found that large proportions of microfilariae Brugia and Wuchereria ingested by anophelines were damaged and amotile. Maximal damage to Brugia occured in Anopheles species which had both the cibarial and pharyngeal armatures. In these species 92 to 96% of the microfilariae were injured and 61 to 83% were amotile after ingestion. Minimal damage to Brugia occurred in Aedes species which have pharyngeal but not cibarial armatures. In these species 9-22% of the microfilariae were injured and 6 to 11% were amotile. An. gambiae s.s injured 51% and immobilized 66% of the ingested W. bancrofti while C. quinquefasciatus and Ae. aegypti

injured and immobilized 2-6%. An examination of the morphology of the cibarial armature showed that the Anopheles species had large sharp teeth and strong pointed spines while C. quinquefasciatus had a weak cibarial armature composed of small delicate teeth. The pharyngeal armatures of all mosquitoes were alike. They also observed that in the anophelines, only a small proportion of ingested microfilariae were capable of penetrating the midgut epithelium while 62% of Brugia microfilariae ingested by Ae. aegypti penetrated the gut wall. Observations on the development of microfilariae injected directly into the thorax of An. gambiae s.s. and Ae. aegypti showed that the susceptibility was comparable, this being 96% in Anopheles and 94% in Aedes. They concluded that the few microfilariae which survive their journey through the gut of An. gambiae A developed to the infective stage. Similar cibarial armatures in Simulium have been found to cause considerable damage to microfilariae of Onchocerca (Bain et al. 1974; Omar and Garms, 1975).

2.5.2 Coagulation of Blood Meal

The rate of bloodmeal coagulation in the mosquito midgut plays a great role in determining the fate of ingested microfilariae. Rapid coagulation can trap the microfilariae in the bloodmeal and thus prevent them from migration. Metcalf (1945) examined the presence or absence of agglutinins and anticoagulins in various mosquito species. She found that the glands of A. quadrimaculatus, A. punctipennis and A. crucians contained both agglutinin and an anticoagulin, but reported that in the other species examined, Ae. aegypti, A. vexans and C. salinarius C. quinquefasciatus, C. restuans and Psorophora discolor, neither an agglutinin nor an anticoagulin for human blood could be detected. She also observed that on examination of Ae. aegypti and A. quadrimaculatus

15 minutes after a bloodmeal, the blood in the stomach of Ae. aegypti was coagulated but not agglutinated, while in A. quadrimaculatus the blood was strongly agglutinated and not coagulated.

Gooding (1975) reported that the ability of salivary gland emulsions to cause agglutination sometimes depends upon the source of the erythrocytes. A. maculi pennis salivary glands agglutinate erythrocytes from man, donkey, rabbit and dogs, but not those from mice, guinea-pigs or monkey. Kartman (1953) showed that anti-coagulants, such as heparin, sodium citrate or a mixture of both, allowed an increase in some mosquito species of the proportion of microfilariae which migrated to the tubules. For example, in donor fed Ae. aegypti only 30.5% of the microfilariae reached the Malphigian tubules whereas in mosquitoes fed through a membrane on blood containing anti-coagulant 78.4% of the microfilariae reached the tubules. In Ae. albopictus, the comparable figures were 63.9% and 87.7%. However anti-coagulant did not affect the proportion of microfilariae which migrated in A. quadrimaculatus for which figures of 95.3% and 94.5% were obtained in donor fed and membrane fed mosquitoes respectively. Kartman concluded that the migration in Aedes mosquitoes is reduced due to quicker clotting of the blood meal.

Obiamiwe (1977) compared the migration of microfilariae in heparinized and non-heparinized bloodmeals in two stocks of C. pipiens selected for susceptibility and refractoriness to infection to B. pahangi. He found that of the ingested microfilariae, 20.7% migrated from the bloodmeals of a selected susceptible strain of C. pipiens compared with only 0.9% in a selected refractory strain. The addition of heparin to the bloodmeal raised the percentage migrating to 20% in the selected refractory stock. This is the level observed in the susceptible strain. The addition of heparin did not increase the migration rate in

susceptible mosquitoes. Owen (1978) attempted to examine the state of the blood meals in intact Ae. aegypti SS mosquitoes and in isolated Ae. aegypti SS midguts in vitro. The results indicated clotting took place faster in vitro than in vivo.

Nayar and Sauerman (1975) reported that in An. quadrimaculatus, A. taeniorhynchus and A. sollicitans microfilariae move freely from the midgut to the Malphigian tubules because of the presence of substantial amounts of anti-coagulins in their salivary glands. However in A. aegypti, C. nigripalpus and C. quinquefasciatus movement of microfilariae from the midgut to the Malphigian tubules was obstructed by the coagulation of blood. Coagulation of blood was followed by formation of oxyhaemoglobin crystals in C. nigripalpus and C. quinquefasciatus. They suggested that these crystals hinder the further movement of microfilariae and kill them.

From the above information, it is clear that the association between anticoagulins in mosquitoes and the migration of microfilariae is mostly speculative. There are many additional factors which facilitate microfilarial migration from the midgut. If these were clearly understood, it would be possible to isolate mosquito species in which no migration took place and use them for vector control.

2.5.3 Digestive Enzymes

Due to the recognition of the importance of mosquitoes as important vectors of human pathogens, several workers have investigated the nature of secretion and regulation of digestive and other proteolytic enzymes and their influence on the parasites acquired during the bloodmeal (Clements, 1963; Wigglesworth, 1974; Gooding, 1975).

No digestive enzymes are secreted by the salivary glands of adult mosquitoes. Metcalf (1945) carried out tests for protease, lipase and

amylase in salivary glands of An. quadrimaculatus females and Fisk (1950) and Fisk and Shambaugh (1954) for protease and invertase in the salivary glands of Ae. aegypti females. All gave negative results. Fisk and Shambaugh (1952) found that in Ae. aegypti fed on a bloodmeal protease activity dropped below the residual level characteristic of unfed mosquitoes in the first few minutes after feeding, then rose steadily reaching a maximum about 18 hours after feeding.

The migration of microfilariae B. pahangi to the thorax of a selected susceptible stock of Ae. aegypti has been observed to occur within 10 minutes after ingestion of an infective bloodmeal (personal observation). Laurence and Pester (1961a, 1961b) also observed the rapid migration of B. patei in M. uniformis. The rapidity with which microfilariae migrate to the thorax in the first few hours after ingestion, before secretion of digestive enzymes reaches its optimum, raises doubts as to whether digestive enzymes play an important role in limiting migration of microfilariae. There have been no investigations into whether the pattern of proteolytic enzyme secretion is the same in refractory and susceptible mosquitoes. It is also not known whether digestive enzymes of mosquitoes can kill otherwise undamaged microfilariae in the midgut.

Kartman (1953) found that all microfilariae left in the midgut of C. quinquefasciatus and C. pipiens 12 to 48 hrs after ingestion were killed and digested but remained alive in the midgut of Ae. aegypti and Ae. albopictus. He suggested that in Culex, the microfilariae might have been killed by other factors in the mosquito gut prior to digestion by enzymes. Laurence and Pester (1961a) reported that in An. gambiae 50-60 minutes after a blood meal some microfilariae of B. patei were sluggish and coiling, whereas in a female Mansonia, killed at the same

time, the microfilariae were still active in the blood clot. At 100-140 minutes, half the microfilariae were hardly moving and had blood cells adhering to the surface of the sheath. More information needs to be gathered on the role if any, of digestive enzymes on parasites ingested by mosquito vectors.

2.5.4 Exsheathment of Microfilariae

The blood stream dwelling first stage microfilariae of certain species of filarial nematodes, such as B. pahangi, B. malayi, W. bancrofti and Loa loa, are enclosed within the egg shell which forms an elongate tubular sheath around the larval body. After ingestion of an infective blood meal by a mosquito, exsheathment of microfilariae is a necessary requirement for the migration and further development of these microfilariae. Ewert (1965a) suggested that microfilarial exsheathment in a particular species of mosquito might serve as a rapid indicator of vector potential. Ewert (1965b) compared exsheathment in species of Anopheles, Aedes and Culex. He found that in two strains of An. quadrimaculatus, 98% of the microfilariae had exsheathed 1 hour after being ingested. In C. quinquefasciatus, there was less than 3% exsheathment 1 hour after ingestion. It was noted a high percentage of microfilariae in the midgut of the species had still retained the sheath one day after ingestion. The sheath appeared rigid and the microfilariae were inactive. The author speculated as to whether migration failed due to the retainment of the sheath, or whether the sheath was retained as a protective response. In Ae. aegypti and Ae. albopictus 45% to 68% of the microfilariae exsheathed and migrated to the thorax, but they did not develop to the infective larval stage. The author concluded that lack of exsheathment was indicative of poor vector potential and vice-versa.

Laurence and Pester (1961a) reported finding sheathed microfilariae of B. patei in the fat body and abdomen of M. uniformis and Ewert (1965b) found 3 sheathed microfilariae in the abdomen and thorax of An. quadrimaculatus. Schacher (1962) also reported the occurrence of sheathed microfilariae B. pahangi in An.quadrimaculatus but their subsequent development was abnormal. These observations suggest that in some cases, sheathed microfilariae do succeed in migrating from the blood meal.

Owen (1978) examined the exsheathment of B. pahangi in Ae. malayensis a refractory species and Ae. tabu a susceptible species. In Ae. tabu only 2.9% of the microfilariae were sheathed while in the refractory species 17.13% were sheathed. He found that migration of microfilariae in the susceptible species after 3 hours was 95% whereas in the refractory species 50% of the microfilariae failed to migrate. He suggested that failure to exsheath was one of the mechanisms limiting migration in the refractory species. Owen (1978b) observed attachment of erythrocytes to sheathed microfilariae of B. pahangi in Ae. malayensis and Ae. tabu. He suggested that erythrocyte attachment may be an integral part of the exsheathment mechanism. To date, there is no information on mechanisms leading to the exsheathment of microfilariae in vivo. However, exsheathment of microfilariae in vitro has been investigated and achieved by several workers (Coultenen, 1929; Weinstein, 1963; Katamine and Aoki, 1970; Sivanandam and Dondero, 1972).

Devaney and Howells (1979) reported the exsheathment of 90% of microfilariae B. pahangi after incubation for 1 hour in 20mM $CaCl_2$ in a phosphate free balanced salt solution, and exsheathment rates of 95-100% occurred after incubation for 30 minutes in solutions of endopeptidase or papaya extract protease. In the former case, they suggested that

exsheathment was either facilitated by Ca^{2+} mediated adherence of the sheath to a fixed substrate or that Ca^{2+} ions induced the secretion of a "moulting fluid". In the latter cases, they speculated that these enzymes exerted a digestive influence on part of the sheath and combined with the vigorous activity of the microfilariae led to rupturing of the sheath. It remains unclear how *in vitro* experiments relate to the *in vivo* state of the midgut. It is unlikely that *in vivo* Ca^{2+} ions play a role similar to that observed *in vitro*, since the concentrations of Ca^{2+} used to bring about exsheathment *in vitro* are very high and would not be found to be prevailing in the mosquito midgut. Neither are the *in vitro* effects of proteases similar to those found *in vivo* since it has been observed that *in vivo*, secretion of proteolytic enzymes drops soon after a blood meal and does not reach its optimum until about 18 hours after the bloodmean (Clements, 1963; Fisk and Shambaugh, 1952). Recently, Yamamoto *et al.* (1983) reported that *B. pahangi* microfilariae exsheathed in the haemocoel of *Armigeres subalbatus*. Sheathed microfilariae managed to penetrate the midgut wall, then proceeded to exsheath, while sheathed microfilariae inoculated into the abdominal haemocoel were also capable of exsheathing.

From the above discussion, it is clear that further information is required about the mechanisms involved in the *in vivo* and *in vitro* exsheathment of microfilariae in the midgut of mosquitoes.

2.5.5 The Peritrophic Membrane

The peritrophic membrane of insects is only known and described morphologically as part of the midgut and its function is not clearly understood. It protects the cells of the midgut from hard or sharp particles of food, though it is also found in many insects which suck blood or fluids from plants. Richards and Richards (1977) have reviewed

the formation, properties and functions of the peritrophic membranes of insects. Stohler (1957) reported that the peritrophic membrane of Aedes mosquitoes was secreted in fluid form following a blood meal and in the course of 20-30 hours it grows more and more solid. He found that up to 30 hours after an infective meal, ookinetes readily penetrated the membrane and reached the cells of the midgut.

Lewis (1950, 1953) studied the role of the peritrophic membrane in Simulium damnosum infected with Onchocerca volvulus. He found that microfilariae of O. volvulus ingested became imprisoned in the peritrophic sac. Only a few microfilariae which remained in the tubular part of the midgut where no membrane was formed made their way to the thoracic muscles.

Not much is known of the role of the peritrophic membrane in mosquitoes in the transmission of filarial parasites. Esslinger (1962) observed that the peritrophic membrane of An. quadrimaculatus, on dissection 5 minutes following engorgment was very thin and soft and the microfilariae passed readily through it to the midgut wall with the aid of the cephalic hook. Laurence and Pester (1961a) suggested that the rapid migration of B. malayi in M. uniformis was to avoid coagulation and digestion of blood rather than the formation of the peritrophic membrane.

The rate at which microfilarial parasites migrate from the midgut to the thorax in susceptible mosquitoes is fast enough for them to avoid being trapped in the peritrophic membrane, which remains soft for several hours. This would seem to suggest that the peritrophic membrane does not play any important role in limiting the migration of microfilariae. Stohler (1961) speculated that the inability of mosquitoes to transmit viruses of Murray Valley encephalitis and western and

and eastern equineencephalitis, unless the gut filled with blood was perforated, was due to an intact peritrophic membrane.

2.5.6 Regulation by the gut wall.

Observations in literature review show that the microfilarial density of the donor, the numbers of microfilariae ingested and the numbers of larvae developing in different mosquitoes vary greatly. In most cases, the factors regulating the density remain poorly understood. It has been shown that an increase in microfilarial density does not always bring about a proportional increase in the percentage of mosquitoes with filarial larvae. Wharton (1957) found that the percentage of M. longipalpis infected with B. malayi rose from 12% to 100% when fed on carriers with 0.025 and 2 or more microfilariae per cmm blood respectively. However Jordan and Goatly (1962), working with C. quinquefasciatus and nocturnally periodic W. bancrofti from East Africa reported that there was no increase in the number of infective microfilariae despite feeds on higher levels of microfilariaemia. They found that there was a considerable loss of ingested microfilariae particularly after feeds on high-density microfilariae carriers. Wharton (1960) found that although C. quinquefasciatus showed a considerable increase in the proportion of mosquitoes with filarial larvae of Singapore strain of urban nocturnally periodic W. bancrofti when the microfilarial density of the donor was increased, there was little or no change when the same mosquito species was infected with blood containing varying microfilarial densities of Malayan rural nocturnally periodic W. bancrofti.

Gelfand (1955) in West Africa, observed that the proportion of An. gambiae withlarvae of nocturnally periodic W. bancrofti increased from 17.2% at 0.65 microfilariae per cmm blood to 55% at 5.8 microfilariae

per cmm blood. Rosen (1955) found that whereas the proportion of A. polynesiensis with larvae of the Tahiti strain of sub-periodic W. bancrofti increased from 5.3% at 0.02 microfilariae per cmm blood to 100% at 27.8% microfilariae per cmm blood, the proportion of C. quinquefasciatus with filariae remained at about 20% between the microfilarial densities of 0.05 mff/cmm to 24.4mff/cmm.

Ramachandran (1966) working with Ae. aegypti and B. malayi found no defined relationship between the microfilarial density of the host and the mean number of infective larvae per mosquito. The greatest number of infective larvae per mosquito were in mosquitoes fed a moderate microfilarial density of between 3.6 to 5.6 mff/cmm blood.

The numerical relationship between the ingested microfilariae and the haemocoel reaching microfilariae has been classified into 3 categories by Bain (1976). In the first kind which she termed the "facilitation phenomenon", the haemocoel reaching microfilariae increase in number with an increase in the number of ingested parasites. This was observed with the combination Wuchereria bancrofti - Anopheles gambiae s.s. The second category was the "limitation phenomenon" in which the number of microfilariae reaching the haemocoel is proportional to the number ingested during a small meal of microfilariae, but when the rate of ingestion increases, this number is limited. This has been observed in 3 combinations : W. bancrofti - Ae. aegypti, Setaria labiatopapillosa - Ae. aegypti, O. volvulus - S. damnosum. The third is that of proportionality as found in the migration of Skrajabinofilaria skrajabini in C. pipiens, O. volvulus in S. ochraceum and D. repens in Ae. aegypti. In these examples, the proportion of microfilariae ingested increased. Denham and McGreevy (1977) suggest that its more likely that these various filariae-vector combinations are a summation

of microfilarial interactions with the pharyngeal and cibarial armatures, the blood clot, digestive enzymes, peritrophic membrane and the gut epithelium.

Applying histological techniques Bain andBrengues (1972) found that in An. gambiae s.s, the parasitized cells were disorganized with basophilic nucleus and pynoctic nuclei and the surrounding epithelium was hypertrophied. They speculated that the creation of such hypertrophied areas by the first penetrating microfilariae, facilitated further migration. Subsequently Bain and Chaubaud (1975) found that in Ae. aegypti although there were no hypertrophied areas, the cells, had pynoctic nuclei and basophilic cytoplasm. They believe this reaction impedes further penetration.

2.6 Factors affecting the development of microfilariae in the vector.

The variation in the ability of mosquitoes to support the development of parasites and the likelihood of the parasites being transmitted to another host is due to a combination of genetic and environmental or extrinsicfactors. In the following review, the latter will be discussed.

2.6.1 Age of mosquitoes

Several workers have examined the role if any of the mosquitoes age on the number and growth of developing filarial larvae. Duxbury et al. (1961) recovered longer larvae of D.uniformis from 12 to 13 day old An. quadrimaculatus than from those 4 to 5 days old. Desowitz and Chellapah (1962) found significantly higher infection rates in 20-22day old C. quinquefasciatus fed on Brugia than in 3 day old mosquitoes. The authors also noted that although prior non-infective blood meals had no significant effect on the infection rates the highest infections were found in older mosquitoes given two such blood meals. Wharton et al.

(1963) on the other hand found no difference in the susceptibility of 5-8 days old and 18-25 days old C. quinquefasciatus fed W. bancrofti. However the susceptibility rate became significantly higher when the older group was given a prior non-infecting meal.

Townson (1964) found no significant change in infection rate with age of Liverpool stock of Ae. aegypti infected with B. pahangi but observed a significantly lower mean intensity of infection in older mosquitoes. The underlying causes of this reduction in number were not discussed but is is possible that smaller blood meal sizes were primarily responsible.

Terwedow and Craig (1977) tested the influence of age in male and female Ae. aegypti, a selected susceptible strain, on W. flexicauda development. The males were found initially to support 3 times as many worms as the females. However there was a significant decrease in susceptibility over 2 weeks in males but not in females.

2.6.2 Encapsulation

Encapsulation is a defensive mechanism by which insects eliminate invading foreign organisms. It is a co-ordinated response involving the aggregation, adhesion and flattening of haemocytes over the foreign surfaces too large to be engulfed by individual cells. Generally, encapsulation reactions in insects are accompanied by the intra-and extra-cellular deposition of the pigment melanin (Salt 1970).

Reports of encapsulation of microfilariae in mosquitoes can be found in the literature. Kartman (1953) found encapsulated D. immitis microfilariae in the Malphigian tubules of Ae. aegypti from the third and successive days following the infective blood meal. In addition to this Ae. aegypti females had 0.04 - 0.2% encapsulated microfilariae in the midgut and haemocoel respectively. Encapsulation did not occur in

C. quinquefasciatus while An. quadrimaculatus showed negligible encapsulation of the microfilariae and first stage larvae. Esslinger (1962) observed pigmental encapsulation as early as 15 minutes after engorgement, forming as brown plaques intermingled with acellular fibrous material. Almost all encapsulated microfilariae were found within the abdominal haemocoel and rarely within the thorax.

Schacher (1962), in a study on the morphology of B. pahangi in eleven species of Louisiana mosquitoes, found encapsulation of some larvae in An. quadrimaculatus and P. confinnis. Burton (1963) found encapsulated microfilariae in seven species of Aedes, Culex, Mansonia and Psorophora from British Guiana. Most of the encapsulated microfilariae were found in the haemocoel and fewer in the stomach. Lindemann (1977) reported finding encapsulated D. immitis microfilariae and first stage larvae in Ae. aegypti from 3 days after the bloodmeal. Encapsulated larvae were associated only with Malphigian tubules exhibiting cellular damage.

Oothuman et al.(1974)observed melanization of B. patei in the abnormal mosquito host An. atroparvus 48-78 hours after ingestion by the mosquito in the anal vesicles.This blockage of vesicles was thought to affect further development of the filarial larvae.

From the above review it is apparent that no definite experiments have shown that encapsulation of microfilariae or larvae reduces the vectorial capacity of mosquitoes. In many of the studies reviewed encapsulated microfilariae were found in mosquitoes with undeveloped microfilariae. This raises doubts as to whether encapsulation plays a significant role in limiting the susceptibility of mosquitoes.

2.6.3 Micro-organisms

McLaren et al (1975) reported unusual bodies in the hypodermal tissues of larval D.immitis and B. pahangi which they thought were gram-negative micro-organisms. They speculated that the presence of these organisms could affect their susceptibility to mosquitoes. Kozek (1971) reported finding similar bodies in mature D. immitis. Similar intracellular organisms were reported in the lateral cords of adult worms and the larval stages of O. volvulus (Kozek and Figueroa, 1977). These organisms appeared to be transmitted transovarially but their significance with respect to the host-parasite relationship and pathogenesis of onchocerciasis remained unknown.

Subsequently, Kozek (1977) found similar organisms also present in the lateral c ords of adult and larval stages of B. malayi but their effect on the filarial worms has not been studied. Hamilton (1975) found that An. quadrimaculatus reared under bacteria-free conditions seemed better vectors of D. immitis than those reared under normal conditions. In sterile mosquitoes, sausage stage larvae appeared within 48 hrs, but took 72 hrs in non sterile mosquitoes. The development of D. immitis in An. quadrimaculatus infested with pure cultures of bacteria was found to be differently retarded. In mosquitoes of the C. pipiens complex and Ae. scutellaris group, a phenomenon known as cytoplasmic incompatibility exists. This term describes the inability of crosses between certain strains of mosquito to produce normal offspring, as a result of cytoplasmic factors. These factors have been identified as symbiotic rickettsial like micro-organisms, which are transmitted trans-ovarially (Yen and Barr 1971,1973). Trpis et al (1981) reported maternally inherited differences in filarial susceptibility to B. malayi and B. pahangi in the Aedes scutellaris group. This suggested that the

rickettsiae might be involved in determining susceptibility of the mosquitoes. To investigate this, Duhrkopf and Trpis (1981), treated Aedes polynesiensis with tetracycline at the larval stages in order to remove the rickettsiae, then infected them with B. malayi. They reported reduced levels of infections in tetracycline treated mosquitoes, suggesting the possibility that the rickettsia provide some necessary component for the development of the filarial parasite.

To see if a similar phenomenon occurs in Culex mosquitoes, Curtis et al.(1983)investigated whether removal of the rickettsia Wolbachia from C. quinquefasciatus affected the development of W. bancrofti. The results showed that strains of C. quinquefasciatus which had been rendered aposymbiotic by tetracycline treatment remained fully susceptible to infection with W. bancrofti.

Data from studies on the inheritance of susceptibility to infection with B. pahangi and W. bancrofti in the Aedes scutellaris group of mosquitoes, carried out by Meek and Macdonald (1982), suggest that nuclear factors are involved in the inheritance of susceptibility to filarial infections, but there was no evidence of a single major gene. In other studies on the crossing relationships among seven members of the group of A. scutellaris mosquitoes, Meek and Macdonald (1984) found that A. alcasidi and A. s. katherinensis were bidirectionally compatible, . They appeared to be aposymbiotic but their crossing relationships with other species conflict with the hypothesis that aposymbiotic males cross successfully with symbiont-infected females, whereas the reciprocal cross is not possible. This hypothesis was found to hold true in crossing relationships of members of the Culex pipiens complex. (Yen and Barr, 1971, 1973).

These conflicting reports on the effects of rickettsia on filarial susceptibility in different mosquitoes and on crossing relationships could be due to the different kinds of rickettsia present in Aedes and Culex mosquitoes. Further information is needed on the nature of the rickettsiae in Aedes mosquitoes before the above differences can be explained.

2.6.4 Temperature

Duxbury et al. (1961) studied the role of temperature on the development of D. uniformis in An. quadrimaculatus mosquitoes maintained at 23,27 and 31°C after feeding on infected rabbits. The number of larvae recovered from mosquitoes maintained at 27°C was considerably higher than in the groups kept at 23°C and 31°C. They noted that although the larvae grew more rapidly during the first 7 days in mosquitoes maintained at 31°C, by the 10th day the larvae at 27°C had attained the greatest mean length. Beam (1967) found that at 15°C, D. immitis did not develop in Ae. sollicitans. However development of parasites was quicker at 30.5°C then at 21.1 to 24.4°C and they also harboured more parasites than those maintained at 15°C. Similarly Kutz and Dobson (1974) found that by transferring An. quadrimaculatus maintained for 30 days at 15°C, to 26°C, the arrested microfilariae resumed growth and developed to the infective stage. Christensen and Hollander (1978) investigated the effects of six different developmental temperatures on the vector-parasite relationships of Ae. trivittatus and D. immitis. Complete parasite development occured at 5 of the temperatures except at 14.5°C. Parasites survived in mosquitoes at 14.5°C for 30 days and completed development in nine days when transferred to 26.5°C. They concluded that the optimum development temperature for D. immitis in Ae. trivittatus was 22.5-26.5°C. Extremes of temperature were found to reduce the number of infected mosquitoes.

2.6.5 Chemical Treatment of Adult Mosquitoes

There is relatively little information on the relation between insecticides and the ability of insects to carry pathogens to humans and animals. Several workers have shown that certain chemosterilants can affect the development of parasites in vectors, the susceptibility to infection and the vector potential of insects. Bertram (1964) found decreased susceptibility of Ae. togoi to B. patei along with adverse effect on the developing parasites when the mosquitoes were exposed to 40mg/sq ft thiotepa for 2 and 3 hours after 24 hours of the infective blood meals. Das et al. (1967) studied the effect of apholate on D. immitis and W. bancrofti in Ae. aegypti and C. quinquefasciatus respectively. The chemosterilant was administered 24 hours before feeding and 72 hours after feeding. Apholate treatment decreased the susceptibility of Ae. aegypti and C. quinquefasciatus to D. immitis and W. bancrofti infections respectively. The effect of apholate was similar in both species despite the different sites of development of the two species of filarial parasite. The adverse effect of apholate on the developing parasites in their hosts was evidenced by the decreased infectivity rates as well as the mean number of infective larvae per infective mosquito. In Ae. aegypti, the deleterious effect of apholate was observed in mosquitoes treated before and after the infective blood meal. However, in C. quinquefasciatus treated 24 hours before the feed, the infectivity rate was significantly higher than those treated after the meal. The authors suggested that such discrepancies could be due to host and parasite specific factors such as differential susceptibility of apholate at the effective locus of the mosquito organ system.

Ahmed (1969) reported that the antibiotics benzyl penicillin, oxytetracycline, tetracycline and dimethylchlortetracycline had no effect on the development of B. pahangi in Ae. togoi and in two naturally refractory strains of Ae. aegypti. However, Wade (1974) found that a mixture of the antibiotics Sodium penicillin G and Streptomycin sulphate depressed microfilarial infectivity. This effect was more pronounced when the antibiotic concentration was above 2,500 international units/ml of blood. In addition, the amount of dejecta and faeces was reduced at high antibiotic levels, suggesting that the digestive process of the mosquitoes was affected.

Jaffe et al. (1978) found that ingestion of a 1.0% solution of antibacterial sulfonamide sulfisoxazole (SXZ) by B. pahangi infected Ae. aegypti females for no less than 3 to 4 consecutive days significantly reduced their filarial larvae burdens and adversely affected the growth and motility of surviving larvae. The authors suggested that sulphonamide was most likely inhibiting de novo synthesis of dihydrofolate (DHF). It remained unclear as to whether SXZ was directly affecting DHF synthesis in the mosquito host, leading to folate-related nutritional deficencies inimical to normal filarial larvae development. They suggested that anti-filarial action of the sulfonamide was expressed only after existing pools of DHF were depleted either in the worms or the mosquito host. The combination of SXZ and methotrexate (a dihydrofolate reductase (DHFR)) was found to exert little or no effect on developing B. pahangi in Ae. aegypti. They accounted for this failure by suggesting that it was due to the inability of the folate analog to reach the target enzyme located within the cells of the host and/or the parasites. Furthermore, Jaffe et al. (1977) had demonstrated that the amount of DHFR per mg total soluble protein increased in Ae. aegypti in response to advanced

infection with B. pahangi and Jaffe and Chrin (1978) reported a more marked increase in the folate-related enzyme serine transhydroxymethylene in the host. Existing evidence strongly suggests that host folate metabolism plays an important role in the mosquito-filaria relationship.

2.6.6 Chemical treatment of mosquito larvae.

Khalil et al. (1974,1975) found that a sublethal dose of DDT, Abate and Sevin applied to larval stages of C. p. molestus had no effect on the infection rate or on the vectorial capacity of the adults to W. bancrofti.

Interesting results were obtained by Gaaboub and Busvine (1975) who studied the effects of Ae. aegypti on the susceptibility to B. pahangi. They found that DDT decreased the percentage of females of the susceptible strain in which B. pahangi reached the infective stage, conversely following DDT treatment a proportion of the refractory strain females became susceptible to infection. In further experiments Gaaboub and Busvine (1976) found that sublethal treatment with chitin inhibitor, Dimilin (PH 60.40) given to the fourth instar larvae of two strains of Ae. aegypti, one DDT susceptible and a good filarial vector and the other DDT resistant and a refractory vector, did not have any appreciable effect on the vectorial capacity of the susceptible strain, but caused the appearance of a moderate level of susceptibility in the refractory strain. Their experiment with heat stress to test the hypothesis that the similar effects of two such different insecticides were due to generalized stress showed negligible effects on the vectorial capacity of both strains so that the effects shown did not seem to be due to simple stress.

2.6.7 Irradiation

Duxbury and Sadun (1963) conducted a series of experiments on the effects of gamma radiation on the development of D. uniformis in An. quadrimaculatus. Mosquitoes irradiated 24 hours prior to infection were found to have more larvae than the non-irradiated controls. This difference was significant only for mosquitoes exposed to 10,000 rep. However, in mosquitoes irradiated 24 hours after infection, there was a marked reduction in numbers and growth of larvae as compared to the controls. On dissection 10 days after infection, the larvae in irradiated mosquitoes were found to be in the sausage stage, which is normally reached in 3 to 4 days. In addition to this, there was no migration of larvae from the abdomen to the thorax and head in any of the irradiated mosquitoes.

Richey and Rodriguez (1976) examined the quantitative effects of cobalt gamma radiation on the development of B. pahangi in a susceptible strain of Ae. aegypti. Females were subjected to unfractionated doses of 3,000 and 5,000 rads before and after simultaneous infections. The authors reported a 56% decrease in susceptibility in females irradiated with 5,000 rads post infection. Likewise, the mean number of infective larvae per female present at both 3,000 and 5,000 rads post infection was reduced significantly whilst the control mosquitoes for both groups had almost twice as many larvae. On the other hand, there was no significant change either in percent susceptibility or in mean number of larvae per female in mosquitoes irradiated before infection.

Ahmed (1969) investigated the influence of X-irradiation on the development of sub-periodic B. pahangi and B. malayi in Ae. togoi and in three naturally refractory and one genetically selected highly

susceptible strain of Ae. aegypti. Irradiation had no effect on the development of B. malayi in Ae. aegypti or of B. pahangi in Ae. togoi and Ae. aegypti. However, in irradiated Ae. togoi infected with B. malayi, the infection rate was significantly higher than in untreated controls. No definite conclusions could be drawn from this single series of experiments due to the heterogeneity of the results.

2.6.8 Enzymes

Lavoipierre (1958) commented that "although evidence that the filarial worm may adversely affect its invertebrate host is based almost entirely on histologic observations and on mortality studies of populations of parasitized arthropods, it should not be forgotten that biochemical investigations, which hitherto have not been undertaken may reveal interference (not otherwise demonstrable) with the normal metabolism of the host."

Jaffe et al. (1977) carried out biochemical investigations to determine whether the characteristic amount or selected properties of lactate dehydrogenase, xanthine dehydrogenase and dihydrofolate reductase in normal mosquitoes would be altered when these mosquitoes become infected with B. pahangi. Results of this study indicated that the amount or turnover of dihydrofolate reductase (DHFR) consistently increased in Ae. aegypti with advanced B. pahangi infections. DHFR catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) mainly in the thorax of Ae. aegypti. Although the authors could not see clearly the significance of these results, they speculated that developing B. pahangi larvae may compete with mosquito host muscle cells for some product(s), the formation of which depended upon the availability of THF factors. The increase in DHFR activity observed would then reflect the attempt by the mosquito host to meet

the increased demand for THF co-factors in this circumstance.

Subsequently Jaffe and Chrin (1978) reported an increase in the activity of methylenetetrahydrofolate reductase but not methylene-tetrahydrofolate dehydrogenase in Ae. aegypti females with advanced B. pahangi infections. They could not tell whether this response was to some sort of nutritional competition between parasite and host or was it merely a host response to mechanical injury of flight muscles caused by the emergence of the third-stage larvae from parasitized muscle cells prior to migration to the mouthparts.

This review on some of the extrinsic and environmental factors which affect the development of filariae in mosquitoes indicates that the factors involved in determining whether a mosquito is an efficient vector or not are many and there is a lot of interaction among them. These studies also indicate that more information is needed on the physiology of normal as well as infected mosquitoes. This kind of information would make it easier to try and elucidate how the genes which determine susceptibility of mosquitoes, translate their message into physiological processes. Such studies would ultimately be important for vector control.

3. MATERIALS AND METHODS

The materials and methods outlined in this chapter refer to the general procedures followed throughout these studies. However, certain chapters will be preceded by specific materials and methods pertaining to the studies reported in that particular section.

3.1 The Mosquitoes

Mosquitoes used in the present studies are as indicated in Table 1.

Where different geographic stocks of the same mosquito species were used great care was taken to avoid contamination by rearing the stocks in different rooms and by providing separate sets of rearing equipment for each stock.

3.2 Rearing and Maintenance of mosquitoes

Mosquitoes were reared and maintained in insectaries at a temperature of 25-28^{o}C and relative humidity of 75-80%. In order to facilitate the feeding by Culex stocks during normal working hours, the lighting was set on 12:12 LD phase, with the dark phase commencing at 13.00 hrs and ending at 1.00hr. Egg rafts from the Culex stocks were collected in plastic cups containing hay infusion, which had been placed in the cages 3 days after a blood meal. Fully engorged females drowned if these cups containing hay infusion were placed in the cages earlier. However, in the case of the autogenous mosquitoes, C. p. molestus, the plastic cups were placed in cages as soon as the adult mosquitoes emerged. This stock did not require a bloodmeal prior to egglaying.

Eggs from Ae. aegypti and An. stephensi were collected on damp filter paper on top of the wet cotton wool placed in plastic cups placed inside the cages 3 days after the bloodmeal. Eggs of Ae. aegypti were dried then stored in labelled self-sealing polythene bags. Eggs of

Table 1: Stocks of Mosquitoes used

Species	Designation	Origin	Date received in Liverpool
1. Culex pipiens quinquefasciatus (Wiedemann)	DAR	Collected as larvae in Dar-es-salaam (Tanzania) reared for one generation and then brought to Liverpool by Dr. J.N. Minjas.	29.4.1978
2. Culex pipiens quinquefasciatus (Wiedemann)	MON	Collected by E.Zielke, Monrovia (Liberia)	19.5.1978
3. Culex pipiens quinquefasciatus (Wiedemann)	THAI	Sent from Bangkok (Thailand) by Dr. S. Sucharit	26.6.1978
4. Culex pipiens quinquefasciatus (Wiedemann)	SUVA	Collected from Suva (Fiji) by Dr. S. Meek	1982
5. Culex pipiens quinquefasciatus (Wiedemann)	D/K5	Received from L.S.H.T.M. (derived from a Kenyan stock see Curtis et al.(1983)	June,1981
6. Culex pipiens molestus (Forskal)	DAGMOL	Received from L.S.H.T.M. Originally collected at Dagenham (U.K.)	21.4.1982
7. Anopheles stephensi (Liston)	STEPH	Received from Parasitology Dept. of L.S.T.M. obtained from Edinburgh.	January 1983
8. Aedes aegypti (Linnaeus)	AMSS	A stock selected from the Liverpool stock of Ae. aegypti for susceptibility to sub-periodic B. malayi by Prof. Macdonald (see Macdonald, W. W., 1962a)	1962

L.S.H.T.M = London School of Hygiene and Tropical Medicine

L.S.T.M. = Liverpool School of Tropical Medicine

An. stephensi cannot be stored for long since they need to be damp all the time. When an egg raft from an individual bloodfed mosquito was required, the mosquito was transferred with a sucking tube into a 7.5 cm x 2.5cm test tube, containing $2cm^3$ of hay infusion and a single feather to prevent drowning of the mosquito. The test-tube was covered with a piece of mosquito netting held in place with an elastic band. Eggs were transferred to adequately labelled rearing dishes containing tap water and hay infusion. Culex and Aedes larvae were fed exclusively on yeast tablets which were placed in the rearing trays. An. stephensi larvae were reared in clean tap water to which only ground yeast was sprinkled every day. C. p. molestus were fed on liver powder plus yeast tablets. Initially one tablet per tray was sufficient for first instar larvae. Subsequently, more tablets per tray were offered as the larvae grew. In order to avoid overcrowding effects, approximately 200 larvae were placed into each tray. Scum formation was prevented by changing the larvae rearing medium every other day. The trays were covered with fine mesh netting to prevent contamination with eggs from escaped mosquitoes. The mosquito pupae were picked daily with small pipettes and transferred into water kept in plastic cups placed in appropriately labelled stock cages into which the adults emerged. Similarly, the pupae from separately reared progenies were transferred to labelled plastic cups containing water then placed into smaller cages which were appropriately labelled.

Adult mosquitoes were maintained in 30 x 30 x 30 cm cages. Each stock was maintained in a different room to avoid contamination. A pad of wet cotton wool and sugar cubes placed on the top of each cage provided moisture and food for adult mosquitoes. The pads were rewetted every second day and replaced when discoloured.

Before offering the mosquitoes a bloodmeal, the pads and sugar cubes were removed for at least 48 hours. The stocks were fed on guinea-pigs anaesthetized with Sagital (0.6ml Kg-1), shaved and placed on top of the cages 4 to 7 days after emergence of the first females.

3.3 The Filarial Parasites.

Brugia pahangi:- These were obtained from infected domestic cats and jirds (Meriones unguiculatus) which were maintained at the Liverpool School of Tropical Medicine.

Wuchereria bancrofti:- The strain used was one from Dar-es-salaam, Tanzania, collected by Dr. H. Townson. The microfilariae were obtained from human donors, then preserved in low H.E.S. and stored in liquid nitrogen refrigerators.

3.4 Mating of mosquitoes

Mass mating was allowed amongst C. quinquefasciatus (DAR) held in stock cages, during the attempt to select for susceptible and refractory species.

Controlled mating between C. p. molestus and C.p. quinquefasciatus (DAR) was achieved by isolating female pupae of C. p. molestus and male pupae of C. quinquefasciatus prior to emergence of the adults. The following procedure was followed in setting up the cross. Pupae of C. p. molestus were sexed by examining the pupal hypopygium and pupal size. The male larvae commenced pupation earlier than the females. The females were generally larger in size but there was always overlap in the pupation time and pupal size in any larval tray. The most reliable feature for separating male and female pupae was the pupal hypopygium. (See Figs. 1 & 2). Ventral to the paddles in the male are two largely

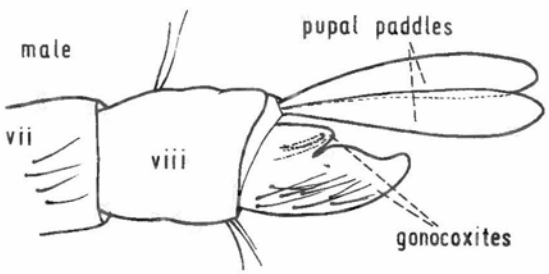


Fig. 1: Showing the morphology of the male hypopgyium of a Culex pupa.

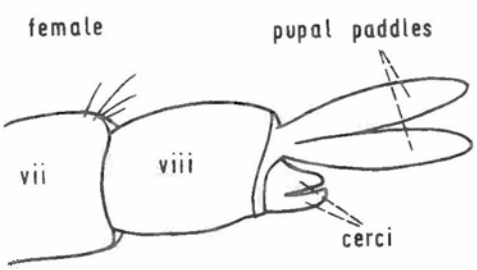


Fig. 2: Showing the morphology of the female hypopgyium of a Culex pupa.

applied gonocoxites. There are two smaller conical projections in the female pupae. Female pupae were divided into groups of 3 or 4 and placed in 7.5 x 2.5cm tubes provided with 2 inches of hay infusion and a feather to provide support for emerging adults. The tubes were covered with mosquito netting held in place by rubber bands. Similarly, male pupae of C. p. quinquefasciatus (DAR) were collected in the above manner. After emergence, the sex of the adults was rechecked and in the case of tubes of intended females where a male had been accidentally placed, all the mosquitoes were discarded to guard against the possibility of insemination of any of the females by the males of their own strain. The checked males and virgin females were released into separate cages. The males were aged for 2 days so as to allow them all to reach sexual maturity and thus to have a chance of taking part in mating when males and virgin female of C. p. quinquefasciatus and C.p. molestus were crossed.

Egg rafts were collected in hay infusion and held individually in tubes of water for two days before scoring for egg hatch. After inspection for hatched larvae, each raft was examined under a dissecting microscope and scored for presence or absence of embryonation. Larvae from hatched rafts were reared to pupal stage and then the pupae were sexed.

3.5 Thawing and Reconstitution

The cryopreservation methods used were those of Minjas and Townson (1981) with minor modifications.

The samples of frozen W. bancrofti blood were thawed by transferring them rapidly from the liquid nitrogen refrigerator to a shaking water bath at 37°C. Minjas (1981) found that leaving the blood to thaw at room temperature was found to be detrimental to the microfilariae.

As soon as thawing was complete, usually in 60-120 seconds, the samples were diluted approximately 10 fold in their own volumes with normal saline previously cooled in ice. The diluted blood was then centrifuged at 3,000g for 5 minutes, the supernatant carefully pipetted out, more saline added and centrifuged once again. This double washing was found necessary after high mosquito mortality rates were observed.

The pellet of blood cells and microfilariae were then resuspended in appropriate volumes of human blood containing 10 iu heparin per ml. This was then incubated at 37°C for 30 minutes, after which 3 smears of $20mm^3$ were prepared to provide a microfilarial count. The remaining blood was placed in a feeder unit and fed to mosquitoes. Minjas (1981) reported that it was necessary to add fresh blood to the thawed blood to compensate for the haemolysis which occurred during the freezing/thawing process. Mosquitoes did not feed well on haemolysed blood.

3.6 Infection of mosquitoes

All the infecting blood offered to mosquitoes in this study was via the artificial membrane feeding system (Wade, 1976). The membrane feeding unit consisted of a water heater and a pump capable of circulating water at 37°C, a glass feeder and an electrically driven stirrer. Stirring the blood prevented sedimentation of erythrocytes and also distributed the microfilariae evenly in the feeder. This led to minimizing the variation of the number of microfilariae ingested per mosquito as well as increasing the chances of each feeding mosquito to pick up microfilariae. The volume of the glass feeder varied depending on the volume of infected blood being used in a particular experiment.

The skins of 7 to 10 day old chicks were used as membranes for the feeders. The chicks were freshly skinned en masse. Individual skins were then sandwiched between two filter papers and stored at -20°C.

These were thawed out individually when required. The skin was washed in cold running tap water and placed between pads of filter paper to remove excess moisture. Any excess fat or feathers were also removed. An elastic band was used for holding the skin to the glass unit. The stretched skin was then shaved with a pair of scissors. To set up for membrane feeding, the heating unit was attached to a plastic bath containing water of 30 cmm^3 depth. By means of flexible rubber tubes, the pump outlet was connected to the inlet of the glass feeding unit. Several glass units could be connected and the outlet of the last glass unit was connected to a length of rubber tube, which returned to the bath. The heating unit was then set to give a bath temperature of $37^{o}C$.

Mosquitoes to be fed were starved and denied free access to water for 24-48hours, before being offered the infective bloodmeal. Mosquitoes were held either in the standard (30 x 30 x 30cm) cages or smaller cylindrical perspex cages (15cm in diameter and 12cm high) depending on the numbers being fed. The latter cages were covered, permanently on one end with plastic netting and the other with nylon netting held in position with elastic rubber bands.

The feeders were placed on the nylon or terylene fabric netting rather than on the brass gauze of the top or sides since the metal conducted the heat away too rapidly thereby inducing the attractiveness of the feeders to the mosquitoes. Occasionally the feeders were moved about to discourage engorged females from hanging onto the membrane and allow other females to probe.

Engorged females were transferred gently with a sucking tube into clean labelled cages 3 hours to 24 hours after feeding. This decreased the number of mosquitoes that suffered handling damages during transfer as the mosquitoes will have had time to loose excess fluid likely to

enhance damage. Fed mosquitoes were provided with water and sugar as mentioned before until they were ready for dissection.

3.7 The Estimation of microfilariae in the blood.

The microfilariae in the infecting blood were estimated from a mean of three $20mm^3$ samples of blood. For each sample, the blood was divided into $10cmm^3$ drops placed on either side of a clean glass slide. This was covered with a glass cover slip and the motile microfilariae counted under X100 magnification of a compound microscope. The mean of three samples gave the number of microfilariae in $20mm^3$ of blood.

3.8 Dissections

The estimation of microfilariae in bloodmeals - The microfilariae ingested by the mosquitoes were counted under a compound microscope at X100 magnification. Detection of microfilariae was aided by the following features.

1. The bloodmeal was teased apart in a drop of Hayes saline (1953) and this reduced the cells and colour intensity per unit volume. Movement of microfilariae made their presence easy to detect.
2. Plastic coverslips divided into 100 small squares were used to cover the teased bloodmeal. By counting in an orderly way repitition or failure to count any area was avoided.

The counting of microfilariae in bloodmeals 24 hours after the bloodmeal required examination of finely teased blood meals at higher magnifications of X400. Microfilariae were dead in the midgut of mosquitoes by this time and larvae more difficult to detect.

Mosquitoes held for more than 24 hours were dissected from day seven after the infective bloodmeal and those dying before were discarded. Mosquitoes were narcotized with carbon dioxide and the head, thorax and abdomen dissected separately in physiological saline under a dissection

microscope at X45, doubtful cases being checked at higher magnifications. Records were kept of the location of the developing larvae and their stage of development. Note was also taken as to whether they were normal or abnormal. Those mosquitoes found to contain second stage and/or infective larvae were scored as positive for infection.

4. THE SUSCEPTIBILITY OF SOME MEMBERS OF THE CULEX PIPIENS COMPLEX TO WUCHERERIA AND BRUGIA FILARIAE

4.1 The development of W. bancrofti in normal and aposymbiotic strains of C. quinquefasciatus (DAR).

4.1.1 Introduction

Variation in the susceptibility of C. quinquefasciatus to different populations of Wuchereria bancrofti, has been reported between West African and Asian populations of this mosquito (Zielke and Kuhlow 1977; Jayasekera et al., 1980). Little or no variation was detected between the F_1 progeny of reciprocal crosses (Zielke and Kuhlow, 1977) which suggested that this variation was due to nuclear genes.

Various workers have demonstrated that the susceptibility of Ae. aegypti to Brugia, Wuchereria, Dirofilaria and Waltonella filarial parasites, is controlled by nuclear genes. Macdonald (1976) reported similar control of susceptibility of Culex pipiens to Brugia.

Recently, data have been presented which suggests that the inheritance of susceptibility to filariae (B. malayi and B. pahangi) in the Aedes scutellaris group, is determined by extra chromosomal factors which are maternally inherited (Trpis et al., 1981). In the Culex pipiens group, crossing type (cytoplasmic incompatibility) follows a pattern of maternal inheritance (Laven, 1967). It would be of interest to know whether maternally inherited variation in filarial susceptibility occurs in this group. The maternal inheritance of crossing type is associated with the rickettsiae Wolbachia pipientis, which can be removed from the line by treatment with tetracycline (Yen and Barr, 1973). The experiments described below, were performed to determine whether differences occur in the development of W. bancrofti in normal (with rickettsia) and aposymbiotic (cured of their rickettsia) C. quinquefasciatus. These experiments

together with others conducted by Curtis and his colleagues at the London School of Hygiene and Tropical Medicine have been reported in Curtis et al. (1983).

4.1.2 Materials and Methods

The aposymbiotic strain of C. quinquefasciatus was derived from a colony originating from Hola, Kenya. Larvae from this colony were treated and retreated with tetracycline hydrochloride and shown to have all the properties of an aposymbiotic strain in crosses and with the electron microscope. This work was carried out by Curtis and his co-workers (Curtis et al. 1983). This strain, designated D/K5 was received in Liverpool and a colony established. Strains of C. quinquefasciatus from Dar-es-salaam (DAR) and Bangkok (THAI) were employed for comparison with the D/K5 strain. The crossing characteristic of these strains show them to be normally infected with Wolbachia. Blood infected with W. bancrofti from donors in Dar-es-salaam had been cryopreserved with HES (hydroxethyl starch) by Minjas and Townson (1980), which has been proven to be a better cryoprotectant than DMSO (Dimethyl-sulphoxide). The blood had been divided in 2ml aliquots and frozen down at $1^{\circ}C$/min to $-40^{\circ}C$ before being plunged into liquid nitrogen.

In preparation for a feed, the blood was thawed rapidly then reconstituted in human blood. The blood plus microfilariae was placed in a membrane feeding apparatus brought to $37^{\circ}C$ and the mosquitoes allowed to engorge. Different stocks of C. quinquefasciatus were fed at different times and the fed females kept in separate containers. Initially, C. quinquefasciatus DAR stock had been infected with W. bancrofti and the development of the parasite was monitored from day 1 after the infective feed. Mosquitoes were anaesthetized using Co_2 and then dissected in Hayes saline. The number of larvae present in the head, thorax and abdomen were

counted and the larval stage was also noted. This allowed study of the morphological changes which occur once the microfilariae have been ingested, up to their infective stage. This experiment was repeated twice using only DAR stock of C. quinquefasciatus and once using the THAI, DAR and D/K5 stocks of C. quinquefasciatus.

In order to study the growth of the parasites, five mosquitoes from the 3 groups were dissected daily, starting from day 8 after the infective feed. The larvae were removed using a drawn out micro pipette, transferred to a well on a glass slide made using nail varnish, then fixed with Bles fixative and mounted using Whartons mountant. Larvae were drawn with the aid of a camera Lucida and the lengths and widths of the images were measured using a map measurer. Their mean, standard error and range were plotted (See figures 3,4, & 5).

4.1.3 Results and Discussion

These results show no significant differences in the rates of development of the larvae nor in the size attained by the infective larvae, in normal and aposymbiotic C. quinquefasciatus. Other experiments carried out in our laboratory showed no reduced susceptibility in the aposymbiotic strain, either in terms of total worm burden, proportion of mosquitoes supporting development, or the proportion of filarial larvae completing their development (See Table 2).

These results suggest that Wolbachia is not a requirement for the successful infection of C. quinquefasciatus by W. bancrofti. This contrasts with a suggestion of rickettsial involvement in the process of filaria infection of a member of the Ae. scutellaris complex made by Duhrkopf and Trpis (1981). The findings of DuhrKopf and Trpis (1981) are not substantiated by the work of Meek and Macdonald (1982). Their data on the inheritance of susceptibility to infection with B. pahangi and W. bancrofti

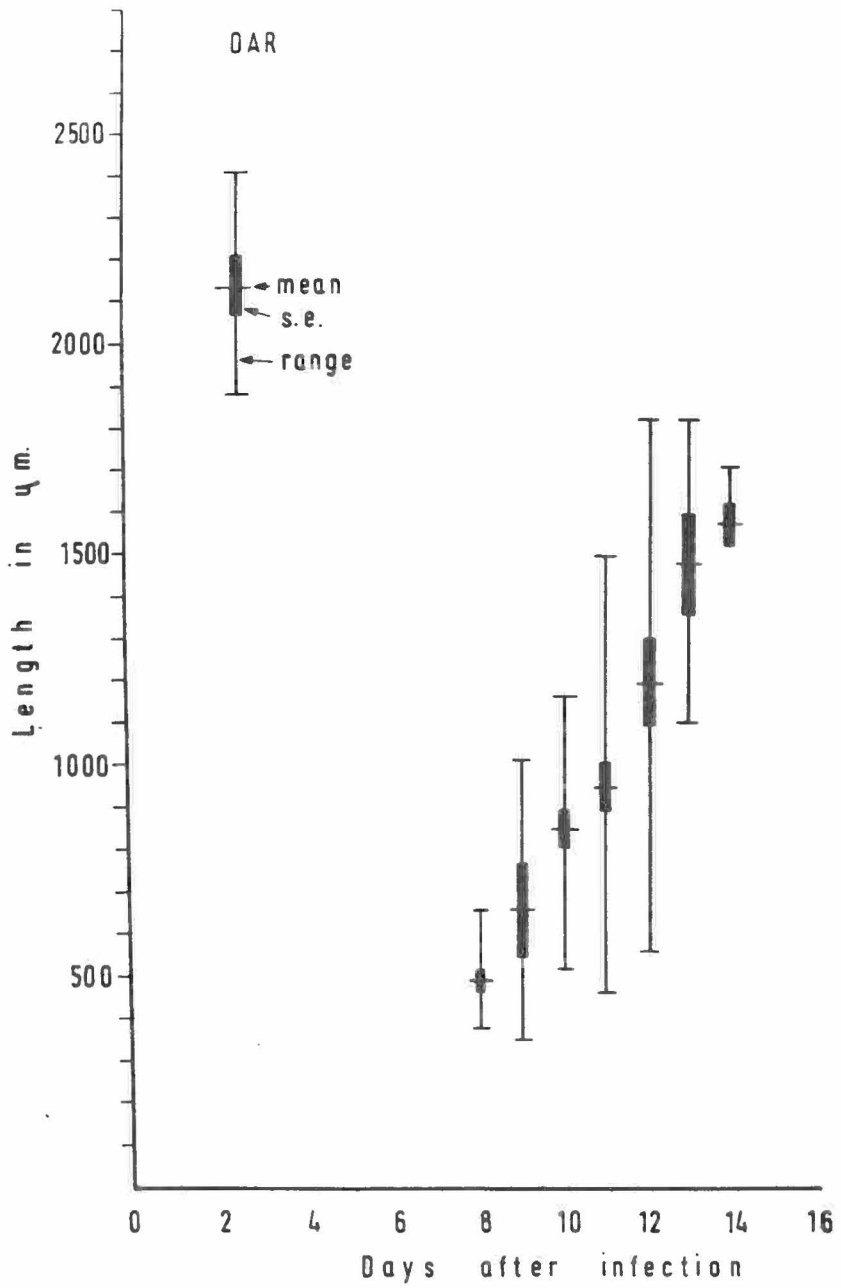


Fig. 3: The growth of larvae of W. bancrofti in Culex quinquefasciatus (DAR) strain.

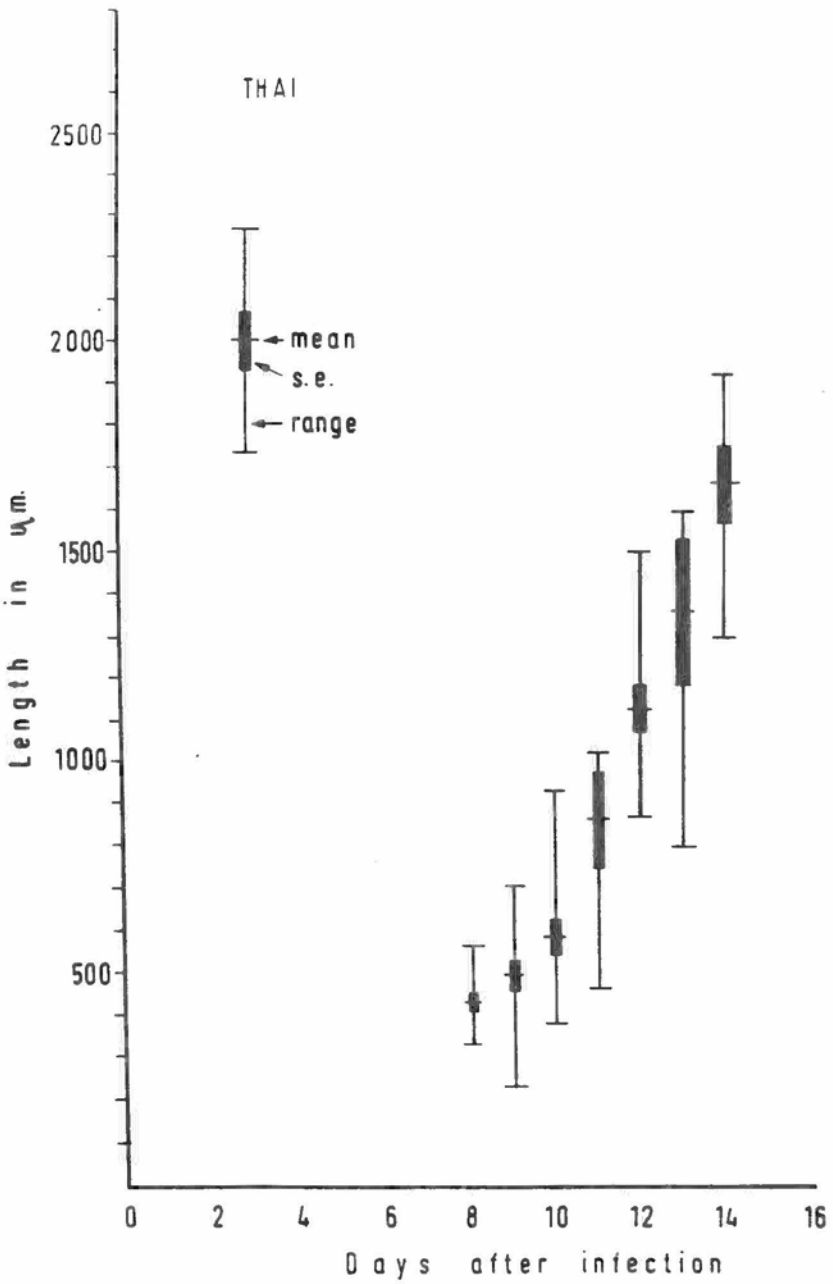


Fig. 4: The growth of larvae of W. bancrofti in Culex quinquefasciatus THAI strain.

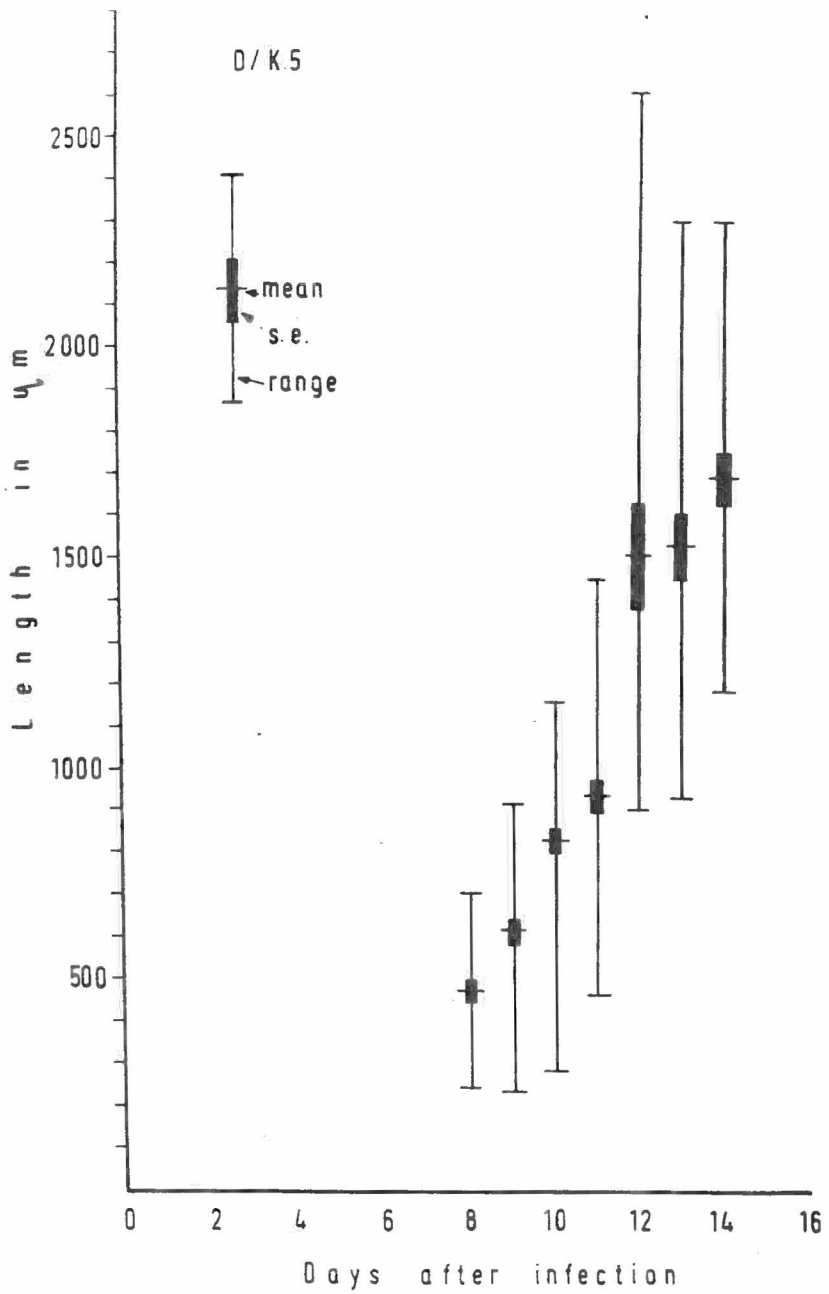


Fig. 5: The growth of larvae of W. bancrofti in Culex quinquefasciatus D/K5 strain.

Table 2: The Results of Feeding Aposymbiotic and Normal Strains of Culex quinquefasciatus on Microfilariae of Wuchereria bancrofti which had been Cryopreserved in Liquid Nitrogen with 15% w/v Hydroxyethyl Starch as Cryoprotectant

Expt. (Date)	mff/ 20µl	Mosquito Strain	No of mosquitoes Fed	Survived	Dissected	Positive	Larvae/mosquito dissected Arithmetic mean	Williams mean	Percentage larvae infective in mosquitoes dissected from Day 14
1. July,1981	46	DAR	15	9	9	6	2.0	1.3	88.9
	46	THAI	101[a]	48	33	32	3.0	2.7	88.3
	46	D/K5	7[b]	7	7	5	5.1	3.3	100.0
2. July, 1981	72	DAR	28	10	10	10	3.4	3.0	91.2
	72	THAI	48	27	27	23	2.2	1.8	92.0
	72	D/K5	2	2	2	2	5.5	5.0	100.0
3. Aug., 1981	80	THAI	80	61	61	61	5.0	4.6	95.5
	80	D/K5	68	68	68	67	10.0	8.0	99.2
4. Aug., 1981	69	THAI	215	195	52	50	4.2	3.6	96.1
	69	D/K5	95	56	56	47	4.4	3.1	98.5

a = Many partially fed

b = Four had taken partial meals.

* Taken from Curtis et al., 1983

in the Aedes scutellaris group of mosquitoes, suggest that nuclear factors are involved in the inheritance of susceptibility to filarial infection, but there was no evidence of a major gene. These conflicting reports on the effect of rickettsia like symbionts on filarial susceptibility in different mosquitoes could be due to the existence of different kinds of rickettsia in Aedes and Culex mosquitoes. That such differences do exist is supported by results from studies on the crossing relationships among seven members of the Ae. scutellaris group by Meek and Macdonald (1984). Their studies showed that males of A. alcasidi and A.s. katherinensis which lack rickettsia-like symbionts (Meek, 1984), were incompatible with the eastern species of A. polynesiensis (aposymbiotic strain), A. polynesiensis (symbiotic strain), A. pseudoscutellaris, and A. cooki, but compatible with the western subspecies A. s. scutellaris. This suggests that the effect of rickettsia-like organisms in the Ae. scutellaris group varies from that observed in the C.pipiens complex. Furthermore this suggests that rickettsia like organisms are sometimes but not always the cause of cytoplasmic incompatibility and that other agents could be causing cytoplasmic incompatibility and could also play a role in determining the susceptibility of these mosquitoes to filariae.

4.2 The susceptibility to B. pahangi of Geographical strains of C. quinquefasciatus

4.2.1 Introduction

Culex quinquefasciatus is a good host for W. bancrofti, but has been found to be a poor host for filarial parasites of the Brugia species. Iyengar (1932) and Raghavan and Krishnan (1949) failed in their attempts to infect Indian strains of C. quinquefasciatus with their local strains of nocturnally periodic B. malayi.

Edeson et al. (1960) reported only a 7% mature larvae rate in Malaysian C. quinquefasciatus infected with B. pahangi. Desowitz and Chellapah (1962), recorded susceptibility rates of 8.5 to 11.2% in three to eleven day old Singapore mosquitoes. Using U.S. strains of C. quinquefasciatus, Ewert (1965a) reported a 4% mature larvae rate of B. pahangi, with very few microfilariae reaching the thorax from the midgut. Obiamiwe (1970) studied the genetic factors affecting the susceptibility to filarial infections in the Culex pipiens group. By selection of susceptible red-eyed and black-eyed strains of Culex pipiens, followed by reciprocal crosses and backcrosses, susceptibility to B. pahangi in the C. pipiens complex was shown to be under the control of a sex linked recessive gene, designated sb by Obiamiwe and Macdonald (1973) and more fully described by Obiamiwe (1977a). This gene has incomplete penetrance. Obiamiwe (1970) also examined the susceptibility to B. pahangi of five geographically separated strains of C. quinquefasciatus in order to measure the frequency of the gene sb in field populations. Similarly in the following experiment, the frequency of the gene sb was examined in four different geographical strains of C. quinquefasciatus and the frequencies obtained were compared to those reported by Obiamiwe (1970).

4.2.2 Materials and Methods

The four strains of C. quinquefasciatus were collected from the following places.

1. Dar-es-salaam, Tanzania
2. Monrovia , Liberia
3. Bangkok , Thailand
4. Suva , Fiji

The strains were maintained as laboratory stocks in different insectaries, in order to avoid contamination. C. quinquefasciatus mosquitoes from DAR, MON and THAI, used in these experiments were in their F_{58}, F_{59}, F_{55}, and F_{54} generations respectively. Those from SUVA had been maintained in the insectary for one year and were approximately in their $F_{12} - F_{15}$ generations. The insectary conditions, rearing of mosquitoes, estimation of microfilariae in infected blood, and the method of membrane feeding were as described under general materials and methods (Chapter 3).

Engorged females were transferred to perspex containers and maintained on sugar and water till day 10, when they were dissected. The number and stages of the filarial larvae in the head, thorax and abdomen of each infected mosquito were recorded. Mosquitoes which had any developing filarial larvae were scored as 'susceptible' and those without larvae as 'refractory'.

B. pahangi microfilariae were removed from the peritoneum of infected jirds, then reconstituted in rabbit blood.

4.2.3 Results and Discussion

Data in Tables 3 and 4 show low susceptibility of four strains of C. quinquefasciatus to B. pahangi, and high susceptibility rates in Ae. aegypti SS. The susceptibility and mature larvae rates ranged from

0-18%. The parasite density of the infective blood meals ranged from 1 to 15 mff/mm^3, but there appears to be no relationship between the parasitaemia and the numbers of susceptible mosquitoes. This is in contrast to Obiamiwe's (1977b) findings, that when parasite density was below 4.1 mff/mm^3, few or none of the microfilariae developed in the thorax, while higher densities (7.4 - 8.6 mff/mm^3) were optimum for successful development, and very high densities (above 9 mff/mm^3) gave lower rates for successful development. In considering the few numbers of mosquitoes found with developing larvae in the thorax, one should bear in mind that the blood meal contained the anti-coagulant heparin at a concentration of 10 iu/ml. This could be responsible for increasing the migration of a few microfilariae to the thorax. If these mosquitoes had been fed directly on an infected cat or other donor, the numbers of microfilariae migrating successfully may have been less, leading to even lower susceptibility levels than those observed.

Obiamiwe (1970), reported that the presence of heparin in the blood allows migration of microfilariae fromthe blood meal to the thorax in genotypically refractory mosquitoes, but that the microfilariae fail to develop. He found that up to 23% of the ingested microfilariae complete their migration, which is the level found to migrate in genotypically susceptible mosquitoes fed on non-heparinized blood.

Ogunba (1969) working with Malaysian C. quinquefasciatus reported virtually no migration of B. pahangi in refractory mosquitoes fed directly on animals. Similarly, Obiamiwe (1977c) observed hardly any migration in a selected refractory strain of C. pipiens. However results from the studies on the effect of different concentrations of heparin on migration of B. pahangi in DAR (Chapter 8) show that the migration rate is not increased at higher concentrations of heparin, the reverse was

found to be true. Not only was migration reduced but mosquitoes did not feed readily.

Due to the high microfilarial densities in all but one experiment, and the low levels of susceptible mosquitoes found, it is difficult to say with certainty that the mosquitoes with filarial larvae carried the gene sb in the homozygous state. It is possible, however that the gene sb is present in the phenotypically susceptible mosquitoes, but has poor penetrance and low expressivity. From the data in Table 3, the frequencies of the gene sb were calculated using the Hardy Weinberg Law and found to be as follows:

Designation of Mosquito Strain	sb frequency
DAR	0.20
THAI	0.28
MON	0.16
SUVA	0.15
(from Obiamiwe 1970)	
Kuala Lumpur	0.33
BoboDioulasso	0.28
Ibadan	0.12
Maracay	0.31
Tanga	0.23

These frequencies are comparable to those found by Obiamiwe (1970). It is interesting to observe that C. quinquefasciatus from Bobo Dioulasso (Upper Volta) and THAI have similar sb frequencies, considering how far apart they are situated geographically. It is possible that the variation in sb gene frequency observed in the various strains of C. quinquefasciatus has occured as a result of colonization of these strains in the laboratory.

As indicated in the materials and methods, mosquitoes used in these studies had been reared for over 50 generations, whereas those used by Obiamiwe (1970) were in their second or third generation as colonized laboratory stocks. The low susceptibility of C. quinquefasciatus to B. pahangi microfilariae, could not be due to reduced infectivity of B. pahangi microfilariae, as control experiments using susceptible black eyed Ae. aegypti had 87-97% infectivity rates (see Table 4). These results are comparable to those reported by other workers (Edeson et al. 1960; Desowitz and Chellapha, 1962; Ewert, 1965; Obiamiwe, 1970) and confirm that C. quinquefasciatus is generally a poor host of B. pahangi.

Table 3: The Susceptibility of four strains of C. quinquefasciatus to B. pahangi

Expt.	Mosquito strain	Mff/mm^3	Nos. of mosquitoes (%) Fed	Dissected	with larvae	with mature larvae	Filarial Larvae in Thorax I	II	III	Abdomen I	II	III	Head III	Proboscis III	A	B
1.	DAR	15	100	42	1(2.4)	1 (2.4)	0	0	0	0	0	0	1	0	1.0	1.0
2.	DAR	6.3	33	11	2(18)	2 (18)	0	0	0	0	0	0	1	2	1.5	1.5
3.	DAR	9.5	80	22	0 (0)	0 (0)	0	0	0	0	0	0	0	0	0	0
4.	THAI	1.0	81	60	1 (2)	1 (2)	0	0	0	0	0	0	0	1	1	1
5.	THAI	10.0	184	85	11(12.9)	10(12.9)	0	1	2	0	0	3	4	4	1.4	1.3
6.	MON	10.0	152	118	3 (2.5)	3 (2.5)	0	1	0	0	0	1	0	2	1.3	1.0
7.	SUVA	8.0	294	254	6 (2.4)	5 (2.0)	0	1*	2	0	0	2	0	1	1.0	1.0

Table 4: The Susceptibility of Ae. aegypti SS to B. pahangi

8.	AMSS	10.0	147	117	102(87.2)	101(99)	70	21	109	2	3	244	71	324	8.3	7.3
9.	AMSS	11.0	33	33	32(96.9)	20(62.5)	0	52	24	0	0	30	20	60	5.6	4.1

A = Total Larvae per susceptible mosquito

B = Mature larvae per susceptible mosquito

* = abnormal larvae

4.3 The susceptibility of C.p. molestus to B. pahangi.

4.3.1 Introduction

Culex pipiens molestus has been reported to be a good host of W. bancrofti. Yoeli (1957) found infection rates of 65%, 86.9% and 82.3% in laboratory bred C.p. molestus from Israel, which had fed on immigrant Indian Jews. The susceptibility of C.p. molestus to Brugia has been found to be higher than that of C. quinquefasciatus. Schacher and Khalil (1965) obtained 14.1% and 19.1% mature larvae rates in Lebanese C.p. molestus infected with B. pahangi. Ogunba (1969) and Obiamiwe (1970) reported susceptibility rates of 36.6% and 33.3% respectively in a London strain of the same mosquito. The strain used by Obiamiwe was red-eyed. In the following studies, the susceptibility of a black-eyed London strain of C.p. molestus to B. pahangi was compared with results reported by other workers. It was hoped that the results would give an indication as to whether it was possible to select susceptible and refractory stocks of C.p. molestus, which would be used in studies designed to determine the gene(s) determining susceptibility of the mosquitoes to B. pahangi.

4.3.2 Materials and Methods

The strain of C.p. molestus used in this experiment was reared from a laboratory colony established at the London School of Hygiene and Tropical Medicine, from eggs collected in Dagenham, London.

This mosquito species of Culex pipiens is black eyed, autogenous and stenogamous. Due to their autogenous stenogamous nature they feed reluctantly on infective blood meals offered to them via a membrane feeder. In order to overcome this difficulty infective blood meals were offered to mosquitoes only after they had laid eggs and been starved of sugar and water for at least 24 hours. Microfilariae B. pahangi used were obtained from peritoneal washings of infected jirds, then mixed with human blood.

It was observed that these mosquitoes preferred human to rabbit blood. Fed females were held separately in perspex containers and provided with sugar and water until day 10 post infection when they were dissected. Mosquitoes with normally developing larvae were designated as 'susceptible' and those without developing larvae as 'refactory'. The location and numbers of larvae in the mosquito was also noted. Ae. aegypti SS stock were used as controls in some of the infective feeds.

4.3.3 Results and Discussion

Results from 7 separate experiments gave susceptibility rates ranging from 0 - 31.3% (see Table 5). Of the total numbers of C. p. molestus dissected, only 5.4% were found to be susceptible. As indicated above, susceptible mosquitoes were those found to harbour normal developing larvae. Of the 25 developing larvae found in 18 mosquitoes, 8 were infective, 9 were in the second larval stage and 8 were in the second larval stage but grossly abnormal. All the abnormal larvae were found in the thorax. The infective larvae were found in the thorax and abdomen and the normal second stage larvae were also found in the thorax. Despite the large numbers of microfilariae present in the blood meals of some experiments, only a small proportion of the ingested larvae succeeded in migrating to the thorax and developing to mature larvae. In experiments 1 and 3, where the microfilarial count in the blood was very high, more normal developing and infective larvae were recovered from mosquitoes.

. These results indicate that C. p. molestus is not a suitable host of B. pahangi. They also suggest that there are some factors in the gut which prevent the normal migration of microfilariae to the thorax. Of those microfilariae that succeed in reaching the thorax, some develop normally and reach the infective stage, others, though normal, develop at a slower rate, while some continue their development abnormally.

This indicates that in the thorax itself, there is some factor(s) which affects the normal development of B. pahangi. This could be one of many unknown factors, such as lack of sufficient nutrients or the presence or absence of certain enzymes or it could be due to genetic factors whose mode of action is unknown. Increasing the numbers of microfilariae B. pahangi in the blood meal seems to increase the chances of mosquitoes becoming susceptible. Ae. aegypti SS stock used as controls were found to have susceptibility rates ranging from 80-100%, removing any doubts as to whether the microfilariae from infected jirds, used in these experiments, were infective or not (see Table 6). All the developing larvae were found in the head, thorax and abdomen of mosquitoes dissected on Day 10 post infection. In contrast, none of the infective L3 larvae found in C. p. molestus had reached the head. This indicates that the development rate of B. pahangi in C. p. molestus is slower than it should be in a suitable host, such as Ae. aegypti SS. Data in Table 6 also indicates that the numbers of microfilariae developing to the infective stage in Ae. aegypti SS decreases with an increase in the number of microfilariae in the infective bloodmeal. The reverse seems true for C. p. molestus. A greater proportion of microfilariae ingested by Ae. aegypti SS, fed on bloodmeals with lower microfilarial counts were recovered at the infective L_3 stage. These mosquitoes also had less L2 larvae. This suggests that the susceptibility of Ae. aegypti SS mosquitoes is enhanced by offering them blood meals with a moderate microfilarial density. A X^2 test was performed to find out whether there is any significant difference in the susceptibility of C. p. molestus and C. quinquefasciatus to B. pahangi. The X^2 value obtained was 1.56, indicating that there is no significant difference in the susceptibility of the 2 species to B. pahangi at the 5% level. The susceptibility rates

of C. p. molestus obtained in these experiments are comparable to those reported by Schacher and Khalil (1965), Ogunba (1969) and Obiamiwe (1970).

The variability of the susceptibility of this mosquito species to B. pahangi indicates that it might be possible to select for strains susceptible or refractory to B. pahangi (See Chapter 6).

Table 5: The Susceptibility of C. p. molestus to B. pahangi

Exp. nos. and date	mff/mm^3	Nos fed	Nos dissected	Nos with developing larvae (%)	Nos with mature larvae (%)	Head			Thorax			Abdomen			Total larvae per susceptible mosquito	Mature larvae per susceptible mosquito
						I	II	III	I	II	III	I	II	III		
1. 4.8.82	15	29	20	4 (20)	4 (100)	0	0	0	0	0	4	0	0	0	1	1
2. 24.8.82	5	9	6	1 (16.7)	0 (0)	0	0	0	0	1	0	0	0	0	1	0
3. 14.9.82	15	18	16	5 (31.3)	0 (0)	0	0	0	0	8	0	0	0	3	2.2	1.6
4. 3.11.82	6	8	6	3 (50)	0 (0)	0	0	0	0	3*	0	0	0	0	1	0
5. 19.11.82	5	67	53	0 (0)	0 (0)	0	0	0	0	0	0	0	0	0	0	0
6. 19.11.82	10	52	45	1 (3)	0 (0)	0	0	0	0	1*	0	0	0	0	1	0
7. 24.11.82	6	65	58	4 (6.8)	1 (25)	0	0	0	0	4*	1	0	0	0	1.25	0

Mosquitoes were dissected on day 10 post infection.

* Abnormal second larval stage.

Table 6: The Susceptibility of Ae. aegypti SS to B. pahangi

Expt nos. and date.	mff/ mm^3	Nos. fed	Nos. dissected	Nos with developing larvae (%)	Nos. with mature larvae (%)	Head			Thorax			Abdomen			A	B
						I	II	III	I	II	III	I	II	III		
1. 4.8.82	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2. 24.8.82	5	56	30	27 (90)	27 (90)	0	0	40	0	20	54	0	0	9	5	4
3. 14.9.82	15	43	30	29 (97)	29 (97)	0	0	86	0	45	83	0	0	27	8	7
4. 3.11.82	6	20	18	18 (100)	18 (100)	0	0	102	0	30	128	0	0	40	17	15
5. 18.11.82	5	24	22	22 (100)	22 (100)	0	0	160	0	10	76	0	0	18	12	12
6. 19.11.82	10	30	20	16 (80)	16 (80)	0	0	115	0	68	161	0	0	32	24	19
7. 24.11.82	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

A = Total larval per susceptible mosquito

B = Mature larvae per susceptible mosquito

Mosquitoes were dissected on day 10 post infection.

5. THE SELECTION OF REFRACTORY AND SUSCEPTIBLE STOCKS OF C.QUINQUEFASCIATUS (DAR) TO B. PAHANGI.

5.1 Introduction

Initial experiments to determine the susceptibility of C. quinquefasciatus (DAR) stocks to B. pahangi gave the susceptibility rates in the range of 2.4 - 18%. On the basis of these results, it was determined to attempt selection for refractory and susceptible stocks. Obiamiwe and Macdonald (1973) reported that susceptibility to infection with B. pahangi in C. 'pipiens' was controlled by a sex linked gene designated sb with a preliminary estimate of the crossover distance between sb and the sex locus in males of 12.8 ± 2. In females, two point linkage data gave an average distance between sb and re of 42. The method used by Obiamiwe (1970) to select a susceptible stock of C. 'pipiens' had some disadvantages in that it introduced refractory genes into the selected progeny. He crossed females of a moderately (33.3%) susceptible strain of C. p. molestus containing red-eyed mutants to males of a refractory black-eyed strain of C. quinquefasciatus from Bobo Dioulasso, which he had tested for susceptibility and found susceptibility rates ranging from 0-16%.

He justified involvement of C. quinquefasciatus (Bobo Dioulasso) in order to increase the feeding vigour and family size of each progeny. Other objectives attained by starting the selection from the female molestus X male quinquefasciatus hybrid, were to breed into the selected mosquito strains stenogamy, autogeny and mating compatibility in both directions. C.p. molestus can be mated with C. quinquefasciatus (Bobo Dioulasso) only when the females were molestus and the males were quinquefasciatus. Stenogamy was desirable in linkage experiments since several controlled matingg could be undertaken in small waxed cups. Since C. quinquefasciatus (Bobo Dioulasso) was refractory, it is

logical to assume it would slow down the selection of both susceptibility and red-eye colour in the C.p. molestus strain. The selection of a black-eyed C. 'pipiens' homozygous for sb was made from a hydrid of black-eyed phenotypically susceptible females of C.p. molestus (Lond) stock and males from the selected C. 'pipiens' strain homozygous for sb and re. Obiamiwe (1976) shows clearly by appropriate crosses of selected refractory and susceptible stocks that both re and sb are both recessive and sex-linked. It is difficult to see why in selecting for refractoriness, he crossed a selected susceptible stock with an already refractory stock; unless he wished the genetic background of the two stocks to be similar. Obiamiwe (1977a)found that the gene sb (filarial susceptibility, B. pahangi) also controls development of sub-periodic B. malayi, but has no influence on the development of periodic W. bancrofti.

5.2 Materials and Methods

Several attempts were made to infect DAR F_{59} generation with B. pahangi, but in all cases the females were found to be refractory. Eventually in one of the infective feeds, susceptible females were obtained and the eggs from these were used to establish a line for selection for susceptibility, while eggs from the refractory females were used to establish into a line for selection for refractoriness. Mosquitoes were infected by membrane feeding as described in Chapter 2.

In this case, human blood was used and the microfilariae were obtained from the peritoneal cavity of infected jirds. Fed female mosquitoes were transferred to a separate cage and provided with sugar and water. On the 5th day, the mosquitoes were transferred to individual tubes provided with a chicken feather for support in order to prevent drowning. The tubes were numbered and covered with netting held in place by elastic bands and then to each tube 2.5mls of hay infusion was added to induce egg laying. Additionally sugar solution was provided on paper wicks for a period of 4 days following the bloodmeals, then removed so that oviposition could take place. All the mosquitoes were dissected individually whether or not they had laid eggs and were also scored for susceptibility or refractoriness. Egg rafts from susceptible mosquitoes were pooled and reared in an enamel tray containing tap water and Hay infusion. Larvae were fed on yeast tablets. Pupae from refractory and susceptible trays were picked, put in separate plastic cups and placed in the appropriate cages for emergence. On the fifth to seventh day after emergence, the adults were offered another infective bloodmeal and the same procedure was followed again. During every infective feed, Ae. aegypti SS stock was used as a control.

5.3 Results and Discussion

Very low susceptibility rates were obtained in an attempt to increase susceptibility of DAR to B. pahangi, using the mass mating method (see Table 7). It appears that there was no response to selection for increased susceptibility. Results from attempts made to select a refractory stock (see Table 8) show that the F_1, F_3, F_4 and F_5 generations were completely refractory. Judging from the initial susceptibility rate obtained, 7.14%, which is low to begin with, the subsequent absence of developing larvae in the F_1, F_3, F_4 and F_5 does not appear to be due

to a response to selection, but is as likely to be due to the innate refractoriness of DAR as to a response to selection. The few mosquitoes found to be susceptible to B. pahangi (2), could be considered to be the few odd mosquitoes in this population of DAR in which the gene sb expresses itself more fully, or it could be that some of the filariae ingested were able to bypass the barriers they encountered in the gut.

Selection by the mass mating procedure was discontinued after the F_5 generation due to the lack of any response in either direction. Instead it was decided to attempt the selection of a susceptible line by sibmating. This method of selection has the advantage of yielding a susceptible or refractory line quicker, depending on which line is being selected.

Having fed and dissected 1,000 DAR females and finding them all refractory, it was impossible to start selection by the sibmating method, hence these investigations were terminated. Ae. aegypti SS used as controls were found to have susceptibility rates ranging from 85-100%, indicating that the blood meals offered to DAR were highly infective if ingested by a susceptible mosquito.

Due to the failure of selecting a susceptible and refractory line of DAR to B. pahangi, linkage experiments could not be conducted, which would have helped elucidate the true location of the gene sb and the sex locus on the chromosomes. It also would have provided crossover values which could be compared to those reported by Obiamiwe and Macdonald (1973) in the hybrid strain of C.'pipiens'. It would have been interesting to note whether the locations of the gene sb in a pure strain of C. quinquefasciatus (DAR) and hybrid C.'pipiens' were any different.

Differences in the ability of Culex mosquitoes to respond to selection for susceptibility and refractoriness to B. pahangi, as shown from Obiamiwe's (1976) data and from the above results, are comparable to the

differences reported by various workers in their attempts to select refractory and susceptible lines of C. quinquefasciatus to W. bancrofti.

Singh and Curtis (1974) failed in their attempt to select a strain of C. quinquefasciatus (Delhi) refractory to periodic W. bancrofti. They sibmated the progeny from refractory female mosquitoes hoping to increase the chance of any recessive genes for non-susceptibility being made homozygous. However, selection was discontinued after 5 generations due to the lack of response to selection and they concluded that the initial population did not contain any gene(s) for refractoriness to W. bancrofti. Similarly, Chakravertty et al. (1977) failed in their attempts to select a refractory strain of C. quinquefasciatus to periodic W. bancrofti after selection for 20 generations. In one experiment, they attempted to select a refractory strain from partially refractory mosquitoes and observed progressive increase of refractory and partially refractory mosquitoes but due to high mortality either in larval or in adult stages, the selected lines were lost. This is most likely due to inbreeding pressure. They suggested that the susceptibility of C. quinquefasciatus to periodic W. bancrofti was polyfactorial, thus making it difficult to select a refractory strain of this species.

However Zielke and Kuhlow (1977) using a colony of C. quinquefasciatus from Monrovia showing 21.6% susceptibility, managed to select a refractory strain but not a highly susceptible one. They also failed to select a refractory strain from a highly susceptible colony from Delhi. Hybrids of crosses between the refractory Monrovia line and the susceptible Delhi line had intermediate susceptibility, showing that in the Monrovia stock refractoriness did not dominate susceptibility whereas this was the case in the Delhi stock. These results led the authors to conclude that at least 2 different factors control the susceptibility of C. quinquefasciatus

to W. bancrofti. Subsequent experiments reported by Jayasekera et al.(1980) showed that a Liberian stock of C. quinquefasciatus did not respond to selection for refractoriness to a Sri Lankan strain of W. bancrofti, showing that the strains of W. bancrofti from Liberia and Sri Lanka differ in their ability to infect specific strains of C. quinquefasciatus.

All these results show that the factors determining the susceptibility of Culex mosquitoes to Brugia and Wuchereria filariae are complex, unlike in Ae. aegypti SS, where the gene f^m controls susceptibility to Brugia and Wuchereria filariae. It appears that more than one gene is involved in determining the susceptibility of C. quinquefasciatus to Wuchereria bancrofti and that these genes differ from those determining its susceptibility to Brugia pahangi, thus making selection for refractory and susceptible strains of either filaria in C. quinquefasciatus much more difficult. In addition, one has to take into account the variability in the infectivity of the various strains of Brugia and Wuchereria to various strains of C. quinquefasciatus.

Table 7: Results showing the selection for susceptibility in C. quinquefasciatus (DAR) using B. pahangi from infected jirds.

Generation	mff/$20mm^3$	Nos mosquitoes dissected	Nos mosquitoes 'susceptible'	Nos of larvae per infected mosquito	% susceptible	Ae. aegypti Control	
						Nos. mosquitoes dissected	% susceptible
P	173	28	2	2.0	7.14	19	89
F1	106	15	0	0	0	30	90
F2	90	38	0	0	0	30	100
F3	236	23	2	1	8.7	12	100
F4	175	56	0	0	0	13	92

' susceptible' mosquitoes are those with normal developing larvae.

Table 8: Results showing the Selection for refractoriness in Culex quinquefasciatus (DAR) using B. pahangi from infected jirds.

Generation	mff/ $20mm^3$	Nos mosq. dissected	Nos of mosq. 'susceptible'	Nos of larvae per infected mosq.	% susceptible	Nos of mosquitoes refractory	Ae. aegypti SS control Nos mosquitoes dissected	Ae. aegypti SS control % susceptible
P	300	28	2	2	7.14	26	19	89
F1	106	74	0	0	0	74	30	100
F2	90	69	2	2	3	67	30	100
F3	236	77	0	0	0	77	12	100
F4	175	123	0	0	0	123	13	92
F5	90	73	0	0	0	73	20	80

'susceptible' mosquitoes are those with normal developing larvae.

6. THE SELECTION OF BLOODFEEDING REFRACTORY AND SUSCEPTIBLE STOCKS OF C.P. MOLESTUS TO B. PAHANGI.

6.1 Introduction

C.p. molestus has been reported to be more susceptible to Brugia than C. quinquefasciatus by various workers. Schacher and Khalil (1965) obtained 14.1% and 19.1% susceptibility rates in Lebanese C.p. molestus infected with B. pahangi while Ogunba (1969) and Obiamiwe (1970) reported susceptibility rates of 36.6% and 33.3% respectively in a red-eyed London strain of the same mosquito. In previous experiments (see Chapter 4.3) a black-eyed London strain of C.p. molestus (=Dagmol) was found to have susceptibility rates ranging from 0-31.3%. Due to the variability observed in the susceptibility of C.p. molestus to B. pahangi, and the failure in the attempts to select a susceptible and refractory stock of C. quinquefasciatus to B. pahangi in previous experiments (see Chapter 5), it was decided to attempt selection of susceptible and refractory lines of C.p. molestus to B. pahangi. If this were possible, then the selected stocks could be used in studies of the genetic mechanisms underlying the inheritance of susceptibility to B. pahangi in Culex mosquitoes.

However, C.p. molestus is autogenous and does not feed readily on blood. Hence attempts were first made to select a stock with an increased blood feeding capability. This would enable a reasonable number of female C.p. molestus to ingest infective blood meals of B. pahangi offered to them via a membrane feeder, and also make it possible to start the selection of susceptible and refractory lines. The results of the above studies are reported in this chapter.

6.2 Materials and Methods

Initially, attempts were made to feed C.p. molestus on guinea pigs, mice, rabbit blood and chicks. All attempts were unsuccessful. Attempts

were then made to feed them on myself. In all cases, the crucial factor was to wait until the mosquitoes has laid their autogenous eggs, and then they were offered a bloodmeal. In order to facilitate the laying of autogenous eggs, the adults were deprived of sugar and only offered water. After several attempts, 20 C.p. molestus females fed on my arm. These were transferred to a separate cage for egg laying. Eggs obtained from these females constituted the F1 generation of bloodfed C.p. molestus. During the larval periods, the larvae were provided with a high protein diet of liver powder and yeast tablets in order to increase the fat body content, leading to production of larger autogenous egg rafts and increasing the chances of a greater number of females ingesting the blood meals offered them. The bloodfed Dag mol selected in the F_1, F_2 and F_3 generations were fed on myself and eggs laid by bloodfed females were used for the next generation. The parental generation for the selection of refractory and susceptible bloodfeeding Dag mol strains was the bloodfed Dag mol F_3 generation. The procedure for selection is similar to that described for C.quinquefasciatus DAR stock. Control experiments were also carried out at every infective feed using Ae. aegypti SS strain.

Despite offering the bloodmeal after the mosquitoes had laid their autogenous eggs and been starved for up to 48hrs, the female mosquitoes were still very reluctant to feed and this made selection very difficult. In order to increase the feeding ability of C.p. molestus it was decided to cross the females with males of C. quinquefasciatus (DAR). Such a cross would have some disadvantages as it would introduce refractory genes into female C.p. molestus. However, it would reduce or help overcome the problem of autogeny and increase bloodfeeding. With a blood feeding stock it would be easier to select a susceptible stock of C.p. molestus.

The following procedure was followed in setting up the cross.

Pupae of C.p. molestus were sexed by examining the hypopygium as indicated in (Chapter 3). Only the female pupae of C.p. molestus were required and the male pupae were collected in a plastic container and transferred to a stock cage. Female pupae were divided into groups of 3 or 4 and placed into 7.5 x 2.5cm tubes provided with 2 inches of Hay infusion and a feather to provide support for emerging adults. The tubes were covered with mosquito netting held in place by elastic bands. Similarly, the male pupae of C. quinquefasciatus (DAR) were collected in the above manner. After emergence, the sex of the adults was rechecked and in the case of intended females where a male had been accidentally placed, all the mosquitoes were discarded to guard against the possibility of insemination of any of the females by males of their own strain. The checked males and virgin females were released into separate cages. The males were aged for 2 days so as to allow them to reach sexual maturity and thus to have a chance of taking part in mating when the males and virgin females of C. quinquefasciatus and C.p. molestus were crossed. Egg rafts were collected in hay infusion and the rafts were held individually in tubes of water for 2 days before scoring for egg hatch. After inspection for hatched larvae, each raft was examined under a dissecting microscope and scored for presence or absence of embryonation. Larvae from hatched rafts were reared to the pupal stage and then the pupae were sexed.

Crosses between C.p. molestus (London) and C. quinquefasciatus (DAR) have been reported to be fully incompatible when the females are Tanzanian and the males London C.p. molestus whereas in the reciprocal cross although most rafts show incompatibility, about 5% of the rafts show moderate to high egg hatch (Curtis and Suya, 1981). For this reason the crosses were made between female C.p. molestus and male

C. quinquefasciatus DAR. Progeny from the above were designated DAG/DAR. They were offered infective blood meals via the membrane feeding method, but it was observed that the mosquitoes were still not attracted to blood. In order to try and increase the ability of DAG/DAR females to feed on blood, they were further backcrossed to male C. quinquefasciatus (DAR). The procedure for the cross was similar to that outlined above. Progeny from this cross were designated DAG/DAR x DAR. Infective blood meals were offered to these mosquitoes via the membrane feeding unit.

6.3 Results

a) Selection

The results of the infections of selected blood fed Dagmol are given in Table 9. The susceptibility rates of the P, F_1 and F_2 generations were 20%, 16.7% and 31.3% respectively. Very few females mosquitoes fed despite having selected this stock for increased blood feeding for 2 generations prior to the infective feeds, hence these rates should be considered cautiously. These rates are within the expected range of susceptibility of C.p. molestus to B. pahangi reported by previous workers and also reported in Chapter 4.3 of this thesis. They do not indicate any noticeable positive response to selection for increased susceptibility to B. pahangi. Ae. aegypti SS used as controls had high susceptibility rates of 90% and 97% respectively, showing that the microfilariae were highly infective.

Results of the selection for refractoriness in C.p. molestus to B. pahangi shown in Table 10 show a decrease in the percentage of susceptible mosquitoes in the F_2 and F_3 generations, followed by two generations of completely refractory mosquitoes (F_4 and F_5) suggesting a response to selection for refractoriness. However, X^2 tests showed no significant difference in the decrease of susceptibility between

F_2 and F_3 and F_3 and F_4 generations ($P > 0.05$). Ae. aegypti SS used as controls were highly susceptible (89-100%) to B. pahangi microfilariae in the infective blood meals.

Due to the great difficulty experienced in trying to persuade the female mosquitoes to feed on infective blood meals, selection of refractory and susceptible lines of C.p. molestus was discontinued and efforts were concentrated in trying to select for increased blood feeding.

b) Results of Crosses

Results of crosses made between C.p. molestus and C. quinquefasciatus (DAR) are shown in Table 11. In both crosses the number of rafts that hatched were very few, these being 7 out of 103 and 13 out of 81 respectively. The % hatch in the first cross was not calculated but from the total number of offspring obtained, it is evident that % hatch was not high. In the second cross there was an increase in the number of rafts from which hatching was observed, but the number of offspring were fewer than in the first cross. The % hatch was not very high, this being only 33.3%. Both sexes were produced from the egg rafts that hatched, showing that insemination had occurred and that some C. quinquefasciatus males were compatible to female C.p. molestus. There was a slight preponderance of females among the offspring.

In both crosses, a greater number of the egg rafts were incompatible, these being 90 and 68 respectively. The mean percentage of embryonated eggs in these rafts was 30.3% and 31.5% in the first and second cross respectively, indicating not much difference in the 2 crosses. In the second cross, none of the rafts were found to be totally embryonated, whereas in the first cross there were 6 totally unembryonated rafts. These could be the result of lack of insemination or an extreme expression of incompatibility. There were no significant differences in the two crosses as expected since compatibility is cytoplasmically

determined.

Attempts to feed the progeny of these 2 crosses on infective bloodmeals were unsuccessful. There was no increase in their attraction to blood and they remained autogenous. In addition there was a high mortality rate of hybrid females once they had laid autogenous eggs. These results led to the termination of this selection attempt.

6.4 Discussion

The failure in attempts to select susceptible and refractory stocks of C.p. molestus, suggests the absence of any genes controlling the susceptibility of this species of mosquito to B. pahangi or its presence at a very low frequency. On the other hand, it is possible that susceptibility is under the control of more than one gene, thus making it more difficult to select stocks of C.p. molestus with increased or decreased susceptibility to B. pahangi. The lack of response to selection observed in these experiments, is similar to that observed in C. quinquefasciatus (DAR) to B. pahangi reported in Chapter 5 of this thesis and is also comparable to that reported by various workers in their attempts to select susceptible and refractory stocks of C. quinquefasciatus to W. bancrofti (Singh and Curtis, 1974; Chakravertty et al., 1977). Obiamiwe (1970) was able to select stocks of C.'pipiens' susceptible and refractory to B. pahangi, from the hybrid of a cross between red-eyed females of C.p. molestus (London) showing 33.3% susceptibility to B. pahangi and black-eyed males of C.p. quinquefasciatus (Bobo-Dioulasso) refractory to B. pahangi. He appears to have had no problems with blood feeding in his hybrid C.'pipiens' considering that his stock of C.p. molestus was autogenous. The crosses reported above were very similar to those of Obiamiwe (1970), yet it is surprising how different the results are, considering that the strain of C.p. molestus

used in both cases were from London, his being red-eyed, whilst the one used in these studies was black eyed. Although he used a West African strain of C. quinquefasciatus in his selection procedure, whereas an East African stock was used in these studies, they were both refractory. It is difficult to explain the failure of reproducibility of his crosses and selections in these studies.

However the results of the crosses reported here are comparable to those reported by Curtis and Suya (1981) in which they crossed wild C. quinquefasciatus from Tanzania with C.p. molestus (Lon.) and a stock designated LONMON (with London cytoplasm and Monrovia genome) reciprocally. Crosses between Tanzanian females and male LON or LONMON were fully compatible but in the reciprocal cross although most of the egg rafts were incompatible, about 5% showed moderate to high egg hatch. Results from the crosses shown in Table 11 show that although most of rafts were incompatible, about 11% of the total rafts examined showed some egg hatch. This difference in % egg hatch could be due to the difference in the compatibility of wild and colonized C. quinquefasciatus males, to C.p. molestus females.

The hybrid progeny from the crosses remained autogenous suggesting that the genes controlling autogeny are dominant to those controlling anautogeny. Similar results have been observed by other workers. Spielman (1957) observed a high percentage of autogeny in the F_1, 73.9% in crosses between an autogenous and two different anautogenous strains of American Culex pipiens, while Krishnamurthy and Laven (1961) observed a higher ratio of autogeny (83.2%) in the F_1 after crossing an Indian anautogenous strain (Delhi) with an European autogenous strain.

Studies on the genetical inheritance of autogeny suggest that more than one gene is involved. Laven (1967) put forward a two-factor hypothesis

one of them being on the first or sex chromosomes and having a dominance-recessivity co-relation and the other in the third chromosome and being represented by a series of multiple alleles. The way in which these genes interact to control autogeny is not yet fully understood and for this reason it is difficult to explain the outcome of crosses performed in this study. Further investigations are required which could lead to a clearer understanding of the genetic control of autogeny in mosquitoes.

Table 9 : The Selection for Susceptibility of C.p. molestus to B. pahangi. Ae. Aegypti SS were used as controls.

Generation	Mff/$20mm^3$	Nos of mosquito dissected	Nos of mosquitoes with developing larvae (%)	Head			Thorax			Abdomen			Total larvae per 'susceptible' mosquito	Mature larvae per 'susceptible' mosquito
				I	II	III	I	II	III	I	II	III		
P	300	20	4 (20)	0	0	0	0	0	4	0	0	0	1	1
F_1	106	6	1 (16.7)	0	0	0	0	1	0	0	0	0	1	0
(AMSS)	106	30	27 (90)	0	0	40	0	20	54	0	0	9	5	4
F_2	300	16	5 (31.3)	0	0	0	0	8	0	0	0	3	2.2	1.6
(AMSS)	300	30	29 (97)	0	0	86	0	45	83	0	0	27	8	7

AMSS CONTROL WAS NOT FED IN THE PARENTAL GENERATION

Susceptible mosquitoes were those with normal developing larvae.

Table 10: The Selection for Refractoriness of C.p. molestus to B. pahangi. Ae. Aegypti SS were used as controls.

Generation	mff/$_{20mm}^{3}$	Nos mosquitoes dissected	Nos of mosquitoes refractory	% susceptible	Ae. aegypti SS (Control) Nos of mosquitoes dissected	Ae. aegypti SS (Control) % susceptible
P	300	20	16	20	-	-
F_1*	N O T	T E S T E D	-	-	-	-
F_2	65	6	5	17	17	100
F_3	211	36	34	5.5	19	100
F_4	197	29	29	0	19	89
F_5	123	27	27	0	18	100
F_6 *	N O T	T E S T E D	-	-	-	-

* Not tested since none fed.

Table 11: Results of the Crosses between C.p. molestus (London) and C. quinquefasciatus (DAR).

Parents of Cross		Total	Number of Rafts		Totally unembryonated rafts	Nos of Pupae			Mean % embryonated eggs in incompatible rafts
Female	Male		Hatched (% hatch)	Incompatible*		♂	:	♀	
Dagmol	DAR	103	7	90	6	167	:	195	30.3
DAG/DAR	DAR	81	13 (33.3)	68	0	40	:	61	31.15

* Incompatible Rafts Refers to Rafts in which there was no hatching although some eggs within each raft were Embryonated.

7. THE SUSCEPTIBILITY OF SOME MEMBERS OF THE CULEX PIPIENS COMPLEX, AE. AEGYPTI SS AND AN. STEPHENSI INOCULATED WITH EXSHEATHED AND SHEATHED B. PAHANGI.

7.1 Introduction

Nelson (1961) was the first to use a simple system for inoculating Setaria labiatopapillosa microfilariae into the thoracic muscles of Aedes aegypti. He experienced a high mortality and very low infectivity in the mosquitoes. Townson (1974, 1975) and McGreevy et al. (1978) took exsheathed B. pahangi microfiliariae from susceptible strains of Ae. aegypti and injected them into the thoracic muscle of recipient mosquitoes. Sucharit et al. (1982) used an even simpler technique for inoculating sheathed B.pahangi microfilariae into the thorax of Ae. togoi and An. balabacensis. Sheathed microfilariae were inoculated into the thorax of mosquitoes manually, by drawing out a glass capillary tube in a flame until the pointed end was 80 μm - 100μm in diameter; shorter needles were easier to handle. The injection was made into the post spiracular area of the mesothorax of starved mosquitoes and the microfilariae were gently blown down the attached rubber tube. The inoculum was controlled by observing the slightly extended abdomen of the mosquito. The mosquitoes recovered within a few minutes after dissection, then they were fed on a hamster and later held in aninsectary until ready for dissection.

The advantage of inoculating microfilariae directly in the thorax, is that the cibarial and pharyngeal armatures can be by-passed. These armatures, as mentioned before (Chapter 2.5.1), are responsible for killing a good proportion of the ingested microfilariae in certain genera of mosquitoes, before they get to the midgut.

Infection rates observed by using the inoculation technique can be compared to infectivity rates achieved by feeding the mosquitoes on infected hosts or by membrane feeding. This technique could also indicate whether there is another barrier within the thorax of the mosquito. Another advantage of this technique is that it can be used quantitatively, since a known number of microfilariae can be inoculated into the mosquitoes, and by dissecting the mosquitoes at the end of the incubation period, the efficiency of the mosquitoes in supporting the development of microfilariae can be assessed from the numbers of infective larvae recovered. When mosquitoes feed directly on infected hosts via membrane feeding, the numbers of microfilariae ingested are highly variable. This variability being due to the different abilities of various mosquitoes in concentrating mff during ingestion of a bloodmeal and the variation in numbers of microfilariae in equal volumes of blood. The aim of this experiment was to determine the susceptibility of above named species of mosquitoes to B. pahangi, by infecting them using the inoculation technique, and to compare this susceptibility with that achieved by the memberane feeding technique. The susceptibility rates obtained when these mosquitoes were inoculated with exsheathed and sheathed B. pahangi was also examined to see whether exsheathing microfilariae had any effect on the development of B. pahangi in the thorax.

7.2 Materials and Methods

The technique used to inoculate mosquitoes was that described by Townson (1974), itself a refinement of the method described by Nelson (1962). The refinements included an improvement of holding and restraining of the mosquitoes during the process of inoculation.

Mosquitoes to be inoculated were held by the thorax on a small perspex plate. A small hole in the plate connects to the tubing which is attached to a water operated pump. A non-essential but useful addition was the inclusion of a meter to aid control of the force of suction. The mounting of the restraining device in a conventional microscope mechanical stage facilitates the manupulation of the mosquito under a dissecting microscope (See Fig. 6 a & b).

Mosquitoes were inoculated in the membraneous area below the parategite with a fine needle of 60-80μm tip diameter. Fine capillary tubing was pulled to the appropriate diameter on an electrode puller. The needle was connected by tubing to a 1ml Luer fitting syringe. By pressure of the thumb and forefinger, the uptake and expulsion of small quantities of inoculum was controlled. Microfilariae to be inoculated into mosqutioes were suspended in Hayes Saline (Hayes, 1953) to which sodium penicillin G (50μg/ml) streptomycin sulphate (50μg/ml) and nystatin (100μg/ml) were added. Microfilariae B. pahangi used were either sheathed or exsheathed.

Sheathed microfilariae were obtained from peritoneal washings of infected jirds and suspended in Hanks balanced salt solution (H.B.S.S.).

Exsheathed microfilariae were obtained in 2 ways:-

1) by exsheathing them in vitro using the enzyme endopeptidase as by Devaney & Howells (1979). The enzyme was made by dissolving 2.0mg endopeptidase in 10ml of HBSS to give a final concentration of 0.2mg/ml. The enzyme was prepared immediately before use. The mff were then incubated in endopeptidase for 30 minutes. After 30 minutes, a small aliquiot of the larvae was removed from the petri dish and examined for exsheathment using a phase contrast microscope. Exsheathed microfilariae were transferred to a

Fig. 6(a): The inoculating equipment

Fig. 6(b) : A mosquito ready for inoculation.

centrifuge tube and spun down. The supernatant was withdrawn and the microfilariae washed with HBSS. This was centrifuged again, and the supernatant removed. The microfilariae were then resuspended in HBSS and were ready for inoculating into the mosquitoes.

2. The other method used to obtain exsheathed microfilariae B. pahangi was by using Ae. aegypti SS as donors. Ae. aegypti previously fed on blood infected with reasonable densities (150-200 mff/20µl) of microfilariae B. pahangi, were dissected in Hayes saline starting from 15 minutes after the feed. Exsheathed microfilariae found within the teased out thorax tissue were transferred by means of a finely drawn out capillary tube, to the well of a cavity slide containing Hayes Saline. The microfilariae were then ready for use.

Mosquitoes were slightly anaesthetized with CO_2 before being mounted on the perspex plate. Immediately after inoculation, each mosquito was transferred to a 7.5 x 2.5cm tube, provided with damp cotton at its base for a height of about 1-2 inches, and this was numbered carefully, then covered with a piece of mosquito netting held in place by elastic bands. Each tube was provided with a sugar source either in the form of a raisin or small rolled up cotton balls soaked in a 20% sucrose solution. Mosquitoes were dissected on day 10. Any mosquitoes that died between days 7-10 were also dissected and scored for susceptibility. Note was also made of the location and stage of development of any developing larvae found.

In cases where sheathed microfilariae or endopeptidase exsheathed microfilariae were used, each inoculum had approximately 20mff due to the greater numbers available. In cases where donor exsheathed microfilariae were used, 3-8mff were used as fewer numbers of exsheathed microfilariae could be recovered from the thorax. In the experiment

using exsheathed microfilariae, the microfilariae were used from 2-40 minutes after they had been recovered from the donor. They were then discarded and a new donor used. This is because they were observed to become less motile, beyond 40 minutes, hence it is most likely that they also become less viable. In the other cases where endopeptidase exsheathed microfilariae were used, they remained viable for up to 3 hrs post exsheathment, after which they were discarded.

7.3 Results and Discussion

Data in Tables 12,13,14,15,16,17, show that the survival rate of mosquitoes to the infective stage was very variable and the recovery of infective larvae low, even in susceptible stock of the Ae. aegypti SS. This low recovery of developing larvae, could be due to various reasons such as:-

a. the microfilariae failing to enter the thorax during inoculation probably being trapped within the needle.

b. damage to the microfilariae by the needle may affect their viability.

c. the size of the inoculum.

d. the viability of the microfilariae decreases the longer they remain outside the mosquito body after dissection.

e. the susceptibility of the mosquitoes used as recipients.

In Table 13 experiment 5, and Table 15 experiment 2, Ae. aegypti SS inoculated with unexsheathed B. pahangi, support the development of microfilariae to the infective stage, despite them being sheathed. Exsheathment of microfilariae within the gut prior to migration to the thorax has been thought to be an important event in the development of microfilariae to the infective stage. In addition, a number of mosquitoes in these experiments had larvae at various stages of development at day 10.

In previous experiments in which AMSS was used as a control in determining the susceptibility of different geographical strains of C. quinquefasciatus to B. pahangi, a majority of the developing larvae recovered on day 10 post infection were infective 3rd stage larvae.

Results in Table 12 show a slightly higher infection rate of 5-22% of C. quinquefasciatus DAR inoculated with exsheathed B. pahangi, compared to those infected using an artificial membrane, 2-18%. Many intermediate stages were found in the thorax and abdomen, with stage 3 larvae occuring in the thorax, abdomen and head, with only a few in the proboscis. However, C. quinquefasciatus infected via the artificial membrane feeder had less intermediate stages than the inoculated mosquitoes. The stage 3 larvae were found mainly in the head and proboscis with a few occurring in the thorax and abdomen. The slightly higher numbers of mosquitoes that became infected when inoculated with unexsheathed filariae, as opposed to those inoculated with exsheathed filariae, may be due to the greater numbers of unexsheathed filariae inoculated into the former mosquitoes. The presence of more intermediate stages in these mosquitoes, could be due to the initial presence of the sheath slowing down the rate of development. Development of unexsheathed B. pahangi to the infective third stage indicates that the microfilariae shed their sheaths in the thorax. In the experiment using exsheathed microfilariae, they were used from 2 to 40 minutes after they had been removed from the donor Ae. aegypti SS. They were then discarded and a new donor used. Microfilariae from the same donor, inoculated into the recipient after 2-40 minutes, seem to be equally viable. Hence the duration of microfilariae outside the donor cannot be directly related to the low infectivity rates, but may be attributed to the refractoriness of C. quinquefasciatus to B. pahangi.

Table 14 shows the development of exsheathed B. pahangi in the thoraces of C.p. molestus. The susceptibility rates of mosquitoes infected by this method ranged from 9.4 - 40%. These rates seem to be higher than those obtained when C.p. molestus was fed on infected blood. A X^2 test showed there is a significant difference at the 5% level ($P < 0.01$). However, the associated probability level should be interpreted with some caution in view of the variability between experiments. The numbers of filarial larvae developing were found to be less than the numbers of microfilariae inoculated into the mosquitoes. This indicates that a large portion of microfilariae are killed by some other factor(s) in the thorax although random loss into the abdomen during inoculation cannot be ruled out. An abnormal stage 2 larvae was observed in one mosquito thorax. C.p. molestus fed on the infected blood had more abnormal larvae in the thorax. The rate of growth of the inoculated microfilariae appears to be slower than usual for B. pahangi which are normally in the proboscis by day 10 in susceptible mosquitoes. The majority of the mature and developing larvae were found in the thorax. Only two were found in the head and abdomen. No filariae were found in the proboscis on day 10 in the surviving mosquitoes. Mortality rates in the 6 experiments were highest between days 3-7. In one experiment, there was 100% mortality after day 1. The only possible explanation for the mortality of a great number of inoculated mosquitoes is the traumatic effects of inoculation. The deaths appeared not to be due to fungal or bacterial infections, antibiotics having been added to the inoculation medium before hand.

A X^2 test (see Table 18) was carried out to determine whether the variability of the susceptibility rates of C.p. molestus to B. pahangi observed in five of the six inoculation experiments was significant or not. A X^2 value of 7.3 obtained was not found to be significant at the 5%

significance level.

Data in Table 13 show results of inoculating DAR, AMSS, Dagmol and An. stephensi with unexsheathed B. pahangi. These experiments were conducted for comparison purposes with those experiments in which exsheathed B. pahangi was used for inoculation. Ae. aegypti SS acted as controls. In experiment number 6, 36% of AMSS dissected were found to be susceptible. This lower than expected susceptibility rate could be due to any one, or a combination of the reasons mentioned earlier in this discussion.

However in Table 15, experiment 2, 88% of AMSS were susceptible. This latter result confirms that unexsheathed B. pahangi are capable of developing normally in a susceptible species of mosquito. All the developing larvae were normal and the survival rate was reasonable (64%).

Endopeptidase exsheathed B. pahangi inoculated into AMSS (see Table 16) were not as infective as unexsheathed B. pahangi. The mosquito mortality rate before day 7 post inoculation, ranged from 16.6-82% in the 4 separate experiments. The reason for low infectivity of exsheathed B. pahangi to Ae. aegypti SS is not clear. It could be that exsheathed B. pahangi were more vulnerable to injury during inoculation, hence damaged microfilariae were not viable. The injuries could have been inflictedby the drawn out micropipette or possibly damage due to endopeptidase treatment. Other reasons for decreased susceptibility are similar to those mentioned earlier.

An. stephensi inoculated with unexsheathed B. pahangi (see Table 13 experiment 4) had a susceptibility rate of 23%. Majority of the third stage larvae were located in the thorax, a few occurring in the head and one third stage larva in the proboscis. A greater number of L2 were found in the thorax and a few first stage larvae in the thorax. This

susceptibility rate is significantly higher than that obtained when An. stephensi was fed on B. pahangi. In the latter case, the susceptibility rate obtained was 5%. This lower susceptibility rate could be due to the damage caused to microfilariae by the bucco-pharyngeal armature of An. stephensi which is well developed. The fact that not all the microfilariae inoculated into the mosquito thorax developed, indicates that there are other factors in the thorax, which inhibit development of B. pahangi.

C.p. molestus inoculated with unexsheathed B. pahangi were found to have a susceptibility rate of 24% which is less than that of those inoculated with exsheathed B. pahangi (6.3 -40%). However a X^2 test showed there was no significant difference between the two at the 5 per cent level, $X_1^2 = 0.26$. Development of unexsheathed B. pahangi in C.p. molestus seems to be slower than expected. On dissection at day 10, only a few third stage larvae were found in the thorax and abdomen, none occurring in the proboscis. A greater number of stage 2 larvae were found in the thorax, a few in the abdomen and head. Probably the initial presence of a sheath slows down development.

C. quinquefasciatus DAR inoculated with exsheathed and unexsheathed B. pahangi were found to have susceptibility rates of 10-39%. These susceptibility rates are higher than those obtained when C. quinquefasciatus were fed on an infective blood meal via a membrane feeder. A X^2 test gave a X^2 value of 15.84, which is highly significant, $P < 0.001$. This suggests that by bypassing the gut and inoculating B. pahangi directly into the thorax, increases the chances of microfilariae developing to the infective stage. This further shows that some of the major factors responsible for hindering development of B. pahangi are located in the gut of mosquitoes. Since not all inoculated microfilariae

develop in the thorax, this would seem to indicate that the factors in the thorax are lethal to microfilariae B. pahangi. Ae. aegypti SS inoculated with exsheathed B. pahangi from donor Ae. aegypti SS, also had variable susceptibility rates ranging from 3.6-31% (Table 17), which is low compared to susceptibilities of Ae. aegypti SS membrane fed or inoculated with unexsheathed B. pahangi these being 100% and 88% respectively. Mortality rate was also variable and high. The only possible explanation for the low susceptibility rates is injury to exsheathed B. pahangi, during inoculation, high mortality being due to traumatic effects. The high mortality rate of inoculated mosquitoes observed, has been reported by various workers, using the same technique for infecting Ae. aegypti SS.

Bianco et al. (1980) reported that only 11% of Ae. aegypti SS inoculated with O. gutturosa survived the 12 day development period. In a second trial in which the numbers of microfilariae inoculated into the mosquitoes were decreased to 20, the survival rate was better, presumably due to the smaller number of microfilariae given and the smaller volume of inoculum. Lok et al. (1980) also reported high mortality of Ae. aegypti (SS, REFM, Liverpool) stocks inoculated with microfilariae belonging to the bovine Onchocerca species. In other experiments by Devaney and Howells (1979) the viability of endopeptidase and Ca^{2+} exsheathed B. pahangi was tested, by inoculating Ae. aegypti (SS + REFM) strains. Of the mosquitoes surviving until day +8 post inoculation, 58% of the mosquitoes which received endopeptidase exsheathed larvae and 70% of those which received Ca^{+} exsheathed larvae had infective larvae in the head and proboscis. High mortality rates observed in the above experiments could not be due to bacterial or fungal infections since antibiotics were added to the inoculum prior

to infection. The most likely reasons for low susceptibility in Ae. aegypti SS plus high mortality, are experimental technique and traumatic effects.

The experiments in Tables 12,13 and 14 showing the susceptibility of various species of mosquito to exsheathed and unexsheathed B. pahangi by inoculation into the thorax, were performed without the inclusion of a control experiment using Ae. aegypti SS. Hence in these experiments it was not possible to compare the susceptibilities of these refractory species to that of susceptible Ae. aegypti. However the results of experiments shown in Table 15, in which a proper control was used, show clearly that unexsheathed B. pahangi inoculated into susceptible Ae. aegypti are capable of developing normally to the infective stage without prior exsheathment before inoculation. 88% of the total number of mosquitoes dissected were susceptible. Furthermore these results show that unexsheathed B. pahangi also develop in C. quinquefasciatus (DAR), which is highly refractory to B. pahangi and the susceptibility rate of 39% obtained was much higher than that obtained by feeding DAR on B. pahangi. Hence the susceptibility of DAR to B. pahangi can be increased by direct inoculation into the thorax.

Table 12: Inoculation of C.p. quinquefasciatus (DAR) with exsheathed B. pahangi.

Date	Number of experiment	Nos. inoculated	Nos. dissected	% mortality before day 7	% survival beyond day 7	Nos mff inoculated	Nos with larvae (%)	Nos with mature larvae (%)	Thorax I	Thorax II	Thorax III	Abdomen I	Abdomen II	Abdomen III	Head I	Head II	Head III	Proboscis I	Proboscis II	Proboscis III	Total larvae per susceptible mosquito	Mature larvae per susceptible mosquito
20.10.81	1	35	35	94.3	5.71	20	5 (14.3)	0 (0)	9	1	0	0	0	0	0	0	0	0	0	0	2	0
4. 3.82	2	34	20	41	59	6	2 (10)	1 (4)	0	2	0	0	0	0	0	0	0	0	0	2	2	1
8. 3.82	3	27	18	11	89	6	4 (22)	2 (11)	0	2	2	0	0	1	0	0	1	0	0	0	1.5	2
9. 3.82	4	36	28	39	61	6	5 (18)	3 (11)	0	2	2	0	0	1	0	0	0	0	0	0	1	1
11. 3.82	5	33	22	33	67	6	5 (22)	5 (22)	0	0	1	0	0	1	0	0	0	0	0	3	1	1

* Donors of exsheathed microfilariae were selected susceptible stock of Ae. aegypti. In experiment 1 microfilariae were exsheathed using the enzyme endopeptidase.

Inoculum size was in the range of 6-10 mff per mosquito.

Table 13: Inoculation of C.p. quinquefasciatus (DAR) and (THAI) C.p. molestus, An. stephensi, Ae. aegypti SS with unexsheathed B. pahangi dissected day 10 post infection.

Expt. date and strain of mosquito	Expt. No.	Nos inoculated	Nos dissected	% mortality before day 7	% survival beyond day 7	Nos with larvae (%)	Nos with mature larvae (%)	Proboscis			Head			Thorax			Abdomen			Total larvae per mosquito	Mature larvae per susceptible mosquito	% susceptible
								I	II	III	I	II	III	I	II	III	I	II	III			
8.10.81 (DAR)	1	26	26	42.3	57.7	8(30.8)	4(15.4)	0	0	0	0	0	3	5	8	0	0	0	1	2.1	1.0	31
9.10.81 (THAI)	2	28	28	43	57	8(28.5)	6(21.4)	0	0	0	0	0	6	0	0	7	5	9	4	3.9	2.1	29
1.2.83 (DAR)	3	50	50	28	72	12(24)	6(12)	0	0	0	0	0	2	0	5	6	0	0	2	1.3	1.3	24
1.2.83 (DAGMOL)	3	76	76	80	20	18(24)	4(5)	0	0	0	0	0	1	3	13	4	0	0	2	1.3	1.5	24
8.2.83 (An.stephensi)	4	74	74	52	47	17(23)	8(11)	0	0	1	0	0	2	8	42	28	1	0	0	4.8	3.5	23
9.2.83 (AMSS)	5	70	25	7	24	9(36)	3(12)	0	0	0	0	0	0	4	24	5	0	0	0	3.6	1.6	36

Inoculum size approximately 20mff.

Table 14: Inoculation of C.p. molestus (DAGMOL) F12 with exsheathed B. pahangi (Ae. aegypti SS were used as donors).

Expt No. and date of experiment.	Nos inoculated	Nos dissected	% mortality before day 7	% survival beyond day 7	Nos with larvae (%)	Nos with mature larvae (%)	Proboscis			Head			Thorax			Abdomen			Total larvae per susceptible mosquito	Mature larvae per susceptible mosquito	% susceptible
							I	II	III	I	II	III	I	II	III	I	II	III			
18.1.83 (1)	14	11	64	36	3(27.3)	0	0	0	0	0	0	0	0	10	0	0	0	0	3.3	0	27.3
19.1.83 (2)	13	11	54	46	1(9.1)	1(9.1)	0	0	0	0	0	0	0	0	3	0	0	0	3.0	3	9.1
20.1.83 (3)	24	20	33	67	8(40)	7(35)	0	0	0	0	0	2	0	*1	5	0	0	1	1.1	1.1	40
25.1.83 (4)	25	25	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26.1.83 (5)	26	24	46	54	7(29.2)	5(21)	0	0	0	0	0	1	0	4	5	0	0	1	1.6	1	29.2
27.1.83 (6)	16	16	94	6	1(6.3)	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	6.3

* Abnormal larvae

Inoculum size approximately 6-10mff.

Table 15: Inoculation of C.p. quinquefasciatus (DAR) and Ae. aegypti SS with unexsheathed B. pahangi dissected day 8 post infection.

Strain of mosquito and date of experiment	Nos inoculated	Nos dissected	% mortality before day 8	% survival beyond day 8	Nos with larvae (%)	Proboscis			Head			Thorax			Abdomen			Total larvae per infected mosquito	% susceptible
						I	II	III	I	II	III	I	II	III	I	II	III		
DAR 22.3.83	33	28	29	71	11 (39)	0	0	0	0	0	0	0	19	0	0	1	0	1.8	39
AMSS 22.8.83	33	25	36	64	22 (88)	0	0	0	0	0	0	1	41	2	0	0	3	2.1	88

Table 16: Inoculation of Ae. aegypti with Exsheathed B. pahangi (exsheathed using endopeptidase)

No. expt	Nos. inoculated	% with L_1 & L_2 larvae	% with infective larvae	% mortality before day 7	% survival beyond day 7
1	30	10	36.7	16.6	83.4
2	28	14.3	7.14	82	8
3	36	-	3.6	30	70
4	60	-	1.6	57	43

Table 17: Inoculation of Ae. aegypti with exsheathed B. pahangi (using donors).

No. expt	Nos. inoculated	% with L_1 & L_2 larvae	% with infective larvae	% mortality before day 7	% survival beyond day 7
1	20	15	5	85	15
2	34	-	20.6	50	50
3	35	-	12.0	51.43	48.6
4	28	-	3.6	78.6	21.4
5	32	-	6.25	50	50
6	28	-	10.7	57.14	42.9
7	49	-	31	59	41
8	40	-	15	62.5	37.5
9	34	-	8.82	47	53

Table 18: Showing Data of Dagmol inoculated with exsheathed B. pahangi.

Expt. No.	Nos of mosquito dissected	Nos with larvae O	Nos with larvae (E)	Nos without larvae O	Nos without larvae (E)
1	11	3	(2.6)	8	(8.3)
2	11	1	(2.6)	10	(8.3)
3	20	8	(4.8)	12	(15)
4	24	7	(5.8)	17	(18.1)
5	16	1	(3.9)	15	(12.1)
Total	82	20		62	

$X^2_4 = 7.3$

Therefore $P > 0.05$

O = observed

E = expected

8. THE EFFECT OF DIFFERENT CONCENTRATIONS OF HEPARIN ON THE EARLY MIGRATION OF MICROFILARIAE B. PAHANGI FROM C. QUINQUEFASCIATUS BLOODMEALS.

8.1 Introduction

The low susceptibility rates of C. quinquefasciatus to B. pahangi reported in section 4.2, could be due to the lack of migration of microfilariae from the midgut to the thorax, as a result of the rapid clotting of the bloodmeal, in which the microfilariae become trapped. Thus it was decided to examine the effect of various concentrations of the anti-coagulant heparin, on the early migration of B. pahangi from the midgut to the thorax of C. quinquefasciatus. The addition of anti-coagulant to the infected blood during membrane feeding allows some control of the variable clotting time of the blood meals. These investigations are based on the work of Obiamiwe (1970), who examined the effects of different concentrations of heparin on the early migration of B. pahangi in selected susceptible and refractory strains of C.'pipiens'.

8.2 Materials and Methods

One week old unfed C. quinquefasciatus (DAR), THAI, and MON stocks were used. All mosquitoes were membrane fed and the density of microfilariae in the blood was in the range of 3-14 per mm^3. Six concentrations of heparin were prepared, 10, 300, 1,000, 3,000 and 5,000 iu (international units) per ml blood. In the preparation of 300 iu concentration, 290 iu heparin were added by pipette to a watch glass. This was placed in an oven at $60^{\circ}C$ and the contents evaporated to dryness. One ml of rabbit blood, containing the microfilariae and 10 iu heparin per ml was added to the watch glass. The final concentration after agitation was 300 iu per ml. Other concentrations were prepared likewise, following the method described by Obiamiwe (1970). Heparin is not denatured by heating

at 60°C. As a control, mosquitoes were fed directly on a cat infected with B. pahangi, since the bloodmeal would not have any anticoagulant in it. The microfilarial count in the cat was 14/mm^3.

Mosquitoes which fed on the various bloodmeals were maintained for 24 hrs in the insectaries at temperatures of 27-28°C and relative humidity of 70-80%. Mosquitoes were then dissected in Hayes saline. The thorax and abdomen was teased finely in a drop of saline, then covered with plastic coverslips, marked with 64 squares to aid the examination of the preparations using a binocular microscope at X100 magnification.

8.3 Results

The data in Table 19 show that most of the microfilariae remained within the gut contents, very few microfilariae migrating to the thorax (0.3 - 9.7%). In experiment 1, where mosquitoes were fed directly on a cat, very few females of DAR and MON fed, while none of the THAI females fed. None of the microfilariae ingested by these mosquitoes migrated to the thorax. In experiment 2, where the bloodmeal had 10 iu heparin, some of the ingested microfilariae succeeded in migrating to the thorax. In DAR and MON 9.7% and 7.7% of the ingested microfilariae were found in the thorax 24 hrs after the feed. However, in experiments 3 to 6 where there was a great increase in the amount of heparin in the bloodmeal, there was a decrease in the number of microfilariae migrating to the thorax. During membrane feeding, it was also observed that as the heparin concentration in the bloodmeal increased, the mosquitoes became very reluctant to feed and the blood meals ingested became smaller.

The numbers of microfilariae found in the midgut contents of any batch of mosquitoes increased as the density of microfilariae in the blood used for feeding increased in experiments 2 to 4, where the

heparin concentration did not exceed 1,000 iu /ml. However in experiments 5 and 6, at heparin concentrations of 3,000 and 5,000 iu /ml, the numbers of microfilariae found in the bloodmeal were less than expected, considering the microfilarial count in the feeder. Despite the small increase in the numbers of microfilariae migrating to the thorax at heparin concentrations of 10 iu/ml of blood, the overall results show that increasing the heparin concentration in infective bloodmeals produced no significant increase in the migration of B. pahangi to the thorax of various strains of C. quinquefasciatus. In fact, it had the reverse effect.

8.4 Discussion

The lack of migration of microfilariae B. pahangi in females of DAR and MON fed directly on an infected cat, is most likely due to the microfilariae being trapped in the bloodmeal which clotted rapidly, in the absence of heparin. The increase in the migration of microfilariae from the bloodmeals with 10 iu /ml heparin, to the thorax could be a result of the presence of heparin in the bloodmeal, which delayed the rapid clotting of the bloodmeal, thus allowing some microfilariae to escape from the midgut. The decrease in the number of microfilariae migrating to the thorax, as the concentration of heparin increased in the bloodmeal, as observed in experiments 3 to 6 suggests that very high concentrations of heparin somehow prevent migration of microfilariae from the midgut to the thorax. It is possible that the level of migration of microfilariae B. pahangi was increased only up to a certain concentration of heparin, this being 10 iu /ml heparin, beyond which there was no further increase in migration. Rather, the higher concentrations of heparin had adverse effects on the microfilariae, reducing their motility and preventing further migration.

The results obtained in these experiments are similar to those of Obiamiwe (1970). He found that in donor fed (no anticoagulant) refractory C.'pipiens', B. pahangi failed to migrate from the bloodmeals to the thoracic muscles of the mosquitoes. At a concentration of 10 iu/ml heparin in the infecting blood, there was an increase in the microfilarial migration in homozygous refractory C.'pipiens' to the level (25%) observed in susceptible C!pipiens'. There was no change in the microfilarial migration in donor fed (no anticoagulant) and membrane fed (anticoagulant) C. 'pipiens' of the homozygous susceptible strain. High heparin concentrations (300 or more iu per ml blood) decreased the microfilarial migration in C. 'pipiens'. Similarly, Wade (1974) reported that in Ae. aegypti fed on infective bloodmeals of B. pahangi with the heparin concentration of the blood above the base level did not produce an increase in the extent of microfilarial migration.

The manner in which high concentrations of heparin inhibit migration of microfilariae is not clearly known. Obiamiwe (1970) suggested that this might be due to an increase in osmotic pressure or viscosity of the gut contents caused by high heparin concentrations. Such a change might reduce the motility of microfilariae and their ability to penetrate the stomach wall. On the other hand, it could be that high concentrations of heparin prevent microfilariae from migrating, by adversely affecting their metabolism, hence decreasing their motility and viability. The above author also observed that mosquitoes were reluctant to feed on infective blood with heparin concentrations higher than 10 iu /ml, and the higher the concentration, the more reluctant they were. The size of the bloodmeals also decreased when mosquitoes ingested bloodmeals with high concentrations of heparin.

Kartman (1953) found that the presence of anticoagulant in the bloodmeal of Ae. aegypti, allowed a greater percentage migration of microfilariae of D. immitis from the midgut to the Malphigian tubules than was observed in donor fed mosquitoes. Thus the absence or presence of anticoagulant was the deciding factor.

The lack of migration of microfilariae from the midgut of refractory mosquitoes has been reported by various other workers. Ewert(1965a) found that migration of microfilariae B. pahangi to the thorax 1 hr after feeding was less than 1 per cent in C. quinquefasciatus - a poor vector of B. pahangi. There was no substantial increase in the percentage migration in 24hrs. Similarly, Laurence and Pester(1961b) reported lack of migration of B. patei in An. gambiae and Ramachandran et al. (1961) found that only a few microfilariae of B. malayi migrated to the thorax of Ae. aegypti and developed to the infective stage. Ewert(1965a) also observed that although Ae. aegypti, Ae. albopictus and Ae. sollicitans are all poor vectors of B. malayi, a large percentage of the microfilariae migrated to the thorax but only a small number of them completed their development to the infective stage. This suggests that not in all cases is the extent of migration to the thorax a measure of the vectorial efficiency. Owen (1977) observed that in refractory Ae. malayensis, migration of B. pahangi to the thorax lasted only about 1½ hours after the infective bloodmeal, in which 50% of microfilariae reached the thorax, whereas in susceptible Ae. tabu, migration of B. pahangi continued for approximately 3 hrs after the bloodmeal, by which time 92% of the ingested microfilariae could be recovered in the thorax.

The results of experiments reported in this chapter, indicate that lack of migration of microfilariae B. pahangi from the bloodmeal to thorax is not only due to clotting of the bloodmeal, as suggested, but that there

are other factors in the midgut which inhibit migration. However the migration of microfilariae Wuchereria bancrofti appear to be unaffected by these factors and they are highly infective to C. quinquefasciatus. An important factor which could be affecting migration of microfilariae B. pahangi in these experiments, is the lack of exsheathment of these microfilariae. Exsheathment of microfilariae is a prerequisite to the successful migration of B. pahangi in efficient mosquito vectors. Ewert (1965b)suggested that the exsheathment rate of microfilariae in the midgut of mosquito hosts could be used as a criterion for determining the vectorial capacity of a mosquito host. Thus the exsheathment and subsequent migration of B. pahangi from the midgut to thorax of various mosquito species will be examined in subsequent investigations.

Table 19: Showing the Effect of varying Heparin concentrations on the early migration of *B. pahangi* microfilariae from the bloodmeal. Mosquitoes were dissected 24hrs post feeding.

Expt. Nos.	Strain of *C.quinque-fasciatus*	mff/ mm^3	I.U. heparin per ml of blood	Nos mosquitoes fed.	Total no of Filarial larvae in mosquitoes	
					midgut(%)	thorax(%)
	DAR	14	0*	6	290 (100)	0 (0)
1	THAI	14	0	0	0 (0)	0 (0)
	MON	14	0	6	311 (100)	0 (0)
	DAR	6	10	10	120 (90.3)	13 (9.7)
2	THAI	6	10	10	56 (100)	0 (0)
	MON	6	10	10	95 (92.3)	8 (7.7)
	DAR	9	300	8	271 (100)	0 (0)
3	THAI	9	300	10	233 (98.7)	3 (1.3)
	MON	9	300	10	209 (99.5)	1 (0.5)
	DAR	15	1000	10	428 (99.7)	1 (0.3)
4	THAI	15	1000	9	259 (100)	0 (0)
	MON	15	1000	10	411 (100)	0 (0)
	DAR	5	3000	9	11 (100)	0 (0)
5	THAI	5	3000	8	23 (100)	0 (0)
	MON	5	3000	1	4 (100)	0 (0)
	DAR	3	5000	1	0 (100)	0 (0)
6	THAI	3	5000	9	18 (100)	0 (0)
	MON	3	5000	9	2 (100)	0 (0)

* direct feed on infected cat.

9. THE EXSHEATHMENT AND MIGRATION OF B. PAHANGI IN VARIOUS MOSQUITO SPECIES.

9.1 The Exsheathment and Migration of B. pahangi in C. quinquefasciatus (DAR) C.p. molestus, Ae. aegypti SS and An. stephensi.

9.1.1 Introduction

The exsheathment of microfilariae in the midgut and their subsequent migration to the thorax of a mosquito host are two important phenomena which have some value as early indicators of vector ability. Results on the early migration of B. pahangi in three strains of C. quinquefasciatus reported in Chapter 8, show that the migration rate was very low despite increasing the heparin concentration. Thus, clotting of blood was not the only factor inhibiting migration and it is possible that the microfilariae were not exsheathing. For this reason, it was thought necessary to examine the microfilariae for exsheathment and subsequent migration to the thorax, in various mosquito species known to be refractory and susceptible to B. pahangi, and to compare the results.

9.1.2 Materials and Methods

One week old, previously unfed C. quinquefasciatus (DAR), Ae. aegypti SS (AMSS), C.p. molestus (Dagmol) and An. stephensi (Steph) were starved for 48 hrs then offered blood taken from a cat infected with B. pahangi, via the membrane feeder. The microfilariae count of the bloodmeal was 7mff/mm^3. Mosquitoes which fed were then dissected at intervals of 30 minutes post feeding up to 4 hrs. Each mosquito was divided into thorax, midgut and abdomen (exclusive of midgut). Each portion was teased apart in a drop of distilled water on a separate slide and then smear preparations made. These were allowed to dry for at least 24hrs and then fixed in absolute methyl alcohol, air dried and stained with 5% Giemsa stain. They were then examined for

exsheathment and migration using a binocular microscope at X40 magnification.

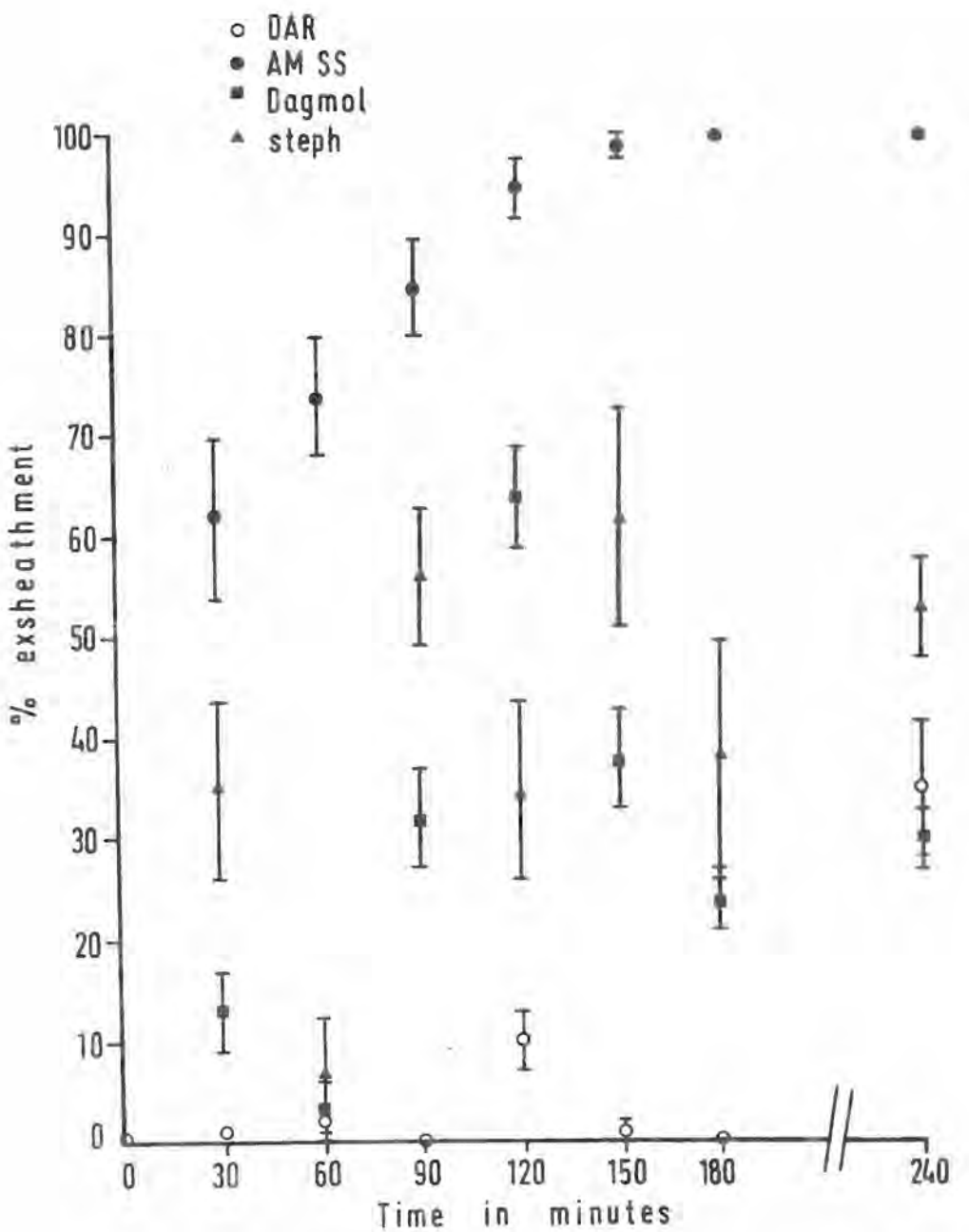
9.1.3 Results

Data in Table 20 and Fig. 7 show the % exsheathment and migration of B. pahangi in DAR, Dagmol, AMSS and Steph. In AMSS, which is highly susceptible to B. pahangi by 30 minutes 62% of the ingested microfilariae had exsheathed but only 5% had migrated to the thorax. There was a rapid increase in the % exsheathment in this mosquito species and by 180 minutes post infection, all the microfilariae examined had exsheathed. However, the migration rate was not proportional to the exsheathment rate. At 4 hours, only 49% of the exsheathed microfilariae had migrated to the thorax. Considering the microfilariae count of the infective blood meal was 7mff/mm^3, the number of microfilariae ingested by these mosquitoes is less than expected, if the average bloodmeal was approximately 3µl.

Exsheathment in DAR was very low during the first 3 hours ranging from (0.8-10%). At 240 minutes postfeed, 35% of the microfilariae examined had exsheathed. However, despite observing that some exsheathment had occurred, there was no corresponding increase in the numbers of microfilariae found in the thorax. Microfilarial uptake was variable, since some batches of mosquitoes were found to have more microfilariae then expected in their bloodmeal and others less than expected. This variability is due to the variation in the size of bloodmeal ingested and unequal distribution of microfilariae in the blood.

In Dagmol, although a reasonable number of microfilariae exsheathed (13-64%) very few microfilariae were found in the thorax. Percentage migration ranged from 0.3-2%. Again, the lack of migration despite exsheathment of some microfilariae, as observed in DAR, suggests that in addition to exsheathment, some other factors in the midgut play a role in determining the migration of microfilariae to the thorax. Microfilarial

Fig. 7: The Exsheathment of B. pahangi in C. quinquefasciatus (DAR), C.p. molestus (Dagmol), Ae. aegypti SS (AMSS) and An. stephensi (Steph).



uptake was also very variable in this species of mosquitoes, some ingesting more, others less than expected.

Percentage exsheathment in An. stephensi ranged from 7%-62% but % migration was very low (3.4-11%) showing that few exsheathed microfilariae migrated. 9.6% of the microfilariae were damaged.

In the case of susceptible AMSS, percentage exsheathment increased with time (see Fig. 7) but in Dagmol and Steph, percentage exsheathment was very variable and the time trend was less well marked.

9.1.4 Discussion

In Ae. aegypti SS, the rate of exsheathment was high and migration to the thorax took place. This facilitates its role as a host of B. pahangi. In C. quinquefasciatus (DAR), the very low exsheathment and migration rates of microfilariae contribute to it being a poor host of B. pahangi. Although in C.p. molestus and An. stephensi some microfilariae exsheathed, the rate of migration to the thorax was very low. Hence, these mosquitoes are also poor hosts of B. pahangi. The damaged microfilariae found in An. stephensi were probably the results of injuries inflicted by the well formed cibarial armature found in Anopheline mosquitoes.

These results are comparable to those of Ewert(1965b) in which he observed that C. quinquefasciatus, a poor host of B. pahangi, exsheathment was less than 3%, while in An. quadrimaculatus, a good host, there was about 100% exsheathment. Owen (1978) studied the exsheathment and migration of B. pahangi microfilariae in susceptible and refractory species of the Ae. scutellaris complex. The % migration was 95% in the susceptible species and 50% in the refracary species. The percentage of microfilariae that did not exsheath was 3% in the susceptible species and 17% in the refractory species. The author

concluded that the failure to exsheath was one of the factors limiting migration since a large percentage of sheathed microfilariae also failed to migrate out of the midgut in the refractory species. The results of the above experiments show that exsheathment does not necessarily lead to migration and that other factors in the midgut play a role in the migration of exsheathed microfilariae.

However, sheathed microfilariae have been reported to migrate. Laurence and Pester (1961a) found sheathed microfilariae of B. patei in the fat body and abdomen of M. uniformis. Schacher (1962) also reported the same occurence of sheathed microfilariae of B. pahangi in An. quadrimaculatus but the subsequent development of microfilariae was abnormal.

Devaney and Howells (1979) were the first to provide evidence that Ca^{2+} ions or hydrolytic enzymes brought about exsheathment of microfilariae in vitro. However, it is unlikely that the conditions created in vitro to bring about exsheathment are similar to those existing in the midgut in vivo. The high concentrations of Ca^{+} used in these in vitro experiments would appear to be considerably greater than the normal physiological range in the mosquito gut. Although the Ca^{2+} ion concentration in the midgut of mosquitoes has not been assayed, that of insect haemolymph is in the range of 3-21 mM/l (Prosser and Brown, 1962) and is considerably higher than that in the midgut. It seems unlikely that proteolytic enzymes play an important direct role since it has been shown that after a blood meal, the level of proteolytic enzymes is depressed to be followed by a gradual build up of proteases which reaches its maximum level 18 hours following a bloodmeal (Clements, 1963). Hence it seems unlikely that these enzymes play a direct role in the exsheathment process, since exsheathment reaches a high level in the susceptible Ae. aegypti SS before protease secretion is maximal.

Yamamoto et al. (1983) reported that sheathed B. pahangi inoculated into the abdominal haemocoel of Armigeres subalbatus exsheathed and continued development to the infective larval stage normally, suggesting that the factors which bring about exsheathment are not confined to the midgut. Results of experiments reported in Chapter 7, in which Ae. aegypti SS was inoculated with sheathed B. pahangi, showed that these microfilariae somehow manage to loose their sheaths within the thorax and proceed to develop normally. The manner in which they exsheath in the thorax is not known, although it is possible that they attach themselves to the muscle fibres and then wriggle free from the sheath.

Further studies are required for the complete understanding of the factors which control exsheathment, migration and development of microfilariae in mosquitoes leading to the determination of vectorial capacity of the mosquito host. It would also be useful to know whether they are confined to a specific organ or whether they occur in the whole mosquito.

Table 20: Showing % exsheathment and migration of *B. pahangi* in *C. quinquefasciatus* (DAR), *C.p. molestus* (DAGMOL) *Ae. aegypti* (AMSS) and *An. stephensi* (STEPH) for the first four hours post infection. (Mff Count of Bloodmeal = 7/mm^3)

Time after feed (mins)	Mosquito species	Nos mosquitoes dissected	Microfilariae in Midgut		Microfilariae in Thorax	
			Total examined	% exsheathed ± S.E.	Total nos of larvae	% migration
30	DAR	5	125	0.8	0	0
	AMSS	5	37	62 ± 0.08	2	5
	DAGMOL	5	80	12 ± 0.04	0	0
	STEPH	5	23	35 ± 0.09	0	0
60	DAR	5	102	2 ± 0.01	0	0
	AMSS	5	46	74 ± 0.06	7	15
	DAGMOL	5	29	3 ± 0.03	0	0
	STEPH	5	30	7 ± 0.05	1	3.4
90	DAR	5	128	0 ± 0	0	0
	AMSS	5	54	85 ± 0.05	25	46
	DAGMOL	5	79	32 ± 0.05	0	0
	STEPH	5	39	56 ± 0.07	0	0
120	DAR	5	84	10 ± 0.03	0	0
	AMSS	5	40	95 ± 0.03	16	40
	DAGMOL	5	98	64 ± 0.05	1	1
	STEPH	5	29	35 ± 0.09	0	0
150	DAR	5	69	1 ± 0.01	0	0
	AMSS	5	80	99 ± 0.01	42	53
	DAGMOL	5	108	38 ± 0.05	4	2
	STEPH	5	21	62 ± 0.11	2	11
180	DAR	5	59	0	0	0
	AMSS	5	94	100	39	42
	DAGMOL	5	186	24 ± 0.03	0	0
	STEPH	5	16	38 ± 0.12	0	0
240	DAR	5	43	35 ± 0.07	0	0
	AMSS	5	39	100	19	49
	DAGMOL	5	313	30 ± 0.03	1	0.3
	STEPH	5	83	48 ± 0.05	0	0

9.2 The migration of endopeptidase exsheathed B. pahangi in C. quinquefasciatus (DAR), Ae. aegypti SS and An. stephensi at various heparin concentrations.

9.2.1 Introduction

Previous results suggest that lack of exsheathment of microfilariae might be one of the factors preventing migration. Hence, in this experiment, mosquitoes were offered bloodmeals with microfilariae of B. pahangi exsheathed prior to the feed and in which the heparin concentration was increased from 10 i.u/ml to 50 i.u/ml of blood. It had been observed in previous experiments (Chapter 8) that very high concentrations of heparin reduced the size of bloodmeals ingested by mosquitoes and reduced the attractiveness of the bloodmeal to mosquitoes. For this reason, the concentrations of heparin used were not as high as those reported in Chapter 8. The aim of this experiment was to find out whether prior exsheathment of microfilariae before the feed and increased blood clotting time affect percentage migration to the thorax.

9.2.2 Materials and Methods

Endopeptidase was used to exsheath B. pahangi microfilariae following the method of Devaney and Howells (1979). A concentration of 0.2mg/ml endopeptidase was used as outlined in Chapter 5.1. Exsheathed microfilariae were resuspended in rabbit blood and offered to mosquitoes which had been starved for 48 hours, via a membrane feeder. The bloodmeals had heparin concentrations of 10,20,30,40 and 50 i.u/ml blood. Smears of the bloodmeal in the midgut and the thorax were made at hourly intervals after feeding for the first 3 hours and then at 24 hours. Slides were stained and examined as mentioned in Chapter 9.1. Earlier experiments in Chapter 9, showed that a proportion of B. pahangi microfilariae ingested by An. stephensi exsheathed, but migration was low when the blood had a heparin concentration of 10 i.u/ml. Hence the

experiment was repeated using higher concentrations of heparin, to see whether this would increase migration of exsheathed microfilariae and sheathed microfilariae B. pahangi to the thorax.

9.2.3 Results

Results in Table 21 show that despite exsheathing B. pahangi prior to feeding, in DAR, migration of microfilariae did not increase significantly compared to migration in DAR fed sheathed B. pahangi. At heparin concentrations of 10 and 20 i.u/ml, a few microfilariae were found in the thorax, percentage migration falling in the range of 0-8% (see Fig. 8). At higher heparin concentration of 30, 40 and 50 i.u/ml, there was no migration. Close examination of microfilariae showed that 9.5% of the total examined were damaged. This damage consisted of lacerations to the body wall, leading to the oozing out of nuclei. No red blood cells were seen in close proximity or attached to the microfilariae. Dissection of surviving mosquitoes at day 10 post infection showed that none had become infected. The results also indicate a decrease in the numbers of microfilariae ingested as the concentration of heparin in the bloodmeal increases. Mosquitoes were also reluctant to feed on bloodmeals with higher heparin concentrations. It was observed that mosquitoes which fed to repletion on bloodmeals with higher heparin concentrations become moribund and could be seen lying at the bottom of the cages. After 24 hours most of these mosquitoes had died.

Data in Tables 22 and 23 show that there was no increase in the migration of exsheathed and sheathed B. pahangi in An. stephensi at higher heparin concentrations. In the case of An. stephensi fed sheathed B. pahangi, some microfilariae did migrate to the thorax (2-5%) at a heparin concentration 10 i.u/ml, but at high concentrations, there was no migration. During feeding, it was observed that these mosquitoes were

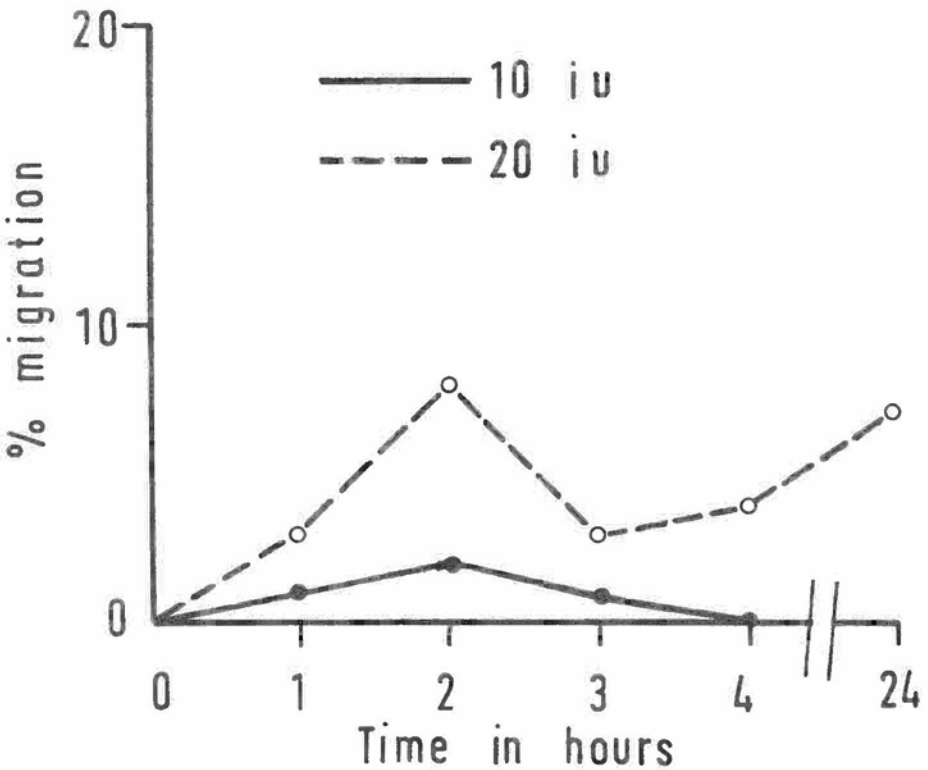


Fig. 8 : The Migration of exsheathed B. pahangi in C. quinquefasciatus (DAR).

reluctant to feed on bloodmeals with higher heparin concentrations and their bloodmeal sizes were smaller. None of the fed mosquitoes survived for dissection on day 10 post infection. Microfilarial uptake was also less than expected in most cases. Results on the migration of exsheathed B. pahangi in Ae. aegypti SS at various heparin concentrations shown in Tables 24 and Fig. 9, show that the migration rate of exsheathed B. pahangi is less than that of unexsheathed B. pahangi. In previous experiments reported in Chapter 9, there was 90% migration of unexsheathed B. pahangi in Ae. aegypti SS at 10 i.u/ml heparin, at 24 hours post feed, whereas the migration rate of exsheathed B. pahangi was in the range of 13-51% 24 hours after the feed. The numbers of microfilariae ingested were less than expected taking into account the microfilarial density of the infective bloodmeal. Mosquitoes fed reluctantly on bloodmeals with higher heparin concentrations and the bloodmeal was smaller in size. There was a high mortality rate of fed females before day 10 post infection. Results from dissections of Ae. aegypti SS day 10 post infection (see Table 25) show that the susceptibility rates at various heparin concentrations range from 36-67%. These rates are lower than those of Ae. aegypti SS fed unexsheathed B. pahangi at 10 i.u/ml heparin, in which case the susceptibility rate was 100%, as observed in those cases where Ae. aegypti SS were used as controls (Chapters 6 & 7). This suggests that the exsheathed B. pahangi were less infective to Ae. aegypti SS then unexsheathed B. pahangi.

Developing larvae were found in the proboscis, head, thorax and abdomen. The average number of developing larvae per infected mosquito at 10,20,30 and 40 i.u/ml heparin were 1.4, 3.5, 6.3 and 4.8 which are lower then expected for the microfilarial density of the infective blood meals. X^2 tests showed there was no significant difference in the

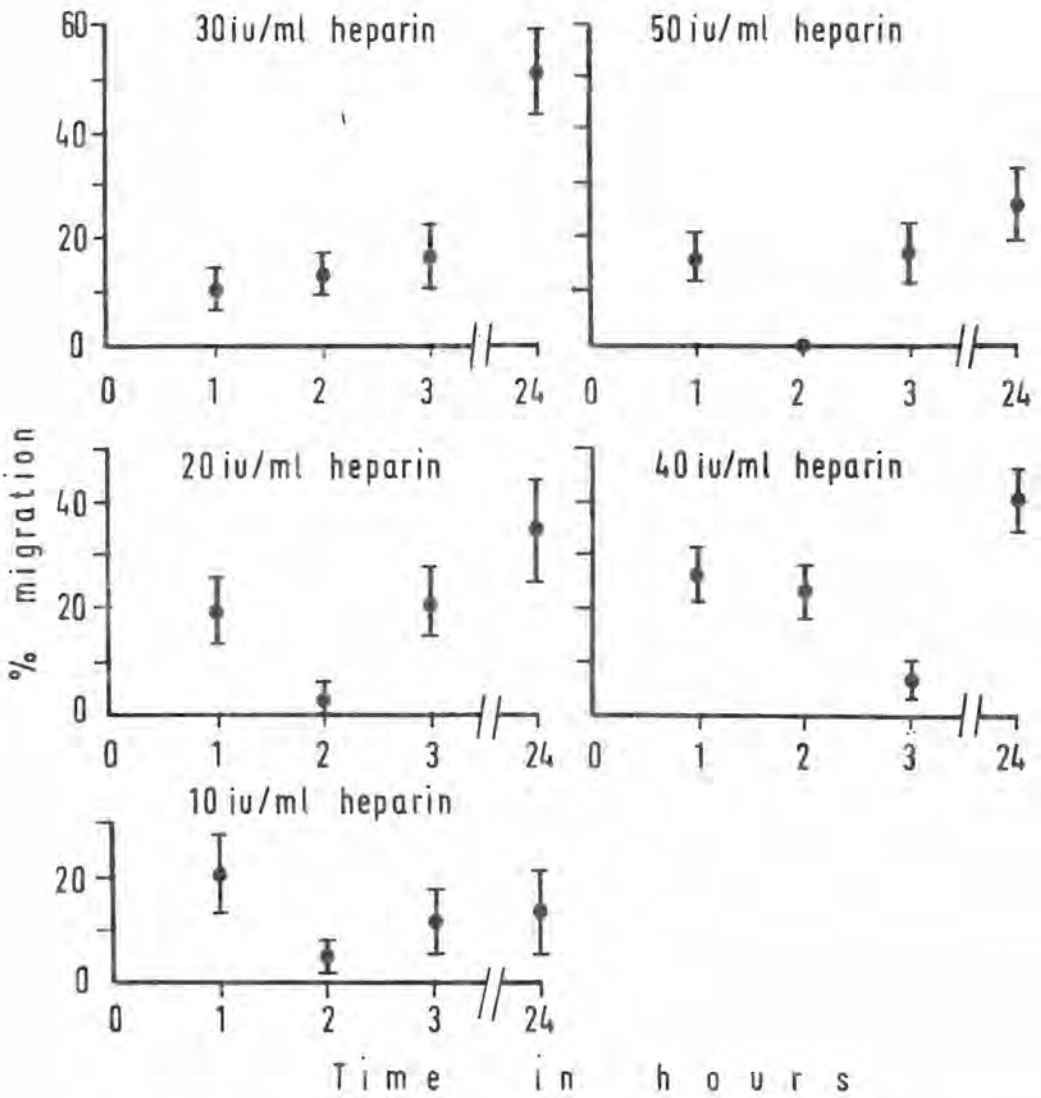


Fig. 9: The Migration of exsheathed B. pahangi in Ae. aegypti SS at various heparin concentrations.

susceptibility rates of Ae. aegypti SS at 10,30 and 40 i.u/ml heparin ($P > 0.05$), but the susceptibility rate at 20 i.u/ml was significantly different from the rest, $P < 0.001$. The significance observed should be treated cautiously as the susceptibility rate is based on the dissection of a very small number of mosquitoes.

9.2.4 Discussion

The results of these experiments show that migration in C. quinquefasciatus (DAR) and An. stephensi is not facilitated by feeding them prior exsheathed microfilariae B. pahangi. This indicates either the absence of an appropriate stimulus to migration or the presence of some factor adversely affecting the microfilariae or both. The apparent drop in the percentage of microfilarial migration at higher heparin concentrations could be due to a combination of the adverse effect of heparin on exsheathed B. pahangi and the adverse factors in the mosquito gut. Obiamiwe (1970) reported a decrease in the migration of B. pahangi in selected stocks of refractory and susceptible C. 'pipiens' and suggested that this was due to the reduced motility and penetration of the gut wall due to an increase in the osmotic pressure and/or viscosity caused by heparin. It is unlikely that in the above experiments, the drop in migration is due to such an effect since the concentrations of heparin used are not high enough to bring about such changes. The decrease in microfilarial uptake and bloodmeal size as the heparin concentration increased in the blood in these experiments, was also observed by Obiamiwe (1970).

The lower susceptibility rates of Ae. aegypti SS fed exsheathed B. pahangi at various heparin concentrations (41-67%) is most likely due to decreased viability of B. pahangi microfilariae as a result of endopeptidase treatment, hence the decrease in the rate of migration.

Devaney and Howells (1979) reported that the susceptibilities of Ae. aegypti (SS + refm strains) inoculated with endopeptidase exsheathed microfilariae directly into the thorax were 58% and 70% respectively, showing that although a good proportion of exsheathed microfilariae are viable, there is some reduction in their viability. Since these microfilariae were inoculated directly into the thorax, there was a greater chance of them infecting the mosquitoes, since they did not have to migrate from the midgut. The susceptibility of Ae. aegypti SS inoculated with unexsheathed B. pahangi (see Chapter 5) was found to be 88%, showing that sheathed B. pahangi are more infective than exsheathed microfiliariae. The reduction in the rate of migration in Ae. aegypti SS could also be related to the loss of important factors located on the sheath which play an important role in triggering exsheathment and subsequent migration of microfilariae to the thorax.

In support of the above suggestion are the findings of Furman and Ash (1983), who investigated the surface carbohydrates in mature in vivo derived and immature in utero derived microfilariae B. pahangi. They reported the presence of certain carbohydrates which bind to lectins in the sheath of mature microfilariae but not in either mature or immature exsheathed microfilariae. They suggested that it was possible that sheath carbohydrates are a component of the molecular trigger which initiates exsheathment and development once the mosquito ingests an infective bloodmeal. However, the role of sheath carbohydrates in the recognition and non recognition of microfilariae by the mosquito vector is unknown.

Further investigations on the properties of the sheath in various microfilariae and the cellular and humoral mosquito immune response might prove valuable in helping us understand the way in which genes , which determine susceptibility in mosquitoes, are manifested physiologically.

Table 21: The migration of exsheathed B. pahangi in C. quinquefasciatus (DAR) at various heparin concentrations.

Time of dissection after feed (hrs)	Heparin Conc. i.u/ml	mff/ mm^3	Nos of mosquitoes examined	Nos of microfilariae in midgut	thorax	% migration
1	10	9	6	168	2	1
2	10	9	6	181	3	2
3	10	9	6	151	2	1
4	10	9	6	79	0	0
24	10	9	6	0	0	0
1	20	8	6	29	1	3
2	20	8	6	34	3	8
3	20	8	6	36	1	3
4	20	8	6	27	0	3
24	20	8	6	26	1	7
1	30	6	6	60	0	0
2	30	6	6	34	0	0
3	30	6	6	44	0	0
24	30	6	6	17	0	0
1	40	6	6	121	0	0
2	40	6	6	52	0	0
3	40	6	6	78	0	0
4	40	6	6	19	0	0
24	40	6	6	0	0	0
1	50	3	5	13	0	0
2	50	3	5	10	0	0
3	50	3	5	13	0	0

Table 22: The migration of exsheathed B. pahangi in An. stephensi at various heparin concentrations.

Time of dissection after feed (hrs)	Heparin Conc. iu/ml	mff/ mm^3	Nos of mosquitoes examined	Nos of microfilariae in midgut	thorax	% migration
1	10	5	5	71	0	0
2	10	5	5	14	1	7
3	10	5	5	44	0	0
24	10	5	5	65	0	0
1	20	5	5	41	0	0
2	20	5	5	57	0	0
3	20	5	5	76	0	0
24	20	5	5	45	1	2
1	30	12	5	123	0	0
2	30	12	5	148	0	0
3	30	12	5	150	0	0
24	30	12	5	93	0	0
1	40	12	5	161	0	0
2	40	12	5	129	2	2
3	40	12	5	141	0	0
24	40	12	5	88	0	0
1	50	11	5	118	0	0
2	50	11	5	144	0	0
3	50	11	5	99	0	0
24	50	11	5	138	0	0

Table 23: The migration of unexsheathed B. pahangi in An.stephensi at various heparin concentrations.

Time of dissection after feed (hrs)	Heparin Conc. i.u/ml	mff/ mm^3	Nos of mosquito examined	Nos of microfilariae in midgut	thorax	% migration
1	10	5	10	52	1	2
2	10	5	10	76	2	3
3	10	5	10	35	2	5
4	10	5	10	113	0	0
1	20	17	6	228	1	0.4
2	20	17	6	102	0	0
3	20	17	6	157	0	0
4	20	17	6	232	1	0.4
1	30	16	6	240	0	0
2	30	16	6	145	0	0
3	30	16	6	167	0	0
4	30	16	6	124	0	0
1	40	8	6	41	0	0
2	40	8	6	47	1	2
3	40	8	6	50	0	0
4	40	8	6	25	0	0
1	50	11	6	190	0	0
2	50	11	6	196	0	0
3	50	11	6	143	0	0
4	50	11	6	69	0	0

Table 24: The migration of exsheathed B. pahangi in Ae. aegypti SS at various heparin concentrations.

Time of dissection after feed (hrs)	Heparin Conc. i.u /ml	mff/ mm^3	Nos of mosquitoes examined	Nos of microfilariae in midgut	thorax	% migration ± S.E.
1	10	5	5	20	5	20 ± 0.08
2	10	5	5	44	2	4 ± 0.03
3	10	5	5	25	3	11 ± 0.06
24	10	5	5	14	2	13 ± 0.08
1	20	5	5	35	9	20 ± 0.06
2	20	5	5	33	1	3 ± 0.03
3	20	5	5	30	8	21 ± 0.07
24	20	5	5	13	7	35 ± 0.10
1	30	12	5	55	6	10 ± 0.04
2	30	12	5	56	8	13 ± 0.04
3	30	12	5	32	6	16 ± 0.06
24	30	12	5	20	21	51 ± 0.08
1	40	5	5	57	20	26 ± 0.05
2	40	5	5	54	16	23 ± 0.05
3	40	5	5	66	5	7 ± 0.03
24	40	5	5	49	23	40 ± 0.06
1	50	4	5	43	8	16 ± 0.05
2	50	4	5	44	0	0
3	50	4	5	30	6	17 ± 0.06
24	50	4	5	29	10	26 ± 0.07

Table 25: Showing the susceptibility of Ae. aegypti SS fed exsheathed B. pahangi at various heparin concentrations and dissected on Day 10 post infection.

Nos of mosquitoes dissected	Heparin Concentration i.u/ml	mff/ mm^3	Developing larvae in				Average nos larvae per mosquito	Total nos. of mosquitoes with larvae	% mosquitoes susceptible
			Proboscis	Head	Thorax	Abdomen			
34	10	5	4	2	12	2	1.4	14	41
6	20	5	2	1	11	0	3.5	4	67
7	30	12	11	1	7	2	6.3	3	43
14	40	5	9	5	10	0	4.8	5	36

* Mosquitoes fed on bloodmeals with 50 i.u/ml heparin all died before day 10.

9.3 The exsheathment and migration of B. pahangi and W. bancrofti in C. quinquefasciatus (DAR) and Ae. aegypti SS fed on mixed infective blood meals.

9.3.1 Introduction

W. bancrofti develops normally in East African C. quinquefasciatus (DAR) whereas B. pahangi does not. These two filariae are very similar morphologically and in some mosquito hosts, notably in the laboratory host Ae. aegypti SS (Macdonald and Ramachandran, 1965) and the natural vectors of the Aedes scutellaris group (Macdonald, 1976; Meek and Macdonald, 1982), the developmental fate of the two species appears to be the same, good hosts of one species being good hosts of the other and vice versa for poor hosts. Hence, it is difficult to explain why B. pahangi does not exsheath and migrate from a bloodmeal taken by DAR. Two of the possibilities which could account for the differences between W. bancrofti and B. pahangi are that

a) different factors are required by the 2 filariae in the mosquito

b) the 2 filariae have different thresholds for the factor(s) which facilitate(s) exsheathment and subsequent migration from the midgut.

The aim of this experiment was to see whether feeding DAR simultaneously on W. bancrofti and B. pahangi, would aid in the exsheathment and migration of B. pahangi. Ae. aegypti SS mosquitoes were used as controls. This stock had been selected for susceptibility to W. bancrofti and B. pahangi (Macdonald and Ramachandran, 1965). Migration and exsheathment in Ae. aegypti SS were compared to that in DAR at the early stages following the bloodmeal. The infective larvae recovered day 10 post infection were also compared.

9.3.2 Materials and Methods

Microfilariae

W. bancrofti : were obtained from storage in liquid nitrogen cryopreserved in low molecular wt H.E.S. The blood had been collected from donors in Tanzania in May 1979.

B. pahangi : were obtained from an infected cat.
Human blood was used for reconstitution.

Mosquitoes : One week old DAR and AMSS were starved for 48hours prior to being offered a bloodmeal.

Smears : Mosquitoes were allowed to feed for 1½-3 hours until they had become fully engorged. Fed mosquitoes were recovered from the cage and transferred to a perspex container. At 30 minute intervals after feeding, smears were made from the midgut and thorax of 5 mosquitoes for each genus , up to 3 hours post feeding. Smears were also made 24 hours post feeding. The remaining mosquitoes were held in perspex cages and offered sugar and water up to 10-14 days when they were dissected. Location, numbers and stage of development of the developing larvae was noted in both DAR and AMSS.

Examination of Smears

Mayers acid haemalum was used for staining slides for 8-10 minutes. Smears of the midgut were first dehaemoglobinized then fixed for 1 minute in methanol. Thorax smears were also fixed. The slides were then stained. During examination of slides, note was made of numbers of microfilariae exsheathed, their identity was checked, their location either in midgut or thorax was also noted.

Identification of Infective Larvae

In mosquitoes (DAR and AMSS) dissected on days 10-14 post infection developing larvae recovered were transferred to TAF. For further examination, they were mounted in alcohol glycerol over slides with ringed wells using the Hanging drop technique. They were then examined at X400 magnification or oil immersion of a compound microscope. Features used for identification of W. bancrofti microfilariae and infective larvae were:-

1. W. bancrofti microfilariae were devoid of nuclei at the tail end.
2. In blood smears, the microfilariae of W. bancrofti show graceful curves.
3. Microfilariae of W. bancrofti (244-296μ) are longer then those of B. pahangi (177-230μ).
4. Infective 3rd stage larvae of W. bancrofti have 3 well developed and rounded caudal papillae at the posterior extremity.

Features used for identification of B. pahangi microfilariae and infective larvae were:-

1. B. pahangi microfilariae have a slender hooked tail containing two or three nuclei.
2. In blood smears, the microfilariae have secondary waves and are kinky in general appearance.
3. Microfilariae of B. pahangi are shorter than W. bancrofti.
4. The infective third stage larvae have 3 ill defined caudal papillae at the posterior extremity.

9.3.3 Results

Data in Tables 26 and 27 show % exsheathment of B. pahangi and W. bancrofti in DAR and AMSS, in 2 similar experiments. In experiment 1, % exsheathment of B. pahangi in DAR over a 24 hour period varied from 15-67%. A Heterogeneity X^2 test showed this variation was not significant $P > 0.05$. The exsheathment rate did not show a significant trend with time. This rate of exsheathment was higher than that found in DAR fed only on B. pahangi (0-35%) reported in Chapter 9. A X^2 test showed this difference to be significnat ($P < 0.05$). In experiment 2, % exsheathment of B. pahangi was very variable, due to the small numbers of microfilariae examined and the variability in the numbers of microfilariae found in the individual mosquitoes thatwere examined. Some mosquitoes had not ingested any microfilariae, whereas in some cases, all the microfilariae examined in a batch of mosquitoes occurred in one mosquito only. There was no significant trend in the exsheathment rate with time. The numbers of microfilariae B. pahangi ingested by DAR mosquitoes in both experiments 1 and 2 were less than expected, considering the microfilarial density in the infective blood offered to these mosquitoes. Figs. 10 and 11 show unexsheathed and exsheathed B. pahangi in the midgut of DAR. The % exsheathment of W. bancrofti in DAR, in experiment 1, increased with time. However, these percentages should be examined cautiously, due to the small numbers of microfilariae on which they are based. In experiment 2, % exsheathment was very variable and over a 24 hours period ranged from 0-76%. A Heterogeneity X^2 test of % exsheathment at the various time intervals was significant ($P < 0.05$).

Ae. aegypti SS mosquitoes in both experiments 1 and 2, had noticeably fewer microfilariae of both B. pahangi and W. bancrofti in the midgut

Fig. 10: Showing a sheathed B. pahangi microfilaria in the midgut of C.quinquefasciatus DAR.

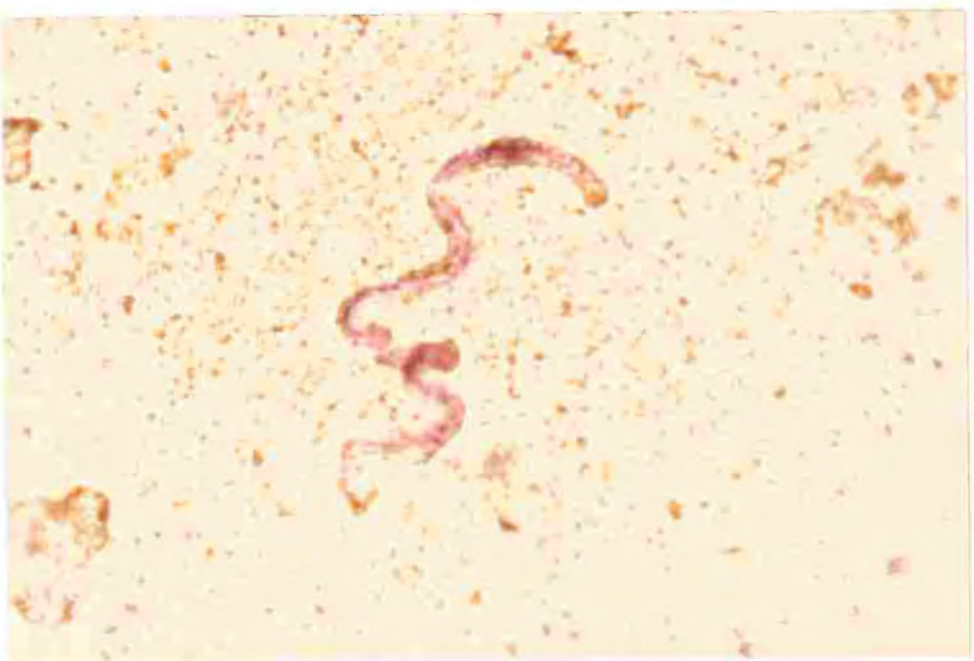


Fig 11: Showing an exsheathed B. pahangi microfilaria in the midgut of C. quinquefasciatus DAR.

than the DAR mosquitoes. The percentage exsheathment of B. pahangi and W. bancrofti in both experiments were very varibale due to the very small numbers of microfilariae found in the midgut and the variability in the distribution of microfilariae among the individual mosquitoes examined at the different time intervals. Thus it was difficult to determine whether the exsheathment rates of the 2 filariae observed followed any trend.

Results of the migration of B. pahangi and W. bancrofti in DAR and AMSS in 2 experiments are shown in Tables 28 and 29 . Migration of B. pahangi in DAR, in experiment 1 over a 24 hour period, varied from 0-50%. Fig 12 shows exsheathed B. pahangi in DAR thorax. These percentages are based on the small numbers of microfilariae found and should be examined cautiously. There seemed to have been an increase in the migration rate with time. Percentage migration of B. pahangi in this experiment was found to differ significantly from that in DAR fed only on B. pahangi, in which no migration took place (see Chapter 9), using a X^2 test ($P < 0.001$). In experiment 2, the migration rate was negligible. The numbers of B. pahangi ingested were less than expected, judging from the microfilariae density of the infective blood. The migration of W. bancrofti in DAR varied from 0-50% over a 24 hour period in the first experiment. There was no trend in the migration rate with time. In experiment 2, the % migration varied from 5-75% over a period of 24 hours, and the migration rate appeared to increase with time up to 3 hours post feeding. The numbers of microfilariae found in these mosquitoes was less than expected and there was a lot of variability in the numbers of microfilariae found in individual mosquitoes.

In AMSS experiment 1, although the numbers of microfilariae examined were very few, there was an increase in the % migration of B. pahangi with time, the migration rate increasing from 17% at 30

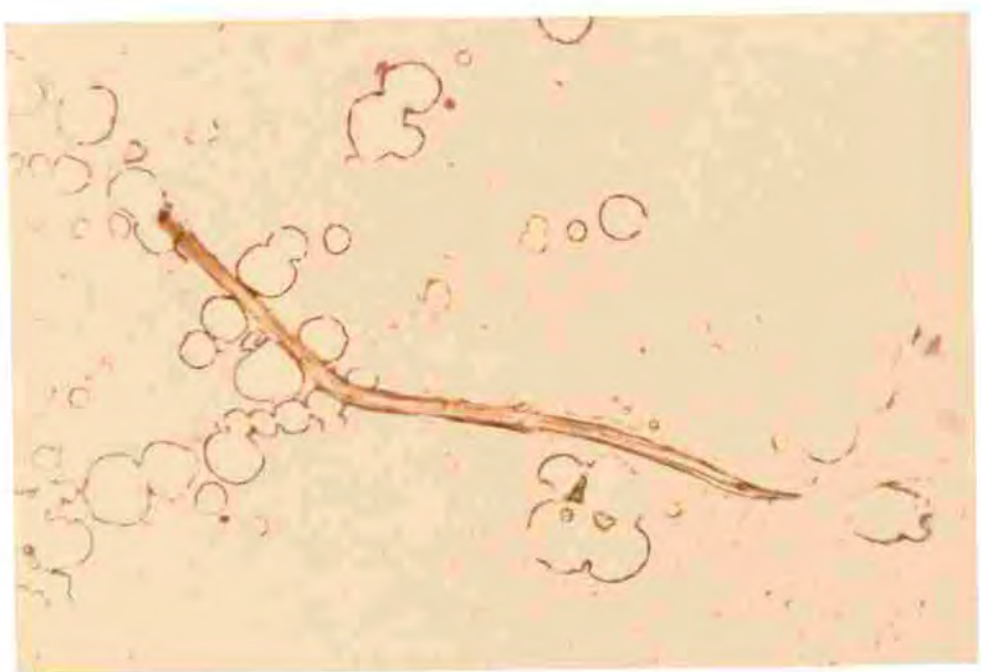


Fig. 12: Showing an exsheathed B. pahangi microfilaria in the thorax of C. quinquefasciatus DAR.

minutes to 100% 3 hours post feeding. However, in experiment 2, most of the mosquitoes examined had no microfilariae and the few mosquitoes found to have microfilariae had ingested very few. Hence it was difficult to determine the migration pattern of B. pahangi in these mosquitoes. The % migration of W. bancrofti in AMSS in the 2 experiments was very variable due to the small numbers of microfilariae found in these mosquitoes on which these percentages were based. This made it difficult to determine the migration pattern of W. bancrofti in AMSS.

Data in Table 30 shows that no developing or infective larvae were recovered in DAR females dissected on days 12 and 14 post infection. The absence of infective larvae is surprising since results in Table 28 show that some microfilariae B. pahangi and W. bancrofti did migrate to the thorax. In the second experiment, some of the DAR mosquitoes dissected were infected. In this case, infected mosquitoes refers to those with normal developing larvae. Results show that the numbers of developing larvae found were less than expected. When one takes into account the microfilarial density of W. bancrofti and B. pahangi in the bloodmeal, however, they do agree with the low microfilarial uptake observed when fed mosquitoes were dissected soon after feeding. Only an average of 2 larvae were found per mosquito. 97% of all larvae examined were infective while the other 3% were stage 2 larvae. Only 27% of DAR mosquitoes were found to be infected.

In experiment 1, 41% of Ae. aegypti SS dissected were infected. The numbers of larvae per infected mosquito was 2. Migration results show that 70 larvae were found in the thorax of 35 mosquitoes examined, that is approximately 2 larvae per mosquito. This coincides with the numbers found in each infected mosquito on dissection day 12 and 14 post infection and shows that all the microfilariae which succeeded in

migrating to the thorax developed normally to the infective stages. Since these mosquitoes were dissected on days 12 and 14 post infection, it is possible that some infective B. pahangi larvae were lost after day 10 during sugar feeding. Most of the infective larvae were located in the head, none being found in the proboscis. In experiment 2, 54% of the mosquitoes were infected. Only an average of 4 larvae were found per mosquito and 91% of all larvae recovered were infective. Comparison of the migration results and infectivity results indicate that all the larvae which migrated to the thorax developed to the infective stage or other developmental stages. Again, some infective stage 3 larvae of B. pahangi could have been lost from the proboscis during sugar feeding after day 10, since mosquitoes were dissected on day 12 and 14 post infection. One would have expected to find more developing larvae and infective stages in AMSS since they are highly susceptible to both B. pahangi and W. bancrofti. The infectivity of AMSS in both experiments were lower than those of AMSS fed only on B. pahangi, in which there was a 100% infectivity rate as reported elsewhere in this thesis.

Results in Table 31 show that in DAR experiment 2, 78% of the total numbers of larvae found were W. bancrofti while 22% were B. pahangi. The mean anal ratios of both types of filariae were approximately the same (3.8:1). The range of the anal ratios for both filariae were from 2.9:1 - 4.6:1. The caudal papillae used for identification purposes were easily seen at X400 magnification. In W. bancrofti, the 3 caudal papillae were distinct well rounded knobs (see Fig. 13) whereas in B. pahangi, they were not well rounded (see Fig. 14).

In Ae aegypti SS experiment 1, 54% of the total infective larvae recovered were W. bancrofti whilst 46% were B. pahangi. In experiment 2, 57% of the total infective larvae were W. bancrofti and 43% were

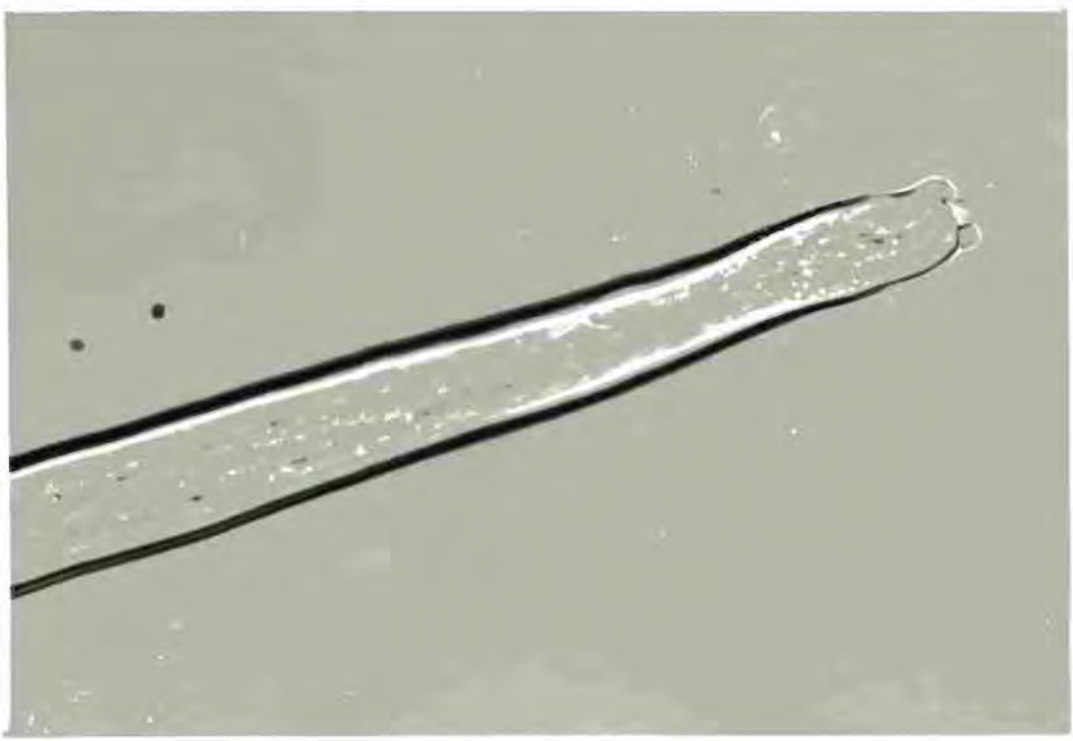


Fig. 13: Showing the well rounded caudal papillae at the posterior end of an infective larva of W. bancrofti.

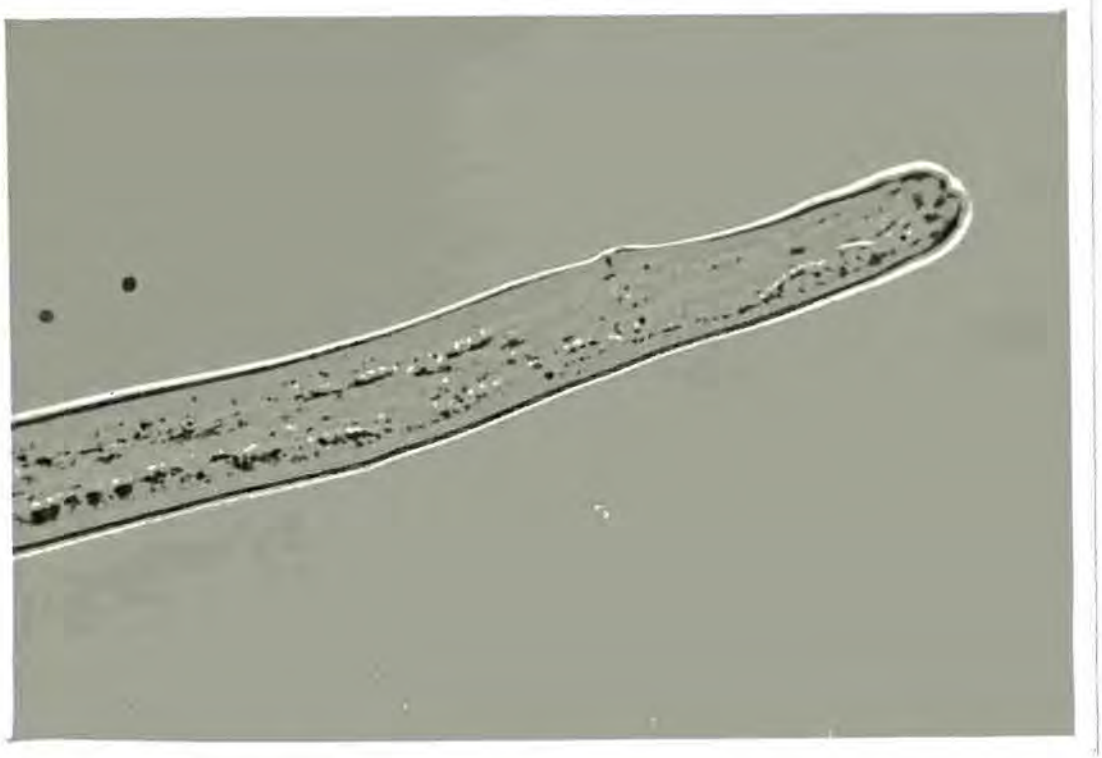


Fig. 14: Showing the indistinct caudal papillae at the posterior end of an infective larva of B. pahangi.

B. pahangi. In experiment 1, the mean and ratio for W. bancrofti was 4:1 while that of B. pahangi was 3:1 with ranges of 2.6-4.4:1 and 2.8-4.4:1 respectively. In experiment 2, the mean anal ratios for W. bancrofti and B. pahangi were 4:1 and the ranges were 2.0-5.5:1 and 2.0-5.8:1 respectively. In the second experiment there was more variation in the sizes of W. bancrofti and B. pahangi.

9.3.4 Discussion

The increase in the % exsheathment of B. pahangi in DAR midgut suggests that the presence of W. bancrofti in the bloodmeal facilitates exsheathment, by altering the physiological conditions prevailing in the midgut. These changes could for instance be the production or inhibition of certain factors e.g. enzymes, which also enable a proportion of B. pahangi microfilariae to exsheath. Probably the stimulus required by W. bancrofti to exsheath and migrate also acts on B. pahangi, allowing a small proportion of these microfilariae to exsheath and migrate. Percentage exsheathment of W. bancrofti was higher than that of B. pahangi in DAR, suggesting that B. pahangi requires a higher threshold of stimulus to exsheath and migrate than do W. bancrofti.

The absence of W. bancrofti in DAR midgut at 24 hours in experiment 1, and the absence of B. pahangi in DAR at 24 hours in experiment 2, could be due to the digestion of microfilariae by peptidases produced in the midgut following the bloodmeal.

The small numbers of microfilariae found in the midgut of AMSS could be due to the order in which mosquitoes were fed, DAR feeding first for 1-2 hours, followed by AMSS. In addition, the variability in the bloodmeal size ingested by these mosquitoes and the uneven distribution of microfilariae in the feeder unit, could have been responsible for these low numbers of microfilariae.

The % exsheathment of W. bancrofti and B. pahangi in AMSS was based on very small numbers of microfilariae and it was not possible to make a real comparison of the exsheathment rate, but there appears to be no significant difference. This is not surprising since Ae. aegypti SS is highly susceptible to both B. pahangi and W. bancrofti and susceptibility was shown by Macdonald and Ramachandran (1965) to be controlled by the same gene f^m (filarial susceptibility to B. malayi).

The significant increase in % migration of B. pahangi to the thorax in DAR mosquitoes, suggests that the migration of W. bancrofti to the thorax in DAR facilitates the migration of B. pahangi. The most likely mechanism which enables B. pahangi to migrate through the gut wall is that these microfilariae gain entrance to the haemocoel than to the thorax via the pores in the gut wall created by migrating W. bancrofti. This speculation can only be confirmed by examining microscopically, the midgut epithelium for such pores at various intervals, after ingestion of a mixed infective bloodmeal. The migration of W. bancrofti in DAR was not as high as expected. This could be due to the loss of motility and viability of a proportion of the W. bancrofti microfilariae as a result of cryopreservation and thawing. In AMSS, % migration was higher in B. pahangi than in W. bancrofti. One would have expected similar rates of migration since AMSS is susceptible to both. Again, the lower migration rate of W. bancrofti could be due to loss of motility and viability as a result of cryopreservation and thawing.

The absence of developing larvae of both B. pahangi and W. bancrofti in DAR (experiment 1) suggests that within the thorax there are factors which kill microfilariae. This suggestion could be true for microfilariae B. pahangi, since DAR mosquitoes are refractory to B. pahangi, but in the case of W. bancrofti, the most likely

explanation is that those microfilariae which migrated to the thorax successfully were unable to undergo further development due to their reduced infectivity as a result of cryopreservation and thawing. Minjas (1980) noted that motility of cryopreserved microfilariae could not be used as a quick indicator of survival of microfilariae, since motile cryopreserved microfilariae will not necessarily develop. Rather, their infectivity to mosquitoes should be used as a final criterion for assessing viability.

The small numbers of developing larvae recovered in DAR (experiment 2) is due to the low migration rate of B. pahangi and reduced infectivity of W. bancrofti due to the adverse effects of cryopreservation. In AMSS, the small numbers of larvae of both filariae recovered and the reduced susceptibility observed is due to the initial small numbers of microfilariae ingested, their reduced rate of migration and in the case of W. bancrofti microfilariae, their reduced motility and viability due to the effects of cryopreservation. In AMSS, in both experiments, the proportion of W. bancrofti and B. pahangi recovered was almost the same, there being a slight preponderance of W. bancrofti. When one takes into consideration that a small proportion of B. pahangi could have been lost after day 10, we can safely say that the proportions of both filarial larvae recovered were similar. This indicates that AMSS is a good host of B. pahangi and W. bancrofti.

However, in DAR mosquitoes, the proportion of W. bancrofti recovered was about $3\frac{1}{2}$ times that of B. pahangi, even after day 10. This result shows DAR is not a good host of B. pahangi. The presence of W. bancrofti in a mixed infective feed does seem to increase exsheathment and migration of B. pahangi which ultimately increases the susceptibility of DAR to B. pahangi.

The anal ratios of B. pahangi and W. bancrofti infective larvae were measured because it was thought they could be used as indicators of any departure from the normal development rate of these microfilariae, as a result of their interaction within AMSS and DAR mosquitoes. The anal ratios of these filariae is usually 4:1, and the results showed no significant departures from this in the 2 filariae, showing that their interaction in the mosquitoes did not have any adverse effects on their rate of development.

It would be worthwhile to repeat this experiment using blood freshly obtained from donors infected with W. bancrofti and dissecting larger numbers of mosquitoes, in order to get a clearer picture of the exsheathment and migration patterns in mosquitoes of DAR and AMSS fed simultaneously on B. pahangi and W. bancrofti. It is difficult to draw any sound conclusions from these experiments due to the small numbers of microfilariae found in the mosquitoes examined and the use of cryopreserved blood infected with W.bancrofti. However, the results do indicate that the exsheathment and migration of B. pahangi in refractory DAR mosquitoes, is increased to a certain extent by the presence of W. bancrofti in the bloodmeal.

Table 2 6: Exsheathment of microfilariae B. pahangi and W. bancrofti in DAR and AMSS

EXPERIMENT 1

Time after feeding (mins)	mosquito	Nos of B. pahangi mff			Nos of W. bancrofti mff		
		Total nos in midgut	Nos exsheathed	% exsheathed	Total nos in midgut	Nos exsheathed	% exsheathed
30	DAR	23	9	39	9	3	33
	AMSS	5	0	0	5	1	22
60	DAR	13	2	15	22	16	73
	AMSS	1	0	0	8	2	25
90	DAR	7	3	43	8	7	88
	AMSS	2	0	0	1	1	100
120	DAR	15	4	36	2	2	100
	AMSS	2	0	0	2	1	50
150	DAR	10	3	30	2	2	100
	AMSS	2	2	100	0	0	0
180	DAR	9	6	67	3	3	100
	AMSS	0	0	0	3	3	100
24hrs	DAR	4	2	50	0	0	0
	AMSS	1	1	100	0	0	0

Microfilarial density of bloodmeal = $\frac{30\text{mff}/20\text{mm}^3}{100\text{mff}/20\text{mm}^3}$ $\frac{\text{W. bancrofti}}{\text{B. pahangi}}$

5 mosquitoes were examined at each time interval.

Table 27: Exsheathment of microfilariae B. pahangi and W. bancrofti in DAR and AMSS

EXPERIMENT 2

Time after feeding (mins)	mosquito	Nos of B. pahangi mff			Nos of W. bancrofti mff		
		Total nos in midgut	Nos exsheathed	% exsheathment	Total nos in midgut	Nos exsheathed	% exsheathed
30	DAR	41	1	2	35	1	3
	AMSS	1	1	100	12	1	8
60	DAR	13	0	0	21	0	0
	AMSS	0	0	0	16	0	0
90	DAR	6	0	0	15	3	20
	AMSS	0	0	0	8	0	0
120	DAR	36	24	67	19	10	53
	AMSS	2	2	100	1	1	100
150	DAR	11	11	100	21	16	76
	AMSS	4	1	25	7	4	57
180	DAR	7	6	86	9	2	28
	AMSS	4	0	0	8	3	38
24hrs	DAR	0	0	0	8	4	50
	AMSS	11	3	27	4	0	0

Microfilarial density of bloodmeal = $\frac{100\text{mff}/20\text{mm}^3}{102\text{mff}/20\text{mm}^3}$ $\frac{\text{W. bancrofti}}{\text{B. pahangi}}$

5 mosquitoes were examined at each time interval.

Table 28: Migration of W. bancrofti and B. pahangi in DAR and AMSS

EXPERIMENT 1

Time after feeding (mins)	mosquito	Nos of B. pahangi mff			Nos of W. bancrofti mff		
		Total nos in midgut	Nos in thorax	% migration	Total nos in midgut	Nos in thorax	% migration
30	DAR	23	0	0	9	3	25
	AMSS	5	1	17	5	0	0
60	DAR	13	2	13	24	5	17
	AMSS	1	8	89	8	1	11
90	DAR	7	1	13	8	0	0
	AMSS	1	11	92	1	1	50
120	DAR	15	2	12	2	0	0
	AMSS	2	7	78	2	1	33
150	DAR	10	10	50	2	2	50
	AMSS	2	12	86	0	1	100
180	DAR	9	0	0	3	1	25
	AMSS	0	5	100	3	5	63
24hrs	DAR	4	4	50	0	0	0
	AMSS	1	17	94	0	0	0

Microfilarial density of bloodmeal = 30 mff/20mm^3 W. bancrofti
100 mff/20mm^3 B. pahangi

5 mosquitoes were dissected at each time interval.

Table 29: Migration of W. bancrofti and B. pahangi in DAR and AMSS

EXPERIMENT 2

Time after feeding (mins)	mosquito	Nos of B. pahangi mff			Nos of W. bancrofti mff		
		Total nos in midgut	nos in thorax	% migration	Total nos in midgut	Nos in thorax	% migration
30	DAR	41	0	0	25	2	5
	AMSS	1	0	0	12	0	0
60	DAR	13	0	0	21	2	9
	AMSS	0	0	0	16	2	11
90	DAR	6	0	0	15	6	29
	AMSS	0	0	0	8	2	20
120	DAR	36	1	3	19	10	34
	AMSS	2	2	50	1	2	67
150	DAR	1	0	0	21	7	25
	AMSS	4	1	20	7	1	13
180	DAR	7	0	0	9	12	75
	AMSS	4	0	0	8	2	20
24hrs	DAR	0	1	100	8	7	47
	AMSS	11	6	35	4	0	0

Microfilarial density of bloodmeal = 100mff/20mm^3 W. bancrofti
= 102mff/20mm^3 B. pahangi

5 mosquitoes were examined at each time interval

Table 30: Results of feeding Ae. aegypti SS and C. quinquefasciatus (DAR) on bloodmeals with W. bancrofti and B. pahangi on dissection day 14 post infection

Expt. date and (Nos)	mff/20mm^3 W.bancrofti	mff/20mm^3 B. pahangi	Mosquito Strain	Mosquito Nos dissected	Developing larvae: Proboscis I	Proboscis II	Proboscis III	Head I	Head II	Head III	Thorax I	Thorax II	Thorax III	Abdomen I	Abdomen II	Abdomen III	Nos of larvae per infected mosquito	% larvae infective *	Mosquitoes Nos Infected **	Mosquitoes % Infected
1.2.84 (1)	30	106	DAR	52	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.2.84 (1)	30	106	AMSS	34	0	0	0	0	0	12	0	1	11	0	0	8	2.3	97	14	41
22.2.84 (2)	100	100	DAR	116	0	0	7	0	0	23	0	4	4	0	0	18	2.1	97	31	27
22.2.84 (2)	100	102	AMSS	83	0	0	68	0	0	30	0	12	20	0	3	35	4.0	91	45	54

* Infective larvae refers to the 3rd larval stage
** Infected mosquito refers to any mosquito with normal developing larvae.

AMSS infected on 22.2.84 were dissected on Day 12 post infection whilst for DAR infected on the same feed, 25 females were dissected on Day 12 post infection and the remainder were dissected on Day 14 post infection.

Table 31: Identification of infective larvae found in C. quinquefasciatus (DAR) and Ae. aegypti (SS) fed on W. bancrofti and B. pahangi and dissected of Day 12 and 14 Post infection

Expt date and Nos	Mosquito species	Nos of infective larvae examined	INFECTIVE LARVAE							
			W. BANCROFTI							
			Total Nos.	%	Meananal Ratio	Range of anal ratio	Total Nos	%	Mean anal Ratio	Range of anal ratio
1.2.84 (1)	DAR	0	0	0	0	0	0	0	0	0
1.2.84 (1)	AMSS	28	15	54	3.5:1	2.6-4.4:1	13	46	2.9:1	2.8-4.4:1
22.2.84 (2)	DAR	39	30	78	3.8:1	2.9-4.6:1	9	23	3.7:1	2.9-4.6:1
22.2.84 (2)	AMSS	86	49	57	3.8:1	2.0-5.8:1	37	43	3.7:1	2.0-5.2:1

9.4 The Exsheathment and Migration of *W. bancrofti* in *C. quinquefasciatus* (DAR) and *Ae. aegypti* SS.

9.4.1 Introduction

C. quinquefasciatus DAR and *Ae. aegypti* SS are good hosts of *W. bancrofti*. Results in Chapter 9.3 suggest that the presence of *W. bancrofti* in an infective bloodmeal containing *B. pahangi*, fed to *C. quinquefasciatus* (DAR), facilitates an increase in the exsheathment and migration of *B. pahangi* in these mosquitoes. The aim of this experiment is to establish the exsheathment and migration patterns of *W. bancrofti* in DAR and AMSS and to compare theseto those observed when these two mosquito species were fed on a mixed infective bloodmeal. This would show whether the presence of *B. pahangi* in the bloodmeal, in any way affects exsheathment and migration of *W. bancrofti* in either DAR or AMSS.

9.4.2 Materials and Methods

The source of microfilariae *W. bancrofti* used in these experiments is similar to that outlined in section 9.3.2. Infected blood was thawed and reconstituted in human blood then offered to one week old DAR and AMSS, previously starved of sugar and water for 24-48 hours. Smears of the midgut and thorax were prepared, stained and examined as outlined in section 9.3.2.

9.4.3 Results

Data in Table 32 show the % exsheathment of *W. bancrofti* in DAR and AMSS in experiment 1. The exsheathment rate in DAR increased with time up to 3 hours post feed, but in AMSS the exsheathment rate did not follow any trend with time. Heterogeneity X^2 tests of % exsheathment within DAR and AMSS were not significant ($P > 0.05$), but X^2 tests showed significant differences in % exsheathment between DAR and AMSS at the various time intervals, ($P < 0.05$). The numbers of microfilariae

ingested by both species of mosquitoes was much lower than expected despite the low microfilarial density in the infective blood in the feeder unit. It was particularly low in AMSS mosquitoes.

Results in Table 33 of % exsheathment of W. bancrofti in DAR and AMSS in a second experiment similar to the previous one show that in both mosquito species, the exsheathment increased with time (see Fig.15). Heterogeneity X^2 tests of % exsheathment within DAR and AMSS showed significant differences ($P < 0.05$) and X^2 tests showed there were also significant differences at the time intervals of 30,60, 150 and 180 minutes in the % exsheathment between DAR and AMSS ($P < 0.05$).

A comparison between the % exsheathment of W. bancrofti in DAR and AMSS fed only on W. bancrofti and that in DAR and AMSS fed on mixed infective feeds (see section 9.3) was found to be significantly different, using the X^2 test $P < 0.001$.

Data in Table 34 show that % migration in DAR and AMSS ranged from 4-45% and 6-83% respectively. There appears to be an increase in % migration with time (see Fig.16). A X^2 test showed that there were significant differences in % migration of W. bancrofti between DAR and AMSS ($P < 0.01$).

Results in Table 35 show that the proportions of DAR and AMSS infected with W. bancrofti were in the range of 22-30%, and these were not found to be significantly different. Most of the developing larvae recovered on day 14 post infection were in the 3rd larval stage, a few being in their 2nd larval stage. Each infected mosquito had an average of 2 larvae. The numbers of larvae recovered per mosquito was as expected on the basis of the low uptake of microfilariae from the blood meal observed during dissection soon after ingestion of the bloodmeal.

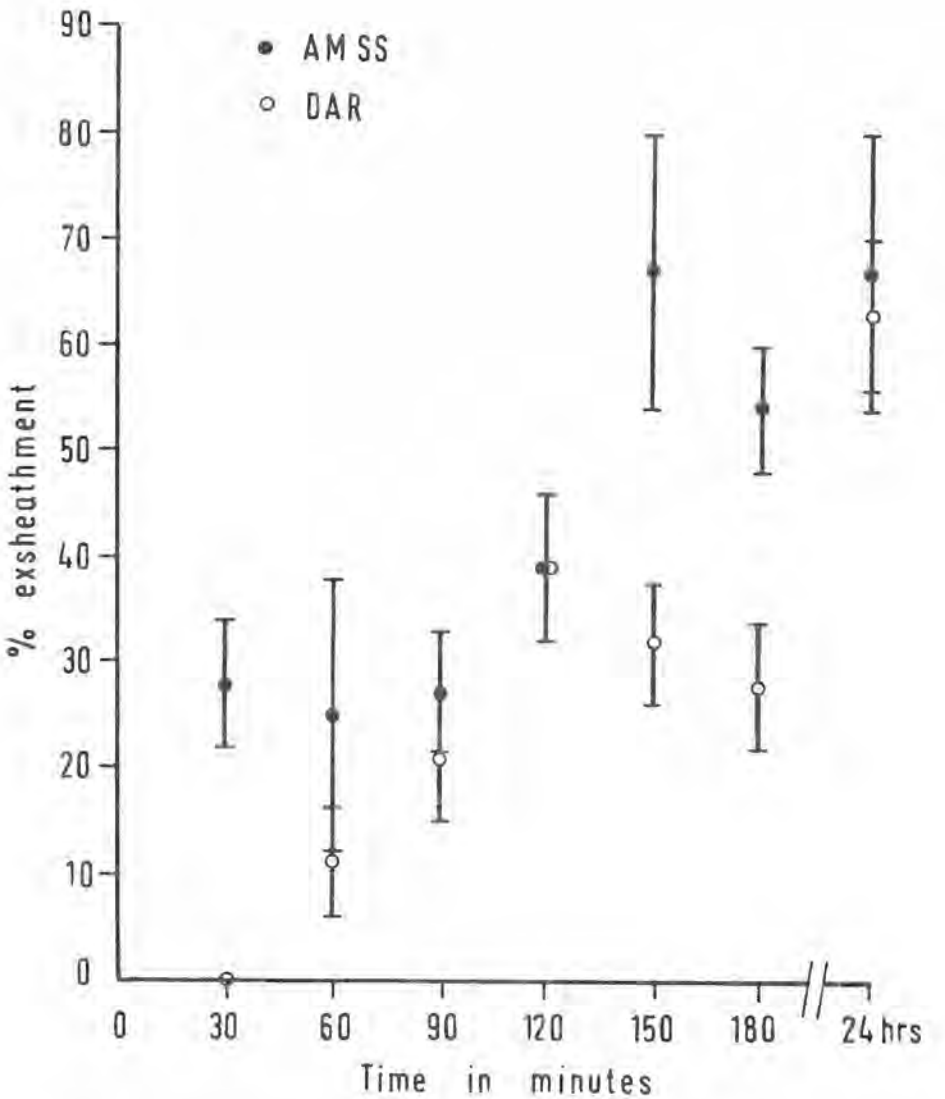


Fig. 15: The Exsheathment of W. bancrofti in C. quinquefasciatus (DAR) and Ae. aegypti SS.

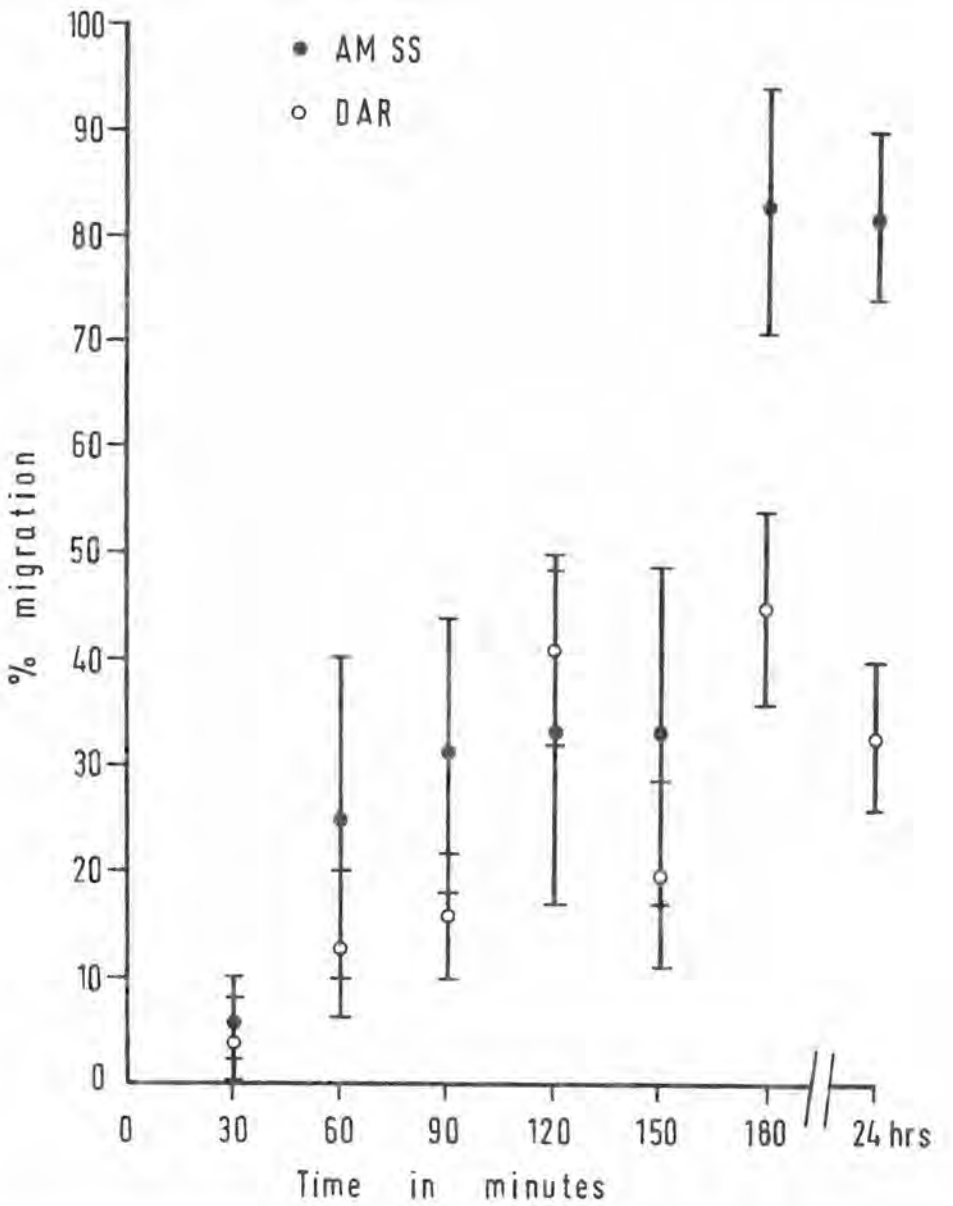


Fig. 16: The Migration of W. bancrofti in C. quinquefasciatus (DAR) and Ae. aegypti SS

9.4.4 Discussion

The variability and lack of any trend with time in the % exsheathment of W. bancrofti in AMSS shown in experiment 1, could be because most of the microfilariae exsheathed within the first 30 minutes and did not exsheath to a great extent there after. The apparent significant difference in the exsheathment of W. bancrofti between DAR and AMSS, should be treated cautiously and is probably due to the small numbers of microfilariae ingested by AMSS, on which % exsheathment was based, compared to those found in DAR. The small numbers of microfilariae ingested by AMSS are probably due to the order in which the mosquitoes were fed. Due to the limited supply of W. bancrofti available, DAR and AMSS were fed on the same feeder unit, DAR feeding first followed by AMSS. The variability in the exsheathment rate could also be due to the inability of a proportion of ingested microfilariae to exsheath, due to some adverse effect on their metabolism, as a result of cryopreservation and thawing.

In experiment 2, exsheathment of W. bancrofti did increase with time in both DAR and AMSS. This trend is similar to that observed for B. pahangi in AMSS, except that in the latter case the exsheathment rate was higher and had reached 100% by 180 minutes post feed (see section 9.1). The lower exsheathment rate of W. bancrofti in the 2 species of mosquito is most likely due to the reduced viability of the microfilariae as a result of cryopreservation. The heterogeneity in the % exsheathment with DAR and AMSS and the significant differences observed between them could be due to a combination of two reasons:-

a) a real difference in the type and threshold of stimulus within DAR and AMSS midguts which facilitate exsheathment of W. bancrofti.

b) the reduced viability of W. bancrofti due to cryopreservation.

Although migration of exsheathed W. bancrofti did take place in DAR and AMSS, the migration rate was not very high, as one would have expected since these 2 mosquitoes are highly susceptible to W. bancrofti. The migration rate is probably lower than expected due to the reduced motility of cryopreserved W. bancrofti. Also the differences observed in the % migration of W. bancrofti in DAR and AMSS in single and mixed infective feeds can be explained as above. In addition, it is possible that some real difference does exist in DAR and AMSS, regarding the rate of migration of W. bancrofti, due to differences in the factor(s) which facilitate migration.

The susceptibility rates in DAR and AMSS to W. bancrofti were much lower than expected. This could be due to the reduced viability of cryopreserved W. bancrofti.

Minjas (1980) obtained high infection rates, ranging from 66% -95% in the DAR stock of C. quinquefasciatus fed on H.E.S. cryopreserved microfilariae W. bancrofti at microfilarial densities of only 3-4/mm^3. These microfilariae had been cryopreserved for a year before use. He recovered an average of 1.4-4.7 larvae per mosquito, whereas in the above experiments both DAR and AMSS had an average of 2 larvae per mosquito. The low susceptibility rates observed in the present experiments are within the range of susceptibility rates (16-54%) obtained when DAR was fed on D.M.S.O. (Di-methyl sulphoxide) cryopreserved microfilariae (Minjas, 1980). In the latter case, the mean number of larvae recovered per mosquito was rarely more than one. The same author obtained high infection rates of THAI, HOLA, and DAR stocks of C. quinquefasciatus fed directly on donors, these being 74.4%, 95.2% and 96.1%. Curtis et al. (1983) in their investigations on the susceptibility of aposymbiotic

and normal strains of C. quinquefasciatus (DAR,THAI) fed some batches of mosquitoes on H.E.S. cryopreserved W. bancrofti and obtained high infectivity rates ranging from 88.3% - 100%. The mean numbers of larvae per infected mosquito ranged from 2-10.5 at microfilarial densities of 46-80/20mm^3. Macdonald and Ramachandran (1965) reported susceptibility rates of the selected susceptible stock of Ae. aegypti to Malayan and Fijian strains of W. bancrofti in the range from 77.6 - 86.9% and 83.6 - 87.5% respectively, showing that they are highly susceptible to W. bancrofti. These findings show clearly that the reduced susceptibilities observed in the present studies were not due to a decrease in the susceptibility of the stocks of DAR and AMSS used to W. bancrofti, but rather due to the reduced infectivity of cryopreserved W. bancrofti to these mosquitoes.

Due to the variability in the rates of exsheathment and migration observed in DAR and AMSS, as a result of using cryopreserved microfilariae W. bancrofti, it is not possible to get a clear picture of the exsheathment and migration patterns of W. bancrofti in the 2 species of mosquitoes, thus making it difficult to compare these two processes in mosquitoes fed on mixed and single infective blood meals. These experiments need to be repeated, feeding the mosquitoes directly on donors or feeding them via membrane feeders on blood taken from donors. By so doing, it will be easier to make such comparisons.

Table 32: Exsheathment of microfilariae W. bancrofti in DAR and AMSS

EXPERIMENT 1

Time after feeding (mins)	Species of mosquito	Nos of W. bancrofti mff		
		Total nos in midgut	Nos exsheathed	% exsheathed ± S.E.
30	DAR	22	8	36 ± 0.08
	AMSS	30	3	10 ± 0.05
60	DAR	21	10	48 ± 0.12
	AMSS	6	2	33 ± 0.15
90	DAR	32	19	59 ± 0.06
	AMSS	9	1	11 ± 0.09
120	DAR	17	10	59 ± 0.08
	AMSS	6	0	0 0
150	DAR	16	6	38 ± 0.09
	AMSS	6	0	0 0
180	DAR	16	10	63 ± 0.10
	AMSS	2	0	0 0
24hrs	DAR	34	9	18 ± 0.07
	AMSS	4	2	33 ± 0.19

Microfilarial density of bloodmeal = 30mff/20mm^3

5 mosquitoes of each species were dissected at each time interval.

Table 33: Exsheathment of microfilariae W. bancrofti in DAR and AMSS

EXPERIMENT 2

Time after feeding (mins)	Species of mosquito	Nos of W. bancrofti mff		
		Total nos in midgut	Nos exsheathed	% exsheathed ± S.E.
30	DAR	29	0	0
	AMSS	32	9	28 ± 0.06
60	DAR	28	3	11 ± 0.05
	AMSS	8	2	25 ± 0.13
90	DAR	34	7	21 ± 0.06
	AMSS	33	9	27 ± 0.06
120	DAR	28	11	39 ± 0.07
	AMSS	28	11	39 ± 0.07
150	DAR	47	15	32 ± 0.06
	AMSS	10	4	67 ± 13
180	DAR	32	9	28 ± 0.06
	AMSS	35	19	54 ± 0.06
24hrs	DAR	27	17	63 ± 0.07
	AMSS	12	8	67 ± 0.13

Microfilarial density of bloodmeal = 62mff/20mm^3

5 mosquitoes of each species were dissected at each time interval.

Table 34: Migration of *W. bancrofti* in DAR and AMSS

Time after feeding (mins)	Species of mosquito	Nos of *W. bancrofti* mff		
		Total nos in midgut	Nos in thorax	% migration ± S.E.
30	DAR	22	1	4 ± 0.04
	AMSS	30	2	6 ± 0.04
60	DAR	21	3	13 ± 0.07
	AMSS	6	2	25 ± 0.15
90	DAR	32	6	16 ± 0.06
	AMSS	9	4	31 ± 0.13
120	DAR	17	12	41 ± 0.09
	AMSS	6	3	33 ± 0.16
150	DAR	16	4	20 ± 0.09
	AMSS	6	3	33 ± 0.16
180	DAR	16	13	45 ± 0.09
	AMSS	2	10	83 ± 0.11
24 hrs	DAR	34	17	33 ± 0.07
	AMSS	4	18	82 ± 0.08

Microfilarial density = 30mff/$20mm^3$

5 mosquitoes were examined at each time interval.

Table 35: Results of feeding Ae. aegypti SS and C. quinquefasciatus (DAR) on W. bancrofti on dissection day 14 post infection.

Expt. Date and numbers	Mosquito			Developing larvae												Nos of larvae per infected mosquito	% larvae infective	Mosquitoes	
	Mff/20mm^3 W. bancrofti	Strain	Nos dissected	Proboscis			Head			Thorax			Abdomen					Nos * infected	% infected
				I	II	III	I	II	III	I	II	III	I	II	III				
13.2.84 (1)	30	DAR	73	0	0	5	0	0	4	0	1	5	0	0	10	2	96	16	30
13.2.84 (1)	30	AMSS	44	0	0	2	0	0	6	0	7	9	0	0	10	3	72	12	27
15.3.84 (2)	62	DAR	118	0	0	14	0	0	19	0	3	6	0	1	8	2	90	29	24
15.3.84 (2)	62	AMSS	27	0	0	0	0	0	2	0	5	1	0	0	2	2	50	6	22

9.5 The Migration and development of exsheathed B. pahangi suspended in serum, fed to C. quinquefasciatus (DAR) and Ae. aegypti SS.

9.5.1 Introduction

The objective of this experiment was to examine the role if any of red blood cells in inhibiting migration of exsheathed B. pahangi to the thorax in refractory C. quinquefasciatus (DAR). It has already been observed that exsheathed B. pahangi in a blood meal, do not migrate from the midguts of DAR mosquitoes. This suggested that there was some factor(s) in the midgut which prevented migration. Possibly, this could be related to the presence of red blood cells, hence this experiment was designed to examine whether exsheathed B. pahangi suspended in serum, migrate to the thorax and develop normally to the infective stage.

9.5.2 Materials and Methods

One week old, unfed C. quinquefasciatus DAR were used. Approximately 50 females were transferred from a large cage into smaller perspex cages. Microfilariae were obtained from infected jirds, then exsheathed using endopeptidase as in previous experiments. Exsheathed microfilariae were resuspended in either human or turkey serum. Counts were made of the number of microfilariae per 20 mm^3 of the serum suspension before setting up the feed. Unfed mosquitoes were removed from the cages, leaving only the fed ones. These were provided with sugar and water until 9-10 days post infection when they were dissected. AMSS females of the same age were used as controls and were fed simultaneously with the DAR mosquitoes.

9.5.3 Results

The mosquitoes of both DAR and AMSS fed with very great difficulty. They did not seem attracted to serum as they are normally to blood, despite starving them for 24-48 hours prior to infection. For this reason, these

feeds had to be repeated several times so as to have a sufficient number of fed mosquitoes. Both DAR and AMSS ingested smaller volumes of serum compared to that of blood.

Results in Table 36 show that by 3 hrs, there were no microfilariae in the midgut of DAR, hence there was no migration to the thorax, whereas in AMSS the % migration to the thorax of microfilariae of B. pahangi at 1, 2, and 3 hours post feed, was 8,23 and 33%.

Data in Table 37 show that none of the DAR females dissected on day 10 post feed were infected. In two mosquitoes in experiments 1 and 2, abnormally developing second stage larvae were found in the thorax. However, AMSS did become infected showing that the exsheathed microfilariae migrated to the thorax and were viable. The susceptibility rates ranged from 71-100%. All the larvae recovered per mosquito were at the 3rd larval stage. The numbers of larvae recovered per mosquito were less than expected. The microfilarial density in the infective serum meals should have given mean recovery rates of larvae per mosquito in the range of 9-15 microfilariae. The mean numbers of larvae per mosquito recovered from AMSS fed on exsheathed B. pahangi suspended in serum were not significantly different from those of AMSS fed on exsheathed B. pahangi suspended in blood, as shown by a paired t test ($P > 0.05$). Some of the DAR and AMSS which ingested serum had fully developed eggs, showing that serum does provide some of the nutrients necessary for egg development. The presence of eggs was a useful indication that serum had been ingested.

9.5.4 Discussion

The lack of migration of exsheathed B. pahangi suspended in serum ingested by DAR mosquitoes shows that the lack of migration of microfilariae in DAR fed on infected blood is not solely due to the clotting

of blood, or the lack of exsheathment of microfilariae as thought to be the case previously. In AMSS, microfilariae succeeded in migrating to the thorax and developing normally. It is possible that the fewer numbers of larvae recovered is due to the smaller volumes of serum ingested and the reduced viability of some of the microfilariae due to the action of endopeptidase. The lack of migration of microfilariae in DAR suggests that within the midgut of DAR, there were other factors which inhibit migration, or it could be due to the lack of some factors which stimulate migration of exsheathed B. pahangi.

Owen (1978) reported that red blood cells appear to play a role in the exsheathment of microfilariae in the midgut of mosquitoes. However, no mention has been made as to whether they affect migration of microfilariae. The results of these experiments suggest that red blood cells play no important role in the migration and subsequent development of B. pahangi, as these processes occurred normally in AMSS. This does not appear to be the case in the development of malarial parasites in mosquitoes. Rosenberg et al (1984), reported that their investigations on the development of P. gallinaceum in Ae. aegypti provided evidence that an erythrocyte substance, released by mosquito digestion, was needed for ookinete invasion of the gut epithelium. Maudlin (1983) reported that certain serum factors were critical in determining whether or not a midgut infection of T. congolense was established and whether such infections matured in Glossina. He observed that the complete removal of serum from cow red cells resulted in very high rates (90%) of T. congolense midgut infections, but very low levels of maturation similarly with pig cells, few flies (2-13%) matured their midgut infections. The removal of all lipids from the serum had the same effect as the complete removal of serum, with only 3% of flies developing mature infections. The above

findings indicate that various components of blood do play a role in certain vector-parasite relationships and it might be worthwhile carrying out further investigations on the role of blood in determining the susceptibility of mosquitoes to filarial parasites.

Table 36: Migration of *B. pahangi* in *C. quinquefasciatus* (DAR) and *Ae. aegypti* SS fed on serum and exsheathed *B. pahangi*.

Time of dissection after feed (hrs)	Mosquito species	Nos mosquitoes examined	Nos (%) of Microfilariae in	
			Thorax	Midgut
1	AMSS	3	3 (8)	40 (92)
	DAR	3	0 (0)	0 (0)
2	AMSS	3	9 (23)	40 (77)
	DAR	3	0 (0)	0 (100)
3	AMSS	3	15 (33)	46 (67)
	DAR	3	0 (0)	0 (0)

Table 37: Showing susceptibility of C. quinquefasciatus DAR and Ae. aegypti SS to exsheathed B. pahangi suspended in serum.

Expt No.	Type of serum used	Species of mosquito	Nos mff per mm^3	Nos of mosquito dissected	Nos of filariae in Proboscis	Head	Thorax	Abdomen	Nos mosquito infected (%)	Mean nos of larvae per mosquito	Nos with eggs (%)
1	Turkey	DAR	5	12	0	0	1*	0	0	0	6 (50)
		AMSS	5	8	5	1	2	0	6 (75)	1	6 (75)
2	Human	DAR	5	26	0	0	1*	0	0	0	12 (46)
		AMSS	5	10	20	8	10	0	9 (90)	3.8	9 (90)
3	Human	DAR	3	32	0	0	0	0	0	0	22 (69)
		AMSS	3	7	15	12	9	5	7 (71)	5.8	6 (38)
4	Human	DAR	4	10	0	0	0	0	0	0	6 (60)
		AMSS	4	29	17	21	18	10	24 (83)	2.3	11 (38)
5	Human	DAR	4	18	0	0	0	0	0	0	9 (50)
		AMSS	4	5	6	0	14	7	5 (100)	5.4	3 (60
6	Human	DAR	10	4	0	0	0	0	0	0	1 (25)
		AMSS	10	0	0	0	0	0	0	0	0

* abnormal second larval stage larvae.

9.6 The development of exsheathed B. pahangi inoculated into the abdominal haemocoel of C. quinquefasciatus (DAR) and Ae. aegypti SS.

9.6.1 Introduction

Experiments reported in section 9.5 showed that there was no migration of microfilariae B. pahangi from the midgut of DAR mosquitoes, even in the absence of red blood cells. This led to the investigation as to whether the abdominal haemocoel had any factors which prevent migration of microfilariae.

9.6.2 Materials and Methods

Microfilariae were obtained from jirds. They were exsheathed in 0.2mg/ml endopeptidase solution by incubation for 30 minutes, then aliquots examined under a Phase Contrast microscopes for exsheathment. Unfed, one week old DAR were divided into two groups. One group was inoculated with 10 $\pm$ 3 microfilariae in the thorax while the other group was inoculated in the abdominal haemocoel. This was done alternately, first inoculating one mosquito with microfilariae in the thorax, then the next in the abdominal haemocoel. Inoculated mosquitoes were transferred individually to small glass vials provided with wet cotton wool and the mosquitoes were maintained on sugar and water till day 10 when they were dissected. Ae. aegypti SS females of the same age were inoculated similarly at the same time and these were used as controls.

Another group of DAR and AMSS mosquitoes were inoculated in the abdomen as described before but they were dissected from 2-7 hours post infection in order to find out whether any of the microfilariae inoculated into the abdomen migrated to the thorax. The state of the microfilariae was also noted.

9.6.3 Results

C. quinquefasciatus DAR inoculated with exsheathed B. pahangi in the thorax and abdominal haemocoel had a high mortality rate which was evident within the first 4 days following inoculation. The high mortality rate was probably due to the traumatic effects of inoculation. The susceptibility rates of mosquitoes inoculated in the thorax and abdominal haemocoel ranged from 0-22% and 0-6% respectively as shown in Tables 39 and 40 .Data in Table 38 show that in DAR inoculated with B. pahangi in the abdomen the percentage of microfilariae found in the thorax rose from 14% - 41% from 2 to 7 hours post inoculation. The microfilariae were found to be active up to 4 hours post inoculation, then they became sluggish or immobile. Of the DAR mosquitoes inoculated with microfilariae in the abdomen and dissected on day 10 post inoculation, only one mosquito was found to have infective larva in the head. A few of the microfilariae inoculated into the thorax developed to the third larval stage.

Results in Tables 41 and 42 show that the infectivity rates of AMSS inoculated in the thorax and abdominal haemocoel ranged from 67-82% and 67-79% respectively. A large proportion of the developing larvae recovered were infective third stage larvae, a few being in the second larval stage. In AMSS inoculated with microfilariae in the abdomen and dissected 2-7 hours post inoculation, the percentage of microfilariae found in the thorax ranged from 33%-55%, as shown in Table 38. Microfilariae were observed to be very active up to 6 hours post inoculation. The mortality rate following inoculation in both the thorax and abdomen was high, mortality being higher in those mosquitoes inoculated in the abdominal haemocoel. X^2 tests showed no significant differences in the susceptibilities of AMSS inoculated with microfilariae B. pahangi in the

thorax and abdomen $P > 0.05$. Paired t tests were used to compare the total and mature larvae rates found in AMSS inoculated in the thorax and abdomen. In experiment 4, there was a significant difference in the total larval rate in both groups of mosquitoes ($P < 0.01$). No significant difference were found in mature larvae ratesof the 2 groups of mosquitoes.

9.6.4 Discussion

These results show that some of the microfilariae inoculated into the abdomen of DAR do migrate to the thorax. However, once they are in the thorax, they do not develop any further. Hence, there are no systemic factors preventing migration in DAR mosquitoes. These results show that the refractory nature of DAR mosquitoes is not only due to a 'gut barrier' but that there are factors in the thorax which play a role in rendering these mosquitoes refractory to B. pahangi. The 'gut barrier' is just a first line of defence against infection and that those microfilariae which do succeed in crossing the gut wall will most likely be killed in the thorax. It appears that the genes that are responsible for refractoriness in DAR manifest their action in more than one part of the mosquito and are not restricted to any one organ.

The susceptibility rates of AMSS are lower than those of membrane fed AMSS, which is usually 100%. The lower rates could be due to damage of microfilariae during inoculation, thus making them less viable or it could be due to loss of some microfilariae during inoculation. Despite the lower susceptibility rate obtained, these results show that exsheathed B. pahangi inoculated into the abdominal haemocoel in AMSS migrate to the thorax and develop normally unlike in DAR. The smaller numbers of larvae recovered in AMSS inoculated in the abdomen in this experiment could be due to one of many reasons such as, loss of some microfilariae during inoculation, damage to microfilariae by the needle during inoculation

or damage to microfilariae caused by the action of endopeptidase.

Results from these experiments suggest that in C. quinquefasciatus (DAR) the gene(s) which control the refractoriness to B. pahangi do not restrict their action to only one area in the mosquito body, rather they manifest their action in the whole mosquito.

Table 38 : Migration of B.pahangi in C. quinquefasciatus (DAR) and Ae. aegypti SS after inoculation into the abdominal haemocoel.

Time of dissection post inoculation (hrs)	Species of mosquito	Nos mosquitoes examined	Nos (%) of microfilariae in	
			Abdomen	Thorax
2-3	DAR	6	114 (86)	19 (14)
	AMSS	6	60 (67)	30 (33)
4-5	DAR	11	154 (62)	95 (38)
	AMSS	7	93 (45)	114 (55)
6-7	DAR	8	96 (59)	66 (41)
	AMSS	7	92 (59)	63 (41)

Table 39: Showing larvae recovered from C. quinquefasciatus (DAR) inoculated with exsheathed B. pahangi in the thorax.

Date of experiment	Experiment number	Nos mosquitoes inoculated	Nos mosquitoes dissected	% mortality before day 10	Nos mff inoculated	Nos with larvae (%)	Proboscis			Head			Thorax			Abdomen			Total larvae per mosquito	Mature larvae per mosquito
							I	II	III	I	II	III	I	II	III	I	II	III		
30.4.84	1	28	11	61	10	0 (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.5.84	2	21	9	57	10	2 (22)	0	0	0	0	0	4	0	0	3	0	0	0	6	6
3.5.84	3	42	20	52	10	0 (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9.5.84	4	56	27	51	10	5 (19)	0	0	3	0	0	5	0	0	0	0	0	2	2	2
15.5.84	5	36	24	33	10	1 (4)	0	0	0	0	0	0	0	2	0	0	0	0	2	0

Table 40 Showing larvae recovered from C. quinquefasciatus (DAR) inoculated with exsheathed B.pahangi in the Abdominal Haemocoel.

Date of experiment	Experiment number	Nos mosquitoes inoculated	Nos mosquitoes dissected	% mortality before day 10	Nos mff inoculated	Nos with larvae (%)	Proboscis			Head			Thorax			Abdomen			Total larvae per mosquito	Mature larvae per mosquito
							I	II	III	I	II	III	I	II	III	I	II	III		
30.4.84	1	28	11	61	10	0 (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.5.84	2	21	4	81	10	0 (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3.5.84	3	42	16	62	10	1 (6)	0	0	0	0	0	1	0	0	0	0	0	0	1	1
9.5.84	4	56	20	64	10	0 (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15.5.84	5	36	14	69	10	0 (0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 41: Showing larvae recovered from Ae. aegypti SS inoculated with exsheathed B. pahanagi in the thorax.

Date of experiment	Experiment number	Nos mosquitoes inoculated	Nos mosquitoes dissected	% mortality before day 10	Nos mff inoculated	Nos with larvae (%)	Proboscis			Head			Thorax			Abdomen			Total larvae per mosquito	Mature larvae per mosquito
							I	II	III	I	II	III	I	II	III	I	II	III		
30.4.84	1	25	10	60	10	7 (70)	0	0	25	0	0	20	0	0	18	0	0	5	9.7	9.7
1.5.84	2	20	12	40	10	8 (67)	0	0	18	0	0	18	0	0	20	0	0	10	9.5	9.5
3.5.84	3	42	22	48	10	18 (82)	0	0	35	0	0	27	0	10	23	0	0	15	6.1	5.5
9.5.84	4	56	30	46	10	24 (80)	0	0	40	0	0	35	0	20	40	0	0	36	7.1	6.2
15.5.84	5	36	18	50	10	14 (78)	0	0	19	0	0	28	0	8	42	0	5	12	8.1	7.2

Table 42: Showing larvae recovered from Ae. aegypti SS inoculated with exsheathed B. pahangi in the Abdominal Haemocoel.

Date of experiment	Experiment Number	Nos mosquitoes inoculated	Nos mosquitoes dissected	% mortality before day 10	Nos mff inoculated	Nos with larvae (%)	Proboscis			Head			Thorax			Abdomen			Total larvae per mosquito	Mature larvae per mosquito
							I	II	III	I	II	III	I	II	III	I	II	III		
30.4.84	1	25	9	64	10	6 (67)	0	0	20	0	0	15	0	0	12	0	0	2	8.2	8.2
1.5.84	2	20	10	50	10	7 (70)	0	0	19	0	0	12	0	8	15	0	0	7	8.7	7.6
3.5.84	3	42	19	55	10	15 (79)	0	0	25	0	0	20	0	9	18	0	0	11	5.5	4.9
9.5.84	4	56	24	57	10	19 (79)	0	0	30	0	0	20	0	11	27	0	0	19	5.6	5.0
15.5.84	5	36	15	58	10	10 (67)	0	0	10	0	0	23	0	7	31	0	0	9	8.0	7.3

10. THE EFFECT OF VARIOUS SUBSTANCES ON THE SUSCEPTIBILITY OF AE AEGYPTI SS AND C. QUINQUEFASCIATUS (DAR) TO B. PAHANGI.

10.1 The effect of Cations on development of B. pahangi in Ae. aegypti SS.

10.1.1 Introduction

It has been shown that calcium, magnesium, manganese and Iron chlorides fed to Ae. aegypti and An. quadrimaculatus inhibited blood digestion to varying degrees (Terzian, 1958). The production of proteolytic enzymes during blood digestion is thought to be involved in the exsheathment and migration of microfilariae in infective blood meals ingested by mosquitoes. In order to investigate if the inhibition of blood digestion interferes with the exsheathment, migration and normal development of microfilariae B. pahangi in Ae. aegypti SS, these mosquitoes were preconditioned with various concentrations of cations prior to offering them infective bloodmeals. They were then examined for exsheathment, migration, and development of microfialriae B. pahangi.

10.1.2 Materials and Methods

Groups of one week old Ae. aegypti SS were fed on 20% sugar solution containing chlorides of calcium and magnesium at a concentration of 0.1M, following the method of Terzian (1958). One batch of AMSS was fed $MgCl_2$ for 48 hours and another batch was fed on $CaCl_2$ for 72 hours before offering them an infective bloodmeal.

Some of the fed females were removed from the cages and transferred to smaller perspex containers. These mosquitoes were maintained on 20% sugar solutions containing $MgCl_2$ and $CaCl_2$ until day 10 when they were dissected. Records were made of the location and stage of development of the larvae recovered. The remaining fed females were dissected 1,3,24, 48 and 72 hours post infection and smear preparations made of the midgut, abdomen and thorax. Five mosquitoes from each batch were dissected

at the required time interval. The slides were then dehaemoglobinized fixed and stained in 5% Giemsa stain, then examined under a compound microscope. As a control, a group of AMSS were fed only on a 20% sugar solution prior to the infective feed, then examined at the time intervals mentioned above.

10.1.3 Results

The bloodmeals of Ae. aegypti SS fed on $CaCl_2$ and $MgCl_2$ prior to the infective feed were bright red, slightly coagulated and surrounded by a well formed peritrophic membrane 3 hours post infection. In the control Ae. aegypti SS, the bloodmeals were dark red. On examination 24 hours post infection, the bloodmeals of AMSS fed on $CaCl_2$ and $MgCl_2$ were dark brown at the periphery adjacent to the peritrophic membrane and had shrunk from it. Bloodmeals of control AMSS were dark brown except for a very small central part which was red. They were well set and had shrunk from the peritrophic membrane. By 48 hours, bloodmeals of Ae. aegypti SS fed on cations were almost all dark brown, except for the central part which was red and they were hard. In AMSS controls the bloodmeals were digested completely and already egg formation had started. By 72 hours post infection, digestion of the bloodmeal was complete in all fed mosquitoes and egg development had started.

Data in Tables 43,44 and 45 show that all the microfilariae found in the midguts of AMSS fed on Cations and in the control AMSS had exsheathed at all the time intervals. There was an increase in the % migration to the thorax, with time, in all the 3 groups of AMSS. There were no significant differences in the exsheathment and migration of B. pahangi in AMSS fed Cations and in Control AMSS.

Results in Table 46 show that more developing larvae were found in control AMSS than in AMSS fed on $CaCl_2$ and $MgCl_2$. Control AMSS

had an average of 9.3 larvae per infected mosquito, whereas AMSS fed $CaCl_2$ and $MgCl_2$ had an average of 5.6 and 4.1 larvae respectively. All the larvae found in AMSS controls were in the infective 3rd larval stage. AMSS fed on cations had a few first and second larval stages, a greater proportion of the larvae being in the third larval stage.

In all three batches of mosquitoes, the numbers of microfilariae ingested was less than expected, considering the microfilarial density in the infecting feed was $7/mm^3$.

10.1.4 Discussion

The observations on the state of the bloodmeal in the midguts of AMSS fed on Cations and in control AMSS, suggest that the process of blood digestion was slower in the former mosquitoes then in control AMSS. This finding agrees with that of Terzian (1958) and Terzian and Stahler (1964) who found that cations inhibited blood digestion in mosquitoes in varying degrees, by interfering with the normal production of proteolytic enzymes. The slowing down of blood digestion did not interfere with the normal exsheathment and migration of microfilariae B. pahangi in AMSS fed $MgCl_2$ and $CaCl_2$ prior to and after the infective bloodmeal.

However, the fewer numbers of larvae found in AMSS fed on cations on day 10 post infection, may be due to adverse effects of these cations on the development of microfilariae after migration to the thorax. In addition, the presence of first and second larval stages in these mosquitoes suggests that the microfilariae develop at a slower rate than those in control AMSS. It is likely that the levels of Ca^{2+} and Mg^{2+} ions present in the experimental AMSS are not physiologically suited for the normal development of larvae in these mosquitoes, and that the effect was enhanced by the antibiotic tetracycline. Further experiments by Terzian and Stahler (1964), in which a wider variety of cations and antibiotics were tested,

either alone or in combination in the mosquito diet of both Ae. aegypti and An. quadrimaculatus gave similar results for the effects of cations. However, antibiotics acted in a more complicated manner and produced a variety of effects. Conclusions which can be drawn from these investigations are that cations do not interfere with exsheathment and migration of B. pahangi microfilariae, but they do inhibit blood digestion and decrease the rate of larval development in the thorax of Ae. aegypti SS.

Table 43: Exsheathment and migration of B. pahangi in Ae. aegypti SS fed on Calcium Chloride.

Time of dissection post feed (hrs)	Nos of mosquitoes examined	Mff/mm^3	Nos of Microfilariae		Nos larvae in thorax	% migration
			Total Nos in midgut	Nos exsheathed (%)		
1	5	7	6	6 (100)	18	75
3	5	7	27	27 (100)	12	31
24	5	7	3	3 (100)	9	75
48	5	7	0	0	6	100
72	5	7	0	0	2	100

Table 44 : Exsheathment and migration of B. pahangi in Ae. aegypti fed on Magnesium Chloride.

Time of dissection post feed (hrs)	Nos of mosquitoes examined	Mff/mm^3	Nos of microfilariae		Nos larvae in thorax	% migration
			Total nos in midgut	Nos exsheathed (%)		
1	5	7	28	28 (100)	5	15
3	5	7	25	25 (100)	36	59
24	5	7	3	3 (100)	24	89
48	5	7	1	1 (100)	10	90

Table 45: Exsheathment and migration of B. pahangi in Ae. aegypti fed on 20% Sugar Solution.

Time of dissection post feed (hrs)	Nos of mosquitoes examined	Mff/mm^3	Nos of microfilariae		Nos larvae in thorax	% migration
			Total nos in midgut	Nos exsheathed (%)		
1	5	7	22	22 (100)	16	42
3	5	7	14	14 (100)	20	59
24	5	7	2	2 (100)	24	92
48	5	7	0	0	14	100

Table 46. Susceptibility of Ae. aegypti SS to B. pahangi after being fed on Sugar, Calcium Chloride and Magnesium Chloride, and dissected day 10 post infection.

Treatment of AMSS	Mff/mm^3	Nos of mosquitoes dissected	Nos of Developing larvae in												Mean Nos. larvae per mosquito	% susceptible
			Proboscis			Head			Thorax			Abdomen				
			I	II	III	I	II	III	I	II	III	I	II	III		
Sugar	7	20	0	0	97	0	0	49	0	0	26	0	0	13	9.3	100
$CaCl_2$	7	20	0	0	29	0	0	31	0	6	33	0	0	13	5.6	100
$MgCl_2$	7	19	0	0	22	0	0	16	2	9	15	0	0	14	4.1	100

10.2 The effect of sulphadiazine, penicillin, ascorbic acid and whole body extracts on the susceptibility of Ae. aegypti SS and C. quinquefasciatus (DAR) to B. pahangi.

10.2.1 Introduction

This experiment was designed to determine if refractoriness of C. quinquefasciatus (DAR) to B. pahangi might be due to the absence of a metabolite or to the prevailing physiological state of these mosquitoes. Hence various substances were administered to mosquitoes with the sugar meal, prior to and after an infective bloodmeal. The purpose was to provide the missing component or to alter the chemical composition from that occurring in the natural state. Earlier experiments by Terzian et al. (1952, 1953) and Terzian and Stahler (1960) determined that alterations in the pattern of infection of Ae. aegypti by P. gallinaceum produced by treatment of mosquitoes with antibiotics, vitamins and a variety of other substances, were a reflection of an interference with the innate immunity mechanism of the host, thereby affecting the inherent physiological balance of the host parasite relationship equilibrium. Specific concentrations of the substances administered were responsible for the changes observed in the susceptibility of Ae. aegypti to P. gallinaceum.

10.2.2 Materials and Methods

The substances selected for evaluation of their effect on the innate immunity of DAR to infection with B. pahangi and the dosages administered were based on the work of Terzian et al. (1952, 1953). Sulphadiazine, Penicillin and ascorbic acid were used. These compounds were added in various concentrations to a 10% sugar solution. Concentrations of sulphadiazine used were 0.01, 0.05 and 0.1%. Penicillin concentrations were 100 units, 500, and 1000 units/ml, and ascorbic acid

concentrations of 0.05, 0.1%. Mosquitoes of the same age derived from the same larval colony were used. These were divided into groups of about 50 mosquitoes in perspex cylind rical cages plus a control group. The sugar and drug solutions were made available to the mosquitoes 24 hours after emergence and maintained on these drugs for 5 days. They were then offered an infective bloodmeal and allowed to feed over a period of 1-3 hours. Following the bloodmeal unfed mosquitoes were removed from the cylinders. The drug was administered for one week after the infective feed. Mosquitoes were dissected on days 10 - 13 following the infective bloodmeal and scored for susceptibility.

Whole body extracts of Ae. aegypti SS and C. quinquefasciatus (DAR) were prepared using the method of Weathersby et al. (1971). Several hundred one week old mosquitoes of Ae. aegypti SS and DAR were anaesthetized in the cold at 4°C. They were weighed and ground up with a mortar and pestle at a ratio of 0.4cc of HBSS per 0.1g of mosquitoes. This solution was ground in a homogenizer then the material was centrifuged at 3,000g and frozen and stored at -20°C until required for use.

Newly emerged AMSS and DAR were fed a mixture of crude whole body extract and sugar of DAR and AMSS respectively for a week prior to the infective bloodmeal. Control groups of mosquitoes were fed on sugar only. Mosquitoes were maintained on the whole body extract and sugar mixture for 3 days after the feed, then fed on sugar only until day 10 when they were dissected.

10.2.3 Results

Mosquitoes maintained on sulphadiazine, penicillin and ascorbic acid prior to the infective feed, did not feed very readily on blood and had to be left for over 3 hours before a sufficient number fed.

Data in Table 47 show that all the C. quinquefasciatus DAR mosquitoes dissected on day 10 post infection remained refractory to B. pahangi. Results in Table 48 show that in Ae. aegypti SS fed crude whole body extract of DAR, microfilariae B. pahangi developed normally. There was an average of 4 larvae per infected mosquito. Control AMSS had 5 larvae per infected mosquito. There was no significant difference in the development of B. pahangi microfilariae in these 2 groups of AMSS. In DAR mosquitoes, no developing larvae were found and the mosquitoes remained refractory to B. pahangi. Control DAR were also found to be refractory to B. pahangi.

10.2.4 Discussion

The above results show that the innate insusceptibility of C. quinquefasciatus (DAR) was not altered by administering various concentrations of sulphadiazine, penicillin, and ascorbic acid. The substances did not alter the refractory state existing within the mosquitoes nor provide any missing component which might enhance the development of microfilariae B. pahangi.

Crude whole body extracts of DAR and AMSS fed to AMSS and DAR respectively also had no effect on the susceptibility of AMSS to B. pahangi, or the refractoriness of DAR to B. pahangi. It is possible that with the crude whole body extracts used, any active substances which are capable of producing an effect, would have been degraded either before or following ingestion. Weathersby et al. (1971) fed extracts from Culex pipiens pipiens refractory to P. gallinaceum to Ae. aegypti susceptible to P. gallinaceum and observed a significant reduction in oocyst counts. However, extracts from susceptible Ae. aegypti also produced significant reductions, making it difficult to draw any conclusions from these results.

The results obtained in these experiments differ from those of Terzian et al. (1952, 1953) and Terzian and Stahler (1960) who succeeded in increasing the innate susceptibility of Ae. aegypti and An. quadrimaculatus to P. gallinaceum by treating them with sulphonamides, antibiotics, vitamins and a variety of other substances at specific concentrations. However, they were unable to determine how this change was effected physiologically, leading to the alteration of the innate immunity mechanism of the host. The differences in the results could be due to the different mechanisms which control the susceptibilities of mosquitoes to microfilariae and malarial parasites.

Sauerman and Nayar (in press) observed no changes in the refractoriness of Ae. aegypti (Vero beach strain) to D. immitis after treating them with a variety of substances. They observed that preconditioning with certain inorganic ions produced gross distension of Malphigian tubule cells and interpreted this as a probable alteration of the physiologic state which was not associated with a change in the pattern of refractoriness.

Several other workers have succeeded in altering the innate immunity of invertebrate systems to infection by various parasites by treatment with various substances. Brewer and Vinson (1971) used phenylthiourea and reduced glutathione to interfere with melanization of challenge material in Heliothis zea larvae. They concluded that the invertebrate immune system might at least in part be a function of the phenoloxidase pathway. By treating Ae. aegypti larvae with phenylthiourea, Beresky and Hall (1977) increased the survival of larvae parasitised by the nematode Neoaplectana carpocapsae; they thought this was due to the prevention of the production of toxic intermediates such as quinones in the synthesis of melanin.

A possible explanation for the difference in the results cited in this section with regard to mosquito extracts and those of Weathersby *et al.* (1971), is that in the latter case, development of the parasite is sensitive to the presence and/or concentration of a substance which is stable under the system of extraction and feeding. This substance need not be the one involved in the facet of susceptibility/ refractoriness controlled by the genetic factors studied by others (see Kilama and Craig, 1969). However in the experiments reported in this section, there is no evidence of a substance in DAR extracts which is stable under the conditions of extraction and feeding which is able to retard or otherwise interfere with development of *B. pahangi* in *Ae. aegypti* SS. This may be because there is no inhibitory substance or that it is present at too low a concentration or that it is unstable during extraction or broken down on ingestion by the mosquitoes. The mechanism by which genes controlling susceptibility of mosquitoes to malaria and filarial parasites act may be different, and so far, success in altering the susceptibility of certain mosquitoes species to filarial parasites has only been achieved by selection for susceptibility or refractoriness in these mosquitoes.

Table 47: Susceptibility of C. quinquefasciatus (DAR) infected with B. pahangi after treatment with Sulphadiazine, Penicillin and Ascorbic Acid at various concentrations.

Concentration of substance used	Nos mosquito dissected	mff/mm^3	Nos of filariae in				Nos mosquitoes susceptible (%)
			Proboscis	Head	Thorax	Abdomen	
Sulphadiazine							
0.01%	38	4	1	0	0	1*	1 (2.6)
0.05%	34	4	0	0	0	0	0
0.1%	44	4	0	0	0	1	1 (2.2)
Control	33	4	0	0	0	0	0
Penicillin							
100 units	21	5	0	0	0	0	0
500 units	30	5	1	0	0	0	1 (3)
1000 units	30	5	0	0	0	0	0
Control	32	5	0	0	0	0	0
Ascorbic Acid							
0.05%	30	5	0	0	0	0	0
0.1%	25	5	0	0	0	0	0
Control	27	5	0	0	0	0	0

* Abnormal developing larvae.

Table 48: Susceptibility of Ae. aegypti SS and C. quinquefasciatus (DAR) fed on whole body extracts of (DAR) and (AMSS) then infected with B. pahangi.

Mosquito strain	Whole body extract used	Nos mosquitoes dissected	Nos infected	Mff/ mm^3	Nos of filariae in Proboscis	Head	Thorax	Abdomen	Mean Nos of larvae ± S.D.	% susceptible
AMSS	DAR	20	16	3	6	8	32	11	4 ± 1.6	80
CONTROL		30	30	3	25	47	58	20	5 ± 0.96	100
DAR	AMSS	42	0	8	0	0	0	0	0	0
CONTROL		24	0	8	0	0	0	0	0	0

11. STUDIES ON THE IN VITRO EXSHEATHMENT OF B. PAHANGI

11.1 The Effect of abdomen homogenate of Ae. aegypti SS, An. stephensi and C. quinquefasciatus (DAR) on endopeptidase induced in vitro exsheathment of B. pahangi.

11.1.1 Introduction

Results in section 9.1 showed that exsheathment of B. pahangi microfilariae in C. quinquefasciatus (DAR) and in An. stephensi was low compared to that in Ae. aegypti SS in which there was 100% exsheathment 24 hours post feeding. These results indicated that certain factors in the midgut of C. quinquefasciatus (DAR) and An. stephensi inhibited exsheathment. Hence this experiment was designed to detect whether these inhibitory factors in C. quinquefasciatus (DAR) and An. stephensi could prevent endopeptidase induced exsheathment of B. pahangi. Devaney and Howells (1979) reported that endopeptidase could be used for the in vitro exsheathment of B. pahangi for routine laboratory use. Ae. aegypti SS were used as controls, since they do not inhibit exsheathment of microfilariae B.pahangi.

11.2.1 Materials and Methods

(i) The in vitro exsheathment of B. pahangi

Preliminary experiments were performed to establish the concentration of endopeptidase to be used for exsheathment. Devaney and Howells (1979) found that the concentration of endopeptidase that brings about maximum exsheathment in the shortest possible time was 0.2mg/ml, 100% exsheathment occurring in 30 minutes. In order to observe if abdomen homogenates of C. quinquefasciatus (DAR) and An. stephensi had any inhibitory effects on in vitro exsheathment of B. pahangi, it was desirable to prolong the rate of exsheathment, to allow enough time for these homogenates to act. A concentration of 0.025 mg/ml endopeptidase

was chosen arbitrarily, and this was tested to see at what rate exsheathment of microfilariae occurred. Endopeptidase was dissolved in H.B.B.S. (Hanks Balanced Salt Solution) and then added to the microfilariae obtained from the peritoneum of infected jirds. Aliquots of the HBSS plus microfilariae were examined at 30 minute intervals after the addition of endopeptidase. At least 100-200 microfilariae were examined for exsheathment under a phase contrast microscope. This experiment and the following experiments outlined below were repeated 3 to 4 times in order to allow for between experiment variability.

(ii) Abdomen homogenates made using varying abdominal wet weight.

50 abdomens of unfed, one week old C. quinquefasciatus (DAR) An. stephensi and Ae. aegypti SS were homogenized in 1ml HBSS until the homogenate was smooth. Microfilariae of B. pahangi were obtained from infected jirds. In order to get rid of the debris and cells in the peritoneal washings, this was centrifuged at 600g for 5 minutes, the supernatant discarded and the white thin layer of microfilariae on top of the cells pipetted off and resuspended in HBSS. 1ml of abdomen homogenate was added to 1ml of a 0.05 mg/ml solution of endopeptidase and sheathed B. pahangi microfilariae in a watch glass. This gave a final concentration of 0.025mg/ml endopeptidase and 25 abdomens/ml. Controls were set up similarly, but with the omission of abdomen homogenate. Aliquots of the suspension were examined at 30 minute intervals for exsheathment. At least 100 microfilariae were examined each time.

(iii) Abdomen homogenate made using equal abdominal wet weight.

Abdomens of C. quinquefasciatus (DAR), An. stephensi and Ae. aegypti of equal wet weight were homogenized in 1ml of HBSS as described previously. Due to the variability in the sizes of the 3 species

of mosquitoes the numbers of abdomens used to make homogenate varied and the consistency of the homogenate varied as well. The homogenate was added to unexsheathed B. pahangi plus endopeptidase as described previously. Controls were set up as above and exsheathment examined as described above.

(iv) Heat inactivated abdomen homogenate made using equal wet weight of abdomens.

Abdominal homogenates of C. quinquefasciatus DAR, An.stephensi and Ae. aegypti SS were prepared as above. Homogenates were then transferred into a glass test-tube which was held in a beaker of boiling water for 5 minutes then allowed to cool before being added to the unexsheathed B. pahangi plus endopeptidase. Exsheathment of microfilariae was determined as indicated above.

(v) Preparation of abdomen homogenate supernatant

Abdomen homogenates of equal wet weight of the 3 species of mosquitoes were prepared as before, then centrifuged at 3,000g for 7 minutes. The supernatant was removed, transferred to a test tube and left to stand in a beaker of ice until the experiment was due to start. This was then added to unexsheathed microfilariae plus endopeptidase as above and the exsheathment rate determined as above.

(vi) Determination of pH

Universal indicator was used to determine pH of the various abdomen homogenate preparations used, by placing a strip of it into the homogenate for a few minutes until the colour changed.

11.1.3 Results

Data in Table 49 show percentage exsheathment of B. pahangi in 2 experiments at an endopeptidase concentration of 0.025mg/ml. There was 100% exsheathment of the microfilariae by 3 hours after incubation. Hence this concentration of endopeptidase was suitable for use in subsequent experiments because it prolonged 100% exsheathment of microfilariae from 30 minutes to 3 hours. Results in Table 50 and Figs. 17, 18 and 19 show % exsheathment of B. pahangi in 3 experiments after 240 minutes in the presence of abdomen homogenates of the 3 species of mosquitoes made using different abdominal wet weights. Exsheathment when Ae. aegypti SS abdomen homogenate was used, reached 100% by 180 minutes post incubation. In the control, 100% exsheathment was observed by 30 minutes post incubation. 53% exsheathment was the highest rate observed after 240 minutes when An. stephensi homogenate was used. Exsheathment was lowest when C. quinquefasciatus (DAR) homogenate was used, the highest rate observed after 240 minutes being 32%.

X^2 tests showed no significant differences in % exsheathment of B. pahangi in experiments 1 and 2 in the presence of C. quinquefasciatus (DAR) and An. stephensi abdomen homogenate ($P > 0.05$), but the differences were significant in experiment 3. There were significant differences in % exsheathment in all 3 experiments between Ae. aegypti SS abdomen homogenate and C. quinquefasciatus (DAR) and An. stephensi homogenate ($P < 0.001$) % exsheathment being much higher in the presence of Ae. aegypti SS abdomen homogenate.

The differences in the rate of exsheathment led to the determination of wet weight of equal numbers of abdomens of the 3 mosquito species. Results in Table 51 show that although the numbers of abdomens weighed were equal, those of C. quinquefasciatus (DAR) were the heaviest,

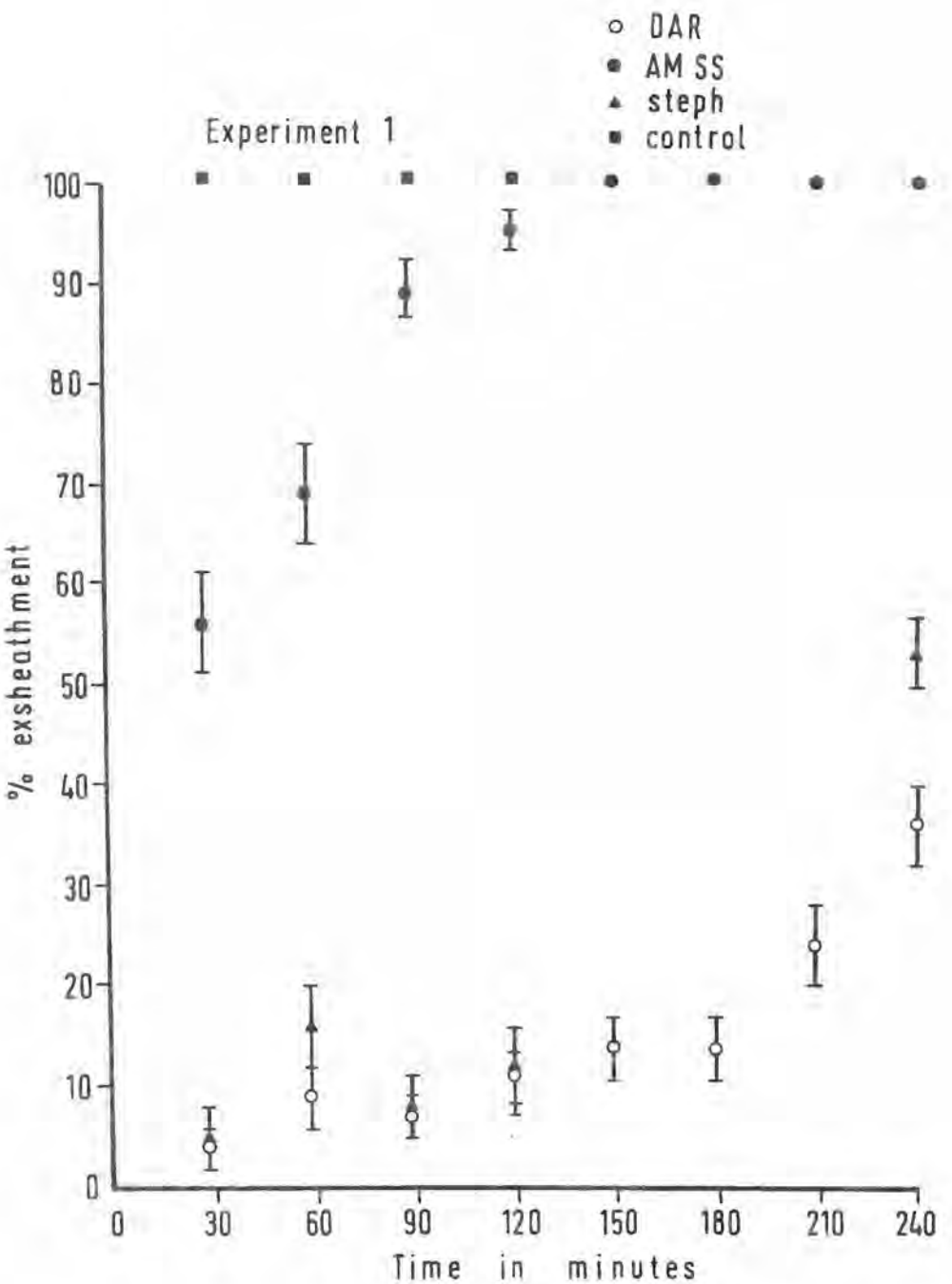


Fig. 17: The Exsheathment of B. pahangi in the presence of abdomen homogenates of C. quinquefasciatus (DAR), Ae. aegypti SS (AMSS) and An. stephensi (Steph) of varying wet weight.

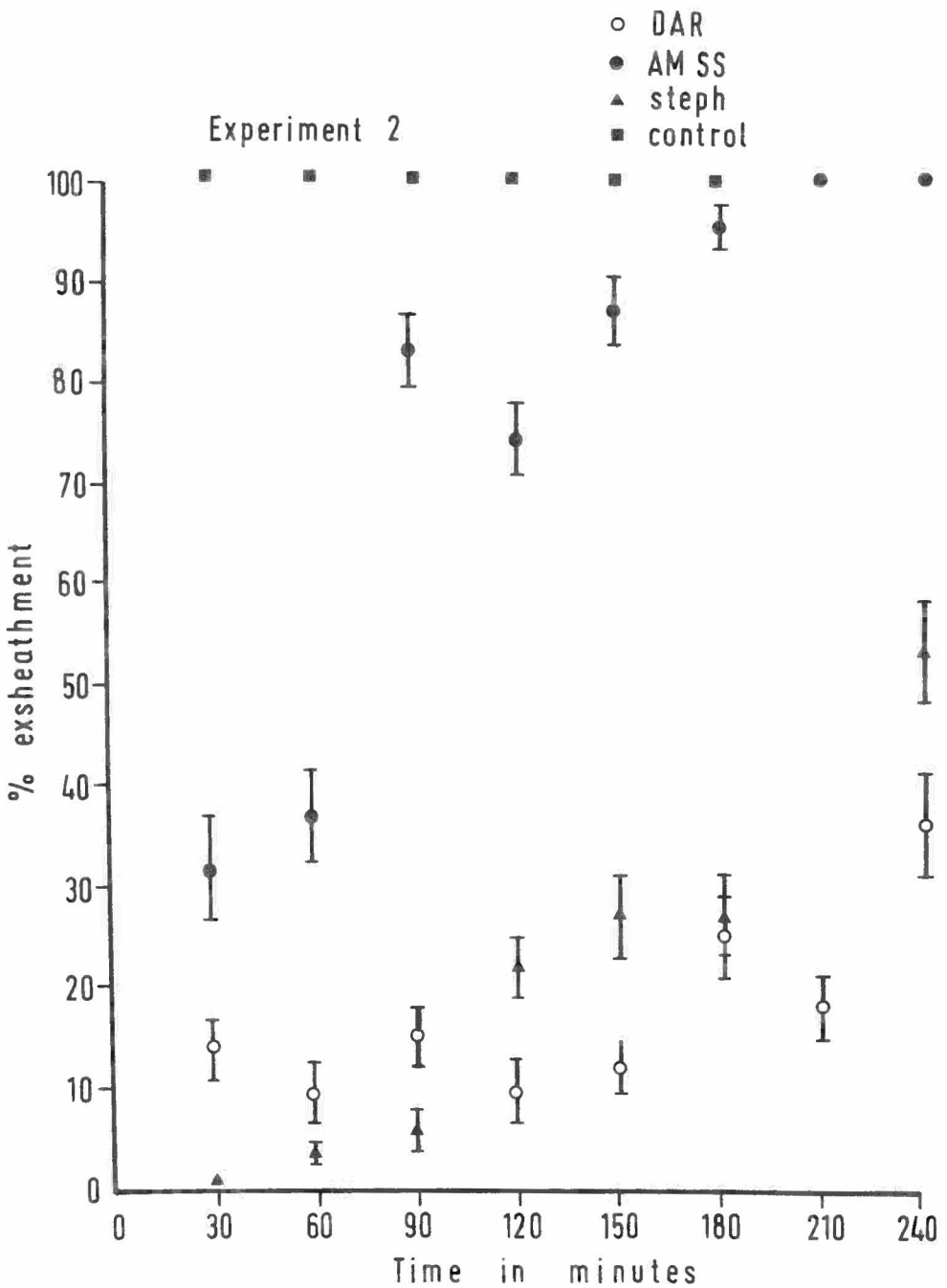


Fig. 18: The Exsheathment of B. pahangi in the presence of abdomen homogenate of C. quinquefasciatus (DAR), Ae. aegypti SS (AMSS) and An. stephensi (Steph) of varying wet weight.

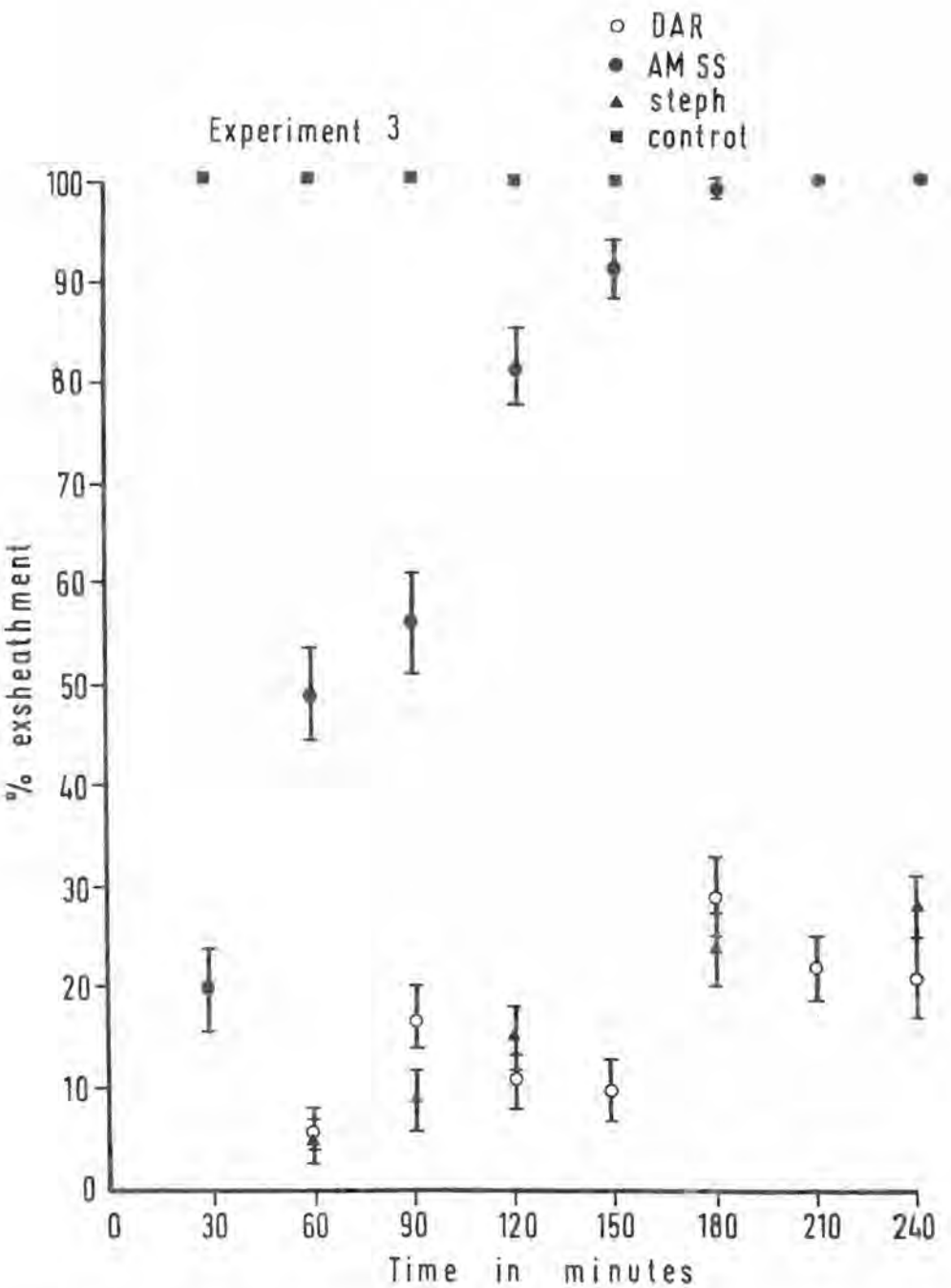


Fig 19: The Exsheathment of B. pahangi in the presence of abdomen homogenates of C. quinquefasciatus (DAR), Ae. aegypti SS (AMSS) and An. stephensi (Steph) of varying wet weight.

followed by Ae. aegypti SS and An. stephensi respectively, suggesting that the abdomen homogenates contained different amounts of protein. The experiment was therefore repeated using abdomen homogenates of equal wet weight, in order to eliminate this as a possible source of differences in the exsheathment rate.

Data in Table 52 and Figs. 20, 21 and 22 show that despite using equal wet weight of abdomens in preparing the 3 homogenates, the rate of exsheathment was highest in Ae. aegypti SS homogenate. However, in this set of experiments, the rate of exsheathment in the presence of Ae. aegypti SS abdomen homogenate was slower than in the previous experiments. The % exsheathment after 240 minutes in the 3 replicate experiments was 53%, 65% and 94% respectively. X^2 tests showed these differences to be highly significant ($P < 0.001$).

The % exsheathment in the presence of C. quinquefasciatus (DAR) and An. stephensi homogenates were not significantly different from the previous results, but X^2 tests showed significant differences ($P < 0.001$), between % exsheathment in the presenece of Ae. aegypti SS homogenates and those of C. quinquefasciatus (DAR) and An. stephensi. In the case of An. stephensi, maximum % exsheathment achieved after 240 minutes was 45% whilst in C. quinquefasciatus (DAR), this was 30%. In all the control experiments, there was 100% exsheathment after 30 minutes.

Results in Table 53 show % exsheathment of B. pahangi when heat inactivated abdomen homogenates were used. % exsheathment achieved was very high, this being 100% in Ae. aegypti after 180 minutes. In the presence of C. quinquefasciatus (DAR) and An. stephensi abdomen homogenates, the % exsheathment was 100% and 70% and 100% and 97% respectively. There were no significant differences in % exsheathment between Ae. aegypti SS, C. quinquefasciatus (DAR) and An. stephensi.

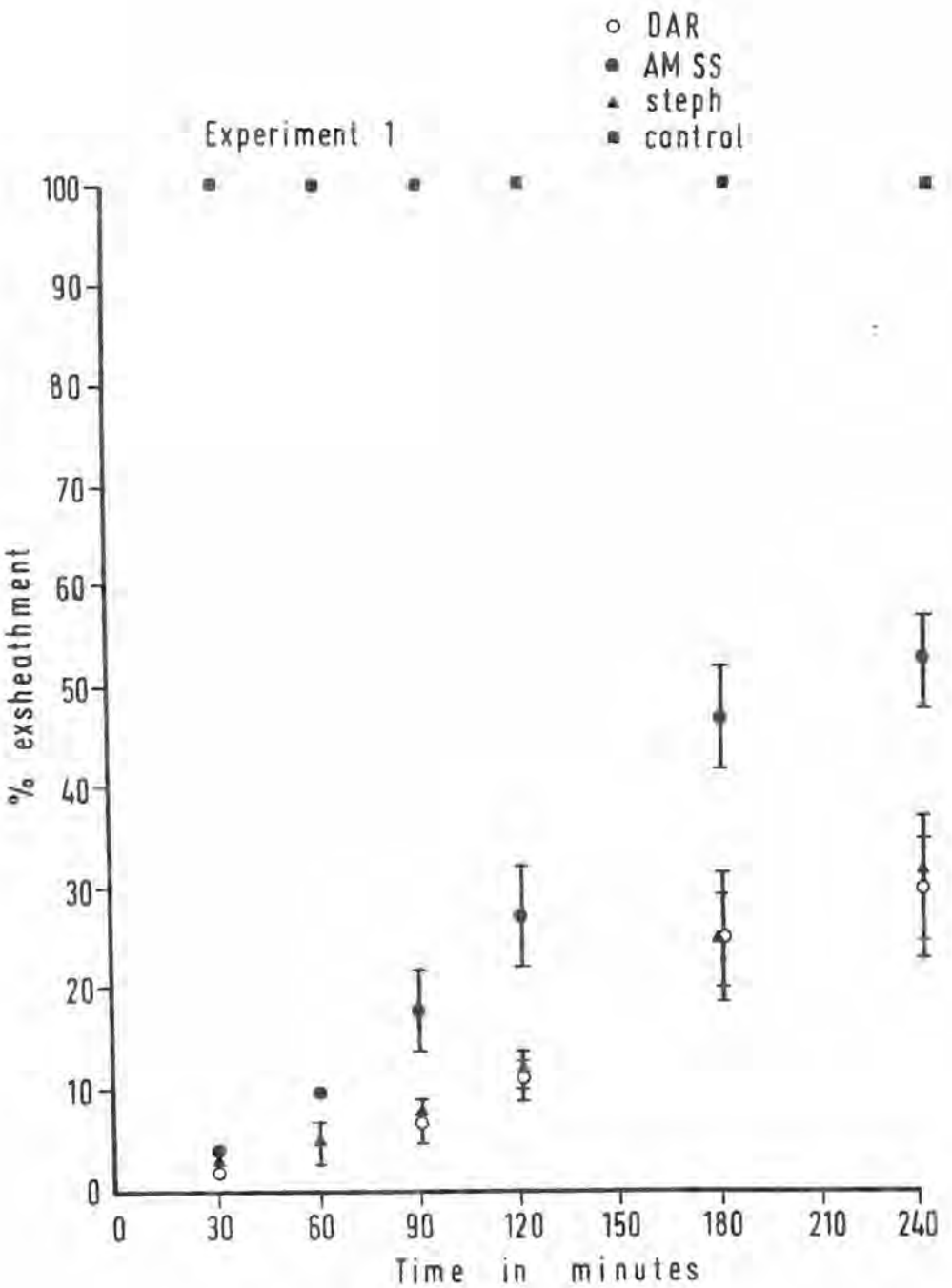


Fig. 20: The Exsheathment of B. pahangi in the presence of abdomen homogenates of C. quinquefasciatus (DAR), Ae. aegypti SS (AMSS) and An. stephensi (Steph) of equal wet weight.

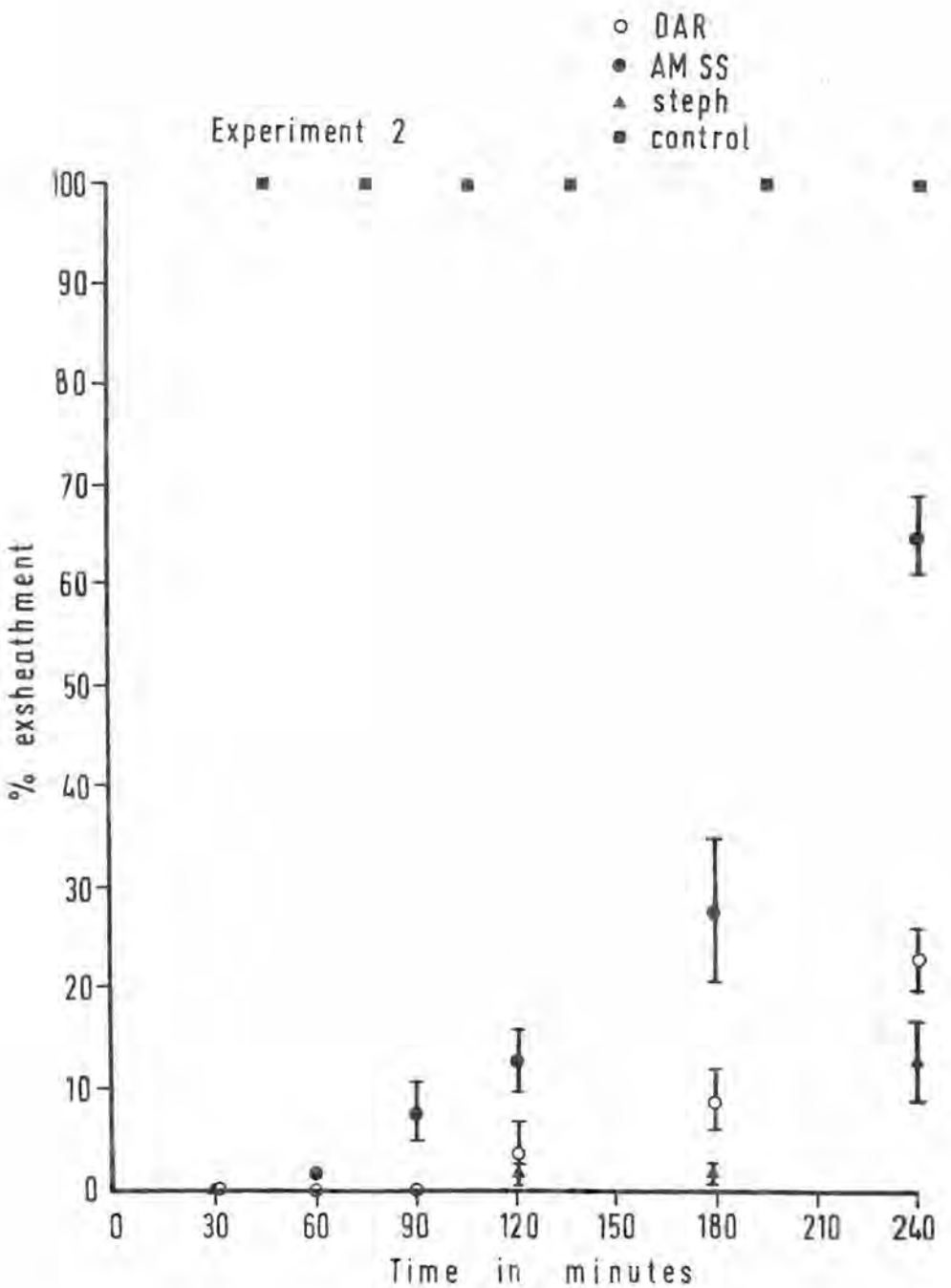


Fig. 21: The Exsheathment of B. pahangi in the presence of abdomen homogenates of C. quinquefasciatus (DAR), Ae. aegypti SS (AMSS) and An. stephensi (Steph) of equal wet weight.

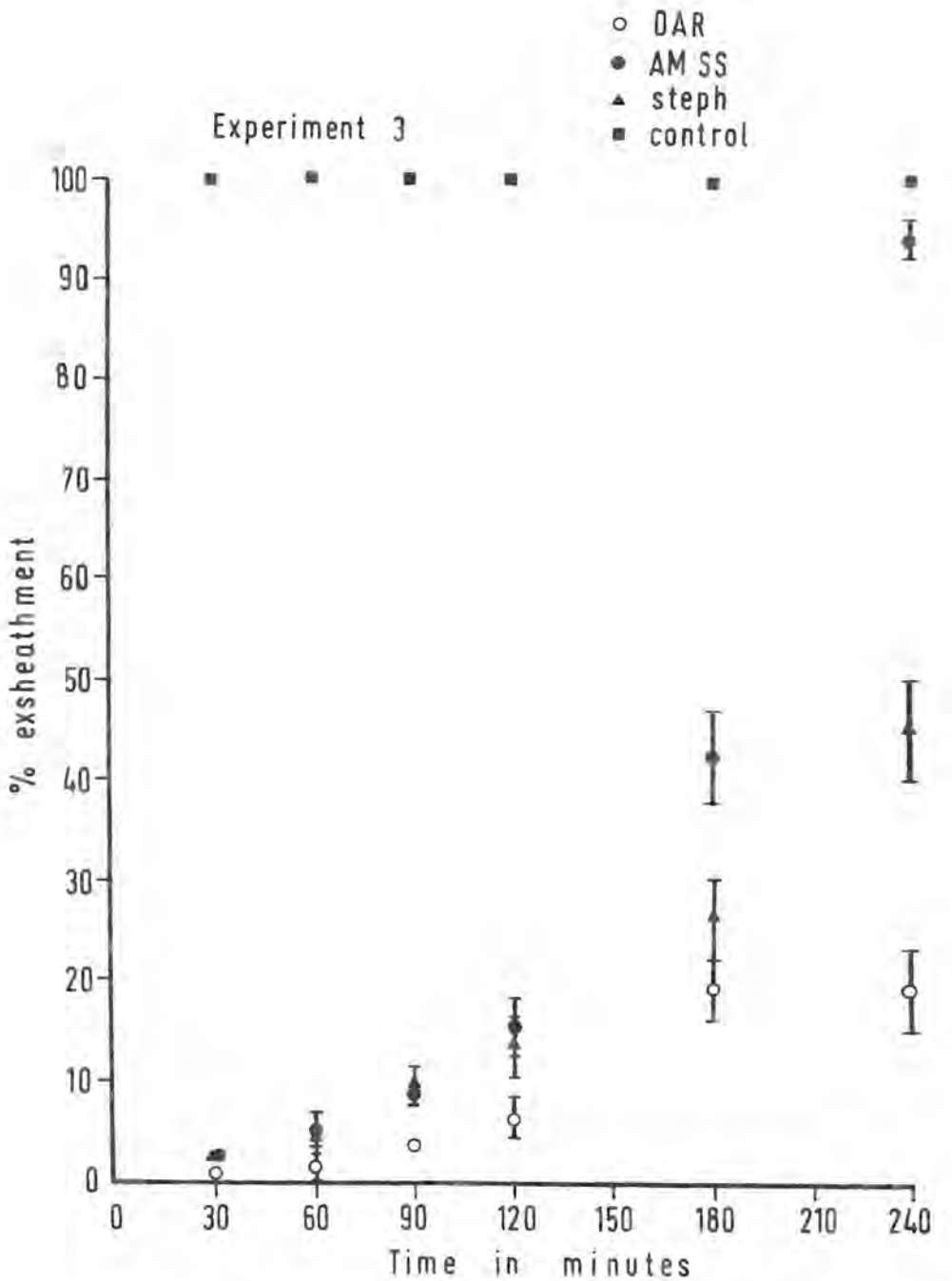


Fig. 22: The Exsheathment of B. pahangi in the presence of abdomen homogenates of C. quinquefasciatus (DAR), Ae. aegypti SS (AMSS) and An. stephensi (Steph) of equal wet weight.

During the heating of the homogenates, it was noticed that the protein material clumped into whitish particles, which precipitated to the bottom of the test-tube, leaving a clear suspension, unlike the whole abdomen homogenate which was thick.

Results from experiments in which abdomen homogenate supernatant was used are shown in Table 54. % exsheathment in the presence of Ae. aegypti SS homogenate ranged from 68-94% in 4 experiments after 180 minutes incubation. Exsheathment in the presence of An. stephensi supernatant varied from 86-98% after 180 minutes incubation. X^2 tests showed no significant differences in % exsheathment in the first 3 experiments. The difference in the fourth experiment was significant ($P < 0.05$). % exsheathment was lower in the presence of C. quinquefasciatus homogenate supernatant, this varying from 29-43% in the 4 experiments and these exsheathment rates were shown to be significantly different from those of exsheathment in Ae. aegypti SS and An. stephensi suspension ($X^2_{(3)} = P < 0.05$).

The pH of abdomen homogenate of C. quinquefasciatus (DAR), Ae. aegypti SS and An. stephensi were all found to be the same, this being pH 7.

11.1.4 Discussion

The low exsheathment rates of B. pahangi observed in the presence of C. quinquefasciatus (DAR) and An. stephensi abdomen homogenates from equal numbers of abdomens compared to the 100% exsheathment in the presence of Ae. aegypti homogenate, could have been due to the presence of inhibitory substances present in C. quinquefasciatus(DAR) and An. stephensi abdomen homogenates, or it is possible that endopeptidase activity was inhibited by protein material in the homogenate binding to it, thus decreasing its activity on the microfilariae.

A hypothesis was put forward that differences in protein content in the homogenates was the cause of the significant differences observed in the exsheathment rate of B. pahangi. This led to the determination of the wet weights of the abdomens of the 3 species of mosquitoes. C. quinquefasciatus (DAR) was found to weigh twice as much as An. stephensi and almost twice as much as Ae. aegypti, the latter two having almost equal wet weight. This suggested that the crude abdomen homogenates of these 3 mosquitoes had unequal protein content. However, despite using equal wet weight of abdomens in preparing homogenates, the results followed a pattern similar to that obtained previously, thus disproving the hypothesis put forward, regarding the role of protein content. The cause of the lower exsheathment in the presence of Ae. aegypti SS homogenate in the first 2 experiments remains unknown. These results suggested the presence of some factors in the homogenates of C. quinquefasciatus (DAR) and An. stephensi which inhibit endopeptidase induced exsheathment.

The high exsheathment rates found in the presence of heat inactivated abdomen homogenates, indicated that heating the abdomen homogenates inactivated the factors which had previously been inhibiting endopeptidase activity in C.quinquefasciatus (DAR) and An. stephensi homogenates. During the heating of the homogenates, it was noticed that the protein material clumped into whitish particles which precipitated to the bottom of the tube. Thus if the protein content of the homogenates had been responsible for inhibiting exsheathment in the previous experiments, its removal (denaturation) by heat in the subsequent experiments, could have been responsible for the increase in the rate of exsheathment.

In experiments where abdomen homogenate supernatant was used, the high exsheathment rate of B. pahangi in the presence of An. stephensi suggested that centrifugation had precipitated out the inhibitory factors in the homogenate. However this was not the case in C. quinquefasciatus (DAR) homogenate, in which % exsheathment remained as low as observed in previous experiments using whole abdomen homogenate. Exsheathment in Ae. aegypti abdomen supernatant remained high. In previous experiments in which wet weight had been estimated An. stephensi had been found to weigh half as much as C. quinquefasciatus (DAR) and less than Ae. aegypti, hence it is possible that by centrifugation, whatever the amount of protein and inhibitory factors present, were sunk to the bottom of the tube, leaving an almost protein free supernatant which allowed exsheathment to proceed.

The pH of abdomen homogenates of C. quinquefasciatus (DAR) and Ae. aegypti SS and An. stephensi were tested to see whether there were any differences which might contribute to the differences in percentage exsheathment of microfilariae in the presence of endopeptidase. All the homogenates had a pH 7, so pH could be eliminated as a possible cause for the differences observed. This pH is similar to that reported by other workers (Clements, 1963; Gooding, 1975).

Observations made in these experiments suggest that some factors present in C. quinquefasciatus (DAR) and An. stephensi abdomens are capable of preventing endopeptidase induced exsheathment of B. pahangi microfilariae. The way in which they act remains unclear, and it would be difficult to determine this since to begin with, the way in which endopeptidase brings about exsheathment of microfilariae of B. pahangi in vitro remains unclear. Devaney and Howells (1979) who were the first to use endopeptidase successfully for routine laboratory

exsheathment of B. pahangi suggested that endopeptidase induced exsheathment by exerting a digestive action on part of the sheath, which together with the vigorous activity of the microfilariae allowed the sheath to rapture.

Since homogenates were prepared using the whole abdomen, it is possible that the inhibiting factors observed, could be located either in the midgut or haemocoel or both. It is therefore necessary to investigate the exact location of the factors inhibiting endopeptidase activity before trying to determine what they are.

Table 49: Exsheathment of *B. pahangi* at endopeptidase concentration of 0.025 mg/ml.

(No of microfilariae scored for exsheathment in all experiments = 100).

Time after incubation (mins)	Nos of mff examined	% Exsheathed	
		EXPT. 1	EXPT. 2
30	100	30	42
60	100	51	54
90	100	86	63
120	100	99	95
180	100	100	100

Table 50: Exsheathment of *B. pahangi* in the presence of abdomen homogenates of *C. quinquefasciatus* (DAR) *Ae. aegypti* SS (AMSS) and *An. stephensi* (STEPH) of varying wet weight.
(50 abdomens in each case).

(No of microfilariae scored for exsheathment in all experiments = 100)

Time after incubation (mins)	Type of abdomen homogenate used	% Exsheathment		
		Expt. 1	Expt. 2	Expt. 3
30	DAR	4	14	3
	STEPH	5	1	0
	AMSS	56	32	25
	CONTROL	100	100	100
60	DAR	9	10	6
	STEPH	16	4	4
	AMSS	69	37	49
	CONTROL	100	100	100
90	DAR	7	15	17
	STEPH	8	6	9
	AMSS	89	83	56
	CONTROL	100	100	100
120	DAR	11	10	11
	STEPH	12	22	15
	AMSS	95	74	81
	CONTROL	100	100	100
150	DAR	14	12	10
	STEPH	28	27	-
	AMSS	100	87	92
	CONTROL	100	100	100
180	DAR	14	25	29
	STEPH	35	27	24
	AMSS	100	95	98
	CONTROL	100	100	100
210	DAR	24	18	22
	STEPH	-	-	-
	AMSS	100	100	100
	CONTROL	100		
240	DAR	36	36	21
	STEPH	53	53	28
	AMSS	100	100	100
	CONTROL	100	100	100

Total Wet weight of DAR = 0.04mg
AMSS = 0.02mg
STEPH= 0.01mg

Table 51: Wet weight of C. quinquefasciatus (DAR), Ae. aegypti SS (AMSS) An. stephensi (STEPH).

Mosquito strain	Wet Weight per abdomen in gms
DAR	0.0013 g
AMSS	0.0008 g
STEPH	0.0006 g

Table 52: Exsheathment of *B. pahangi* in the presence of abdomen homogenates of *C. quinquefasciatus* (DAR), *Ae. aegypti* SS (AMSS) and *An. stephensi* (STEP^{H}) of equal wet weight.

(No. of microfilariae scored for exsheathment in all experiments = 100)

Time after inoculation (mins)	Type of abdomen homogenate used	% Exsheathment		
		Expt. 1	Expt. 2	Expt. 3
30	DAR	2	0	1
	STEPH	3	0	3
	AMSS	4	0	3
	CONTROL	100	100	100
60	DAR	5	0	2
	STEPH	5	0	5
	AMSS	10	2	6
	CONTROL	100	100	100
90	DAR	7	0	4
	STEPH	8	0	10
	AMSS	18	8	9
	CONTROL	100	100	100
120	DAR	11	4	6
	STEPH	12	2	13
	AMSS	27	13	15
	CONTROL	100	100	100
180	DAR	25	9	19
	STEPH	25	2	26
	AMSS	47	28	42
	CONTROL	100	100	100
240	DAR	30	23	19
	STEP H	32	13	45
	AMSS	53	65	94
	CONTROL	100	100	100

Total wet weight of all mosquito species used = 0.03mg

Table 53: The exsheathment of *B. pahangi* in the presence of heat inactivated abdomen homogenates of *C. quinquefasciatus* (DAR) *Ae. aegypti* SS (AMSS) and *An. stephensi* (STEPH)of equal wet weight.

(No. of microfilariae scored for exsheathment in all experiments = 100)

Time after incubation (mins)	Type of abdomen homogenate used	% Exsheathment	
		Expt. 1	Expt. 2
60	DAR	53	58
	STEPH	42	96
	AMSS	94	46
	CONTROL	100	100
120	DAR	96	69
	STEPH	76	97
	AMSS	100	83
	CONTROL	100	100
180	DAR	100	70
	STEPH	100	97
	AMSS	100	100
	CONTROL	100	100

Total wet weight of abdomens of all mosquito species = 0.02mg

Table 54. The exsheathment of *B. pahangi* in the presence of abdomen homogenate supernatant of *C. quinquefasciatus* (DAR), *Ae. aegypti* SS (AMSS) and *An. stephensi* (STEPH) of equal wet weight.

(No. of microfilariae scored for exsheathment in all experiments = 100)

Time after incubation (mins)	Type of Abdomen suspension used	% Exsheathment			
		Expt. 1	Expt. 2	Expt.3	Expt. 4
60	DAR	16	25	7	13
	STEPH	44	44	29	41
	AMSS	6	47	13	3
	CONTROL	100	100	65	100
120	DAR	19	26	18	27
	STEPH	85	89	60	82
	AMSS	83	90	50	67
	CONTROL	100	100	100	100
180	DAR	29	43	30	31
	STEPH	98	90	83	86
	AMSS	84	94	70	68
	CONTROL	100	100	100	100

Total wet weight of abdomens of all mosquito species = 0.02 mg

11.2 The effect of midgut homogenates of C. quinquefasciatus (DAR) An. stephensi and Ae. aegypti SS on endopeptidase and protease induced in vitro exsheathment of B. pahangi.

11.2.1 Introduction

Results of experiments reported in section 11.1 suggest that abdomen homogenate of C. quinquefasciatus (DAR) and An. stephensi contain some factors which inhibit exsheathment of microfilariae of B. pahangi in the presence of endopeptidase. Experiments reported in this section were designed to determine more precisely the location of the inhibiting factors that are present in the abdomen homogenates. In addition some of the experiments were designed to investigate whether the inhibition of exsheathment by abdomen homogenates of C. quinquefasciatus (DAR) and An. stephensi was specific or whether other proteins inhibit endopeptidase induced exsheathment.

11.2.2 Materials and Methods

(i) Exsheathment in the presence and absence of endopeptidase

Midgut homogenate was prepared by removing 30 midguts from one week old mosquitoes of C. quinquefasciatus (DAR) An. stephensi and Ae. aegypti SS. Midguts were homogenized in 100µl HBSS in a 0.1ml all glass homogenizer. Homogenates were kept in crushed ice prior to being used. Each homogenate was divided equally into 4 separate aliquots of 25µl and each aliquot was put in a well of a microtitre plate. To each well, 25µl of microfilariae of B. pahangi in HBSS and 25µl of an endopeptidase solution at a concentration of 0.075 mg/ml was added, giving a final concentration of 0.025mg/ml endopeptidase per well. Control wells were set up in which only endopeptidase and microfilariae of B. pahangi were placed. At intervals of 60 minutes, aliquots from each well were examined up to 180 minutes after incubation for exsheathment. This experiment was repeated 2 times.

Due to the significant differences in the results obtained in experiments where abdomen and midgut homogenates were used, the two experiments were performed simultaneously to see whether differences observed were due to experimental technique or whether they were real. In this experiment, equal wet weight of abdomens were homogenized in 100µl of HBSS. Another experiment was performed in which the procedure followed is similar to that mentioned above except that endopeptidase was not added to the microtitre wells.

(ii) Exsheathment in the presence of Proteases

Proteases have been reported to be good exsheathing agents of microfilariae of B. pahangi by Devaney (1979). Hence protease types III, IV, VII, VIII and X from Sigma Chemical Company (see Table 55) were tested at concentrations of 0.2mg/ml in HBSS, by examining at least 100 microfilariae of B. pahangi under a Phase contrast microscope for exsheathment, at 30 minute intervals after incubation. Experiments were carried out at room temperature 23°C. The source of enzymes used is shown in Table 55. The two proteases which gave a good exsheathment rate were then used in an experiment in which the effect of midgut homogenates of C. quinquefasciatus (DAR) Ae. aegypti SS and An. stephensi on protease induced exsheathment was examined. The procedure followed is similar to that mentioned previously.

(iii) Determination of pH

To determine the pH of midgut homogenate, universal indicator sticks were left to stand in wells of homogenate for several minutes.

(iv) The effect of Foetal calf serum and Bovine serum albumin on exsheathment of B. pahangi by endopeptidase.

The procedure for this experiment is similar to the one mentioned previously in section 11.2.2, except that Foetal calf serum (F.C.S.) and

Bovine serum albumin (B.S.A.) were added to the wells containing endopeptidase and microfilariae of B. pahangi in H.B.S.S. Exsheathment was monitored as mentioned in previous experiments. To determine the protein content in F.C.S. and B.S.A., the Ultra-violet absorption method was used. Undiluted F.C.S. and B.S.A. were diluted X100 with distilled water before estimation of protein content. The optical density was read at 280nm and 260nm. The following formula gave the protein concentration:-

1.55 x absorbance at 280nm - 0.77 x absorbance at 260nm

Values obtained were then multiplied by the dilution factor to give the protein concentration of the original solution. Protein content estimation for abdomens of C. quinquefasciatus (DAR), Ae. aegypti SS and An. stephensi were made from estimations of average protein content of Ae. aegypti SS thorax given by Birtwistle (1971). These were compared to those of F.C.S. and B.S.A.

11.2.3 Results

Results in Table 56 show that the exsheathment rate of B. pahangi microfilariae was high in the presence of all three midgut homogenates in experiment 1, and by 180 minutes post incubation, there was almost 100% exsheathment. In the second experiment, the exsheathment rates were lower compared to the previous ones, these being 73%, 53% and 80% in the presence of C. quinquefasciatus (DAR), Ae. aegypti SS and An. stephensi midgut homogenates respectively. X^2 tests showed them to be significantly different ($P < 0.001$). The surprising result was the 53% exsheathment observed in the case of Ae. aegypti SS midgut homogenate, which was the lowest rate.

Data in Table 57 show the exsheathment rates in the presence of midgut and abdomen homogenates of the 3 mosquito species. These experiments were performed simultaneously side by side. The exsheathment rates are

similar to those observed in previous experiments when they had been performed at different times. Percentage exsheathment was high when midgut homogenate was used. There was a decrease in % exsheathment in the presence of Ae. aegypti SS abdomen homogenate (62% and 28%) compared to previous experiments in which there was 100% exsheathment.

Results in Table 58 show that exsheathment of microfilariae B. pahangi did not occur in the absence of endopeptidase. The exsheathment rates of microfilariae of B. pahangi in the presence of Protease types III,VIII and X, shown in Table 59 were high, these being 81%, 98% and 99% respectively by 60 minutes post incubation. On the other hand protease types IV and VII gave lower exsheathment rates. Data in Table 60 show there was no inhibition of exsheathment of microfilariae of B. pahangi by the three midgut homogenates in the presence of Protease types VIII and X.

In experiments where F.C.S. and B.S.A. were used instead of midgut homogenates, see Table 61, there was no exsheathment of microfilariae of B. pahangi. Protein content of F.C.S. and B.S.A. was 78.25mg/ml and 58.26mg/ml respectively, whereas for Ae. aegypti SS, C. quinquefasciatus (DAR) and An. stephensi homogenates, the protein content was 0.114mg/ml, 0.2mg/ml and 0.05mg/ml respectively.

11.2.4 Discussion

The high rate of exsheathment of B. pahangi microfilariae in the presence of midgut homogenates of C. quinquefasciatus (DAR), Ae. aegypti SS and An. stephensi observed could be due to a considerable dilution of the homogenates to the extent that whatever inhibitory factors that might have been present in the midgut in vivo, were no longer detectable or present in sufficient quantities that could prevent endopeptidase activity. These results do not suggest the absence of an inhibitory

factor in the midgut of C. quinquefasciatus (DAR) and An. stephensi, but call for a change in the design of the experiment in order to create conditions comparable to those in vivo. The significantly lower exsheathment rates observed in the second experiment might be due to the condition of microfilariae used. In both experiments, microfilariae were obtained from different jirds and this could be the source of variation in the behaviour of the microfilariae rather than it being due to the midgut homogenates. Results of the exsheathment rate of microfilariae B. pahangi in the presence of midgut and abdomen homogenates determined simultaneously showed that the differences observed in earlier experiments performed at different times were real and not due to experimental technique. The decrease in % exsheathment in the presence of Ae. aegypti SS abdomen homogenate could be due to the higher protein concentrations present in the homogenates used, compared to those used in section 11.1.2. In the latter case, the concentration of abdomen homogenate was 25 abdomens/ml, whilst in the former, it was 11 abdomens/ 100µl. Hence, the higher concentration of protein in the homogenates could be responsible for the decrease in the rate of exsheathment. The source of microfilariae used and their condition could be responsible to some degree for the decrease in the rate of exsheathment.

Exsheathment did not occur in the absence of endopeptidase, not even in the presence of Ae. aegypti SS midgut homogenate showing that whatever factors cause exsheathment in vivo are not present in the midgut homogenate at high enough concentrations to cause exsheathment in vitro. It is possible that addition of 100µl of HBSS to the midguts, in the preparation of the homogenates, was responsible for diluting factors which might have induced exsheathment.

The lack of inhibition of exsheathment by protease types VIII and X by midgut homogenates of the 3 mosquito species could again be due to the dilution of the inhibitory factors present in C. quinquefasciatus (DAR) and An. stephensi midgut homogenates. The fact that all the midgut homogenates had a pH of 7 eliminated the chances of pH being a source of any of the variation observed.

The complete inhibition of exsheathment by F.C.S. and B.S.A. in the presence of endopeptidase was much greater than that of C. quinquefasciatus (DAR) and An. stephensi abdomen homogenates. The protein concentrations of F.C.S. and B.S.A. were much higher than those of C. quinquefasciatus (DAR), Ae. aegypti SS and An. stephensi abdomens. This large difference in protein content is the most likely explanation for the extent of inhibition observed when F.C.S. and B.S.A. were used. It is likely that endopeptidase activity was completely swamped by the binding of protein to it, thus inhibiting exsheathment. These results also show that inhibition is not specific to abdomen homogenate, but occurs in the presence of other non-specific proteinaceous substances. The extent of inhibition appears to depend on the concentration of protein material present in the homogenate or other proteinaceous substance.

The conclusion that can be drawn from results in section 11.1 and 11.2 is that the inhibitory action of C. quinquefasciatus (DAR) and An. stephensi abdomen homogenates on endopeptidase induced exsheathment of microfilariae B. pahangi is partly due to some specific inhibitory factor in the abdomen and partly due to the protein content, inhibiting endopeptidase activity. However the exact location of this factor(s) in the abdomen remains unclear. We cannot rule out its presence in the midgut simply because in vitro experiments using midgut homogenate did

not inhibit exsheathment. It could be that the experimental set up *in vitro* did not simulate the *in vivo* situation adequately enough for the inhibitory factor(s) to manifest itself. It is possible that the abdomen homogenate of *Ae. aegypti* SS did not entirely inhibit endopeptidase induced exsheathment of *B. pahangi* microfilariae because it lacks this factor(s) and that the inhibition observed in some experiments was due to protein content in the homogenate. In the case where midgut homogenates were used, exsheathment took place simply because whatever factor(s) that causes inhibition of exsheathment in *C. quinquefasciatus* (DAR) and *An. stephensi* plus the protein concentration of these homogenates, was not high enough to inhibit endopeptidase activity. There have been suggestions as to how enzymes and ions induce *in vitro* exsheathment of microfilariae. Devaney and Howells (1979) speculated that Ca^{2+} induced exsheathment of microfilariae *B. pahangi* was facilitated by Ca^{2+} mediated adherence of the sheath to a fixed substrate, followed by escape from the anterior tip brought about by a cephalic hook (Laurence and Simpson, 1968). They also thought that both endopeptidase and papaya extract exert a digestive action on part of the sheath which then ruptures freeing the microfilariae.

Despite these speculations as to the cause of exsheathment of microfilariae *in vitro*, it remains unclear what facilitates or inhibits exsheathment *in vivo* in susceptible and refractory mosquito hosts.

Table 55: Source of Enzyme, the temperature, pH of incubation and the maximum concentration of enzyme employed

Enzyme	Maximum concentration mgm/ml	Temperature	pH	Description *
Endopeptidase	0.025	23	8	Bacterial crude powder
Protease III	0.2	23	7.5	From Papaya
Protease IV	0.2	23	7.5	From Streptomyces caespitosus
Protease VII	0.2	23	7.5	From Bacillus amyloliquefaciens
Protease VIII	0.2	23	7.5	From a strain of Bacillus subtilus
Protease X	0.2	23	7.5	From Bacillus thermoproteolyticus rokko

* All reagents were from the Sigma Chemical Company

Table 56 : Exsheathment of B. pahangi in the presence of midgut homogenates of C. quinquefasciatus(DAR) Ae. aegypti SS (AMSS) and An. stephensi (STEPH).

Expt. No.	Concentration of Endopeptidase (mg/ml)	Nos midgut/ 75µl HBSS	% Exsheathment at 180 minutes			
			DAR	AMSS	STEPH	CONTROL
1	0.025	10	93	91	99	100
2	0.025	10	73	53	80	100

Table 57 : Exsheathment of B.pahangi in the presence of midgut and abdomen homogenates of C. quinquefasciatus (DAR) Ae. aegypti (AMSS) and An. stephensi (STEPH).

Expt. No.	Concentration of Endopeptidase (mg/ml)	Nos midgut/ 75µl HBSS	% Exsheathment at 180 minutes			
			DAR	AMSS	STEPH	CONTROL
1	0.025	10	100	100	100	100
2	0.025	10	100	100	100	100
		Wet wt of abdomens				
1	0.025	0.01	53	62	58	100
2	0.025	0.01	27	28	24	100

Table 58. Exsheathment of B. pahangi (in the absence of Endopeptidase) in the presence of midgut homogenates of C. quinquefasciatus (DAR), Ae. aegypti (AMSS) An. stephensi (STEPH).

Expt. No.	Nos midgut/75µl HBSS	% Exsheathment at 180 minutes			
		DAR	AMSS	STEPH	CONTROL
1	10	0	0	0	0
2	10	0	0	0	0

Table 59. Exsheathment of B. pahangi by various proteases

Expt. No.	Concentration Protease mg/ml	% of exshathment at 60 minutes Protease type				
		III	IV	VII	VIII	X
1	2	81	5	49	98	99

Table 60: Exsheathment of B. pahangi in the presence of midgut homogenates of C. quinquefasciatus (DAR) Ae. aegypti (AMSS) An. stephensi (STEPH).

Expt. No.	Concentration protease mg/ml	Type of Protease	% Exsheathment at 180 minutes			
			DAR	AMSS	STEPH	CONTROL
1	2	VIII	100	100	100	100
	2	X	100	100	100	100
2	2	VIII	100	100	100	100
	2	X	100	100	100	100

Table 61: Exsheathment of B. pahangi in the presence of foetal calf serum and bovine serum albumin.

Expt. No.	Serum used	Protein concentration mg/ml	% of exsheathment at 180 minutes
1	F.C.S.	78.25	0
	B.S.A.	58.26	0
2	F.C.S.	78.25	0
	B.S.A.	58.26	0

11.3 The exsheathment, motility and migration of B. pahangi incubated in vitro with midguts of C. quinquefasciatus (DAR) Ae. aegypti SS and An. stephensi.

11.3.1 Introduction

The in vitro exsheathment of microfilariae has been achieved by various workers using methods which most probably do not resemble those occuring in vivo in the mosquito midgut. Weinstein (1963) obtained the exsheathment of W. bancrofti microfilariae in vitro by sequential treatment of the larvae with sapomin, streptolysin O and trypsin, while Katamine and Aoki (1970) induced exsheathment of microfilariae W. bancrofti and B. malayi by incubating wet films of infected human blood on agar plates. Devaney and Howells (1979) succeeded in exsheathing B. pahangi, B. malayi, W. bancrofti and L. carinii microfilariae by using Ca^{2+}, endopeptidase and papaya extract protease. The aim of the investigations reported in this section was to simulate the conditions occurring in the mosquito midgut in vivo before and after a bloodmeal and to study the exsheathment, motility and migration of microfilariae under such conditions and to compare these results with observations which have been made in vivo in the midgut of refractory and susceptible mosquitoes.

11.3.2 Materials and Methods

(i) Motility and exsheathment of microfilariae B. pahangi incubated in midguts of unfed and fed C. quinquefasciatus (DAR), Ae. aegypti SS and An. stephensi.

Midguts were dissected from unfed 1-2 week old C. quinquefasciatus DAR, Ae. aegypti SS and An. stephensi then transferred to a 10µl drop of physiological saline on a siliconized glass coverslip. Three midguts were placed in each drop. To this, microfilariae were added. A drawn out capillary tube with a narrow tip (0.05mm diameter) was used for

introducing the microfilariae into the drop. The number of microfilariae introduced into each drop were counted by looking down through a dissecting microscope. The cover slip was then inverted over a clean cavity slide and the edges of the coverslip were sealed with vaseline. This experiment was repeated using* Weymouths MB 721/1 medium as the incubating medium. In another similar experiment, the midguts were removed from mosquitoes 30 minutes after a bloodmeal on guinea pigs. The midguts were punctured to allow blood to ooze out into the incubation medium plus to allow the leaking out of any other substances which might have been secreted into the midgut following ingestion of the blood meal. Controls were set up similarly with the exception of midguts. Motility was scored on a scale ranging from 0-3 as follows:-

0 - dead or immobile microfilariae

\+ - sluggish microfilariae

++ - moderately active microfilariae

+++ - active microfilariae

Two slides of each were prepared. Observations were made every hour after incubation for a period of 3 hours. Microfilariae were also examined for exsheathment.

(ii) Motility of ingested microfilariae B. pahangi.

For comparison purposes with experiments on in vitro motility of B. pahangi, observations were also made on the motility of microfilariae soon after ingestion of an infective bloodmeal by one week old C. quinquefasciatus (DAR) and Ae. aegypti SS mosquitoes. Approximately 30 minutes after feeding, fed mosquitoes were transferred to a smaller container. The midguts of fed Ae. aegypti SS and C. quinquefasciatus (DAR) were removed from the abdomen, placed in a drop of distilled water and gently teased apart until the bloodmeal was homogenous. A glass coverslip

* received from Flow laboratories

was placed over a drop of the bloodmeal and examined under a Phase Contrast microscope. Motility of microfilariae observed was scored as mentioned above. Fed. C. quinquefasciatus (DAR) and Ae. aegypti SS females were examined over a 2 hour period post feeding.

(iii) In vitro migration of microfilariae B. pahangi from midguts of C. quinquefasciatus (DAR) and Ae. aegypti SS mosquitoes.

This experiment was carried out to examine whether B. pahangi microfilariae ingested with the bloodmeal would proceed to migrate from the midgut when it was removed from the surrounding abdominal haemocoel, indicating whether the stimulus for migration originates from within the midgut or the abdominal haemocoel. One week old C. quinquefasciatus (DAR) and Ae. aegypti SS were fed on an infective bloodmeal with a high microfilariae count. Fed mosquitoes were dissected 15-30 minutes after feeding, the midgut was removed and transferred to 20µl in HBSS in a cavity slide. The cavity slides were examined 2 hours later for microfilariae, under a dissecting microscope. Any microfilariae observed were recorded.

(iv) The effect of CO_2 on exsheathment of B. pahangi

CO_2 has been shown to affect the development of malarial parasites in in vitro experiments. In particular, the concentrations of CO_2 present in vitro have been shown to inhibit exflagellation and development of gametocytes of both sexes. For this reason, this experiment was carried out to see whether varying the CO_2 concentration in vitro would have any effect on exsheathment of B. pahangi microfilariae. Midguts of unfed and fed C. quinquefasciatus (DAR), Ae. aegypti SS and An. stephensi were dissected from one week old mosquitoes. Three to four midguts were placed in 20µl HBSS in a microtitre plate. Three replicates

of each were made. To each well microfilariae of B. pahangi were added. Controls were also set up in which no midguts were present in the wells just HBSS and jird washings. Two microtitre plates, one with fed and the other unfed midguts was placed in a gas chamber with 3% CO_2 at room temperature (22-23°C). The other two plates were put in a CO_2 incubator in which the CO_2 content was 8% and temperature 37°C. After an incubation period of 3 hours, aliquots were removed from the wells and microfilariae examined for exsheathment under a phase contrast microscope.

(v) Exsheathment of B. pahangi microfilariae incubated in various media and tissues in hanging drop preparations.

This experiment was designed to simulate in vitro, conditions prevailing in the midgut in vivo, to see whether exsheathment would occur in such in vitro conditions. For incubation purposes the hanging drop technique was used. The volume of the drops of medium used was measured with an Eppendorf pipette. Initially, in a preliminary experiment varying volumes of media were used to test whether the size of the drop used had any effect on exsheathment. Due to the variability in % exsheathment observed in these experiments, a constant volume of medium was used in the subsequent experiments in order to reduce sources of variation. Media used for incubation purposes were Hayes Saline, Weymouths, and MK/VP 12 + 20% FBS. In the preliminary experiment ATP was added to some drops to see whether it had any effect on % exsheathment. Since no marked effects were observed, ATP was omitted in subsequent experiments. As well as varying the medium used, various tissues were added to the drop, such as blood, blood clots, intact and slit midguts, blood filled midguts, thorax tissue and salivary glands. Microfilariae were examined for exsheathment over a 3 hour period post incubation under a Phase contrast microscope.

11.3 Results

Results in Table 62 show that a great proportion of microfilariae B. pahangi remained motile over the 3 hour incubation period in saline plus midguts of unfed An. stephensi and Ae. aegypti SS, then those incubated in Hayes saline. In the control experiment only 16% of the microfilariae remained moderately motile after 3 hours incubation while 83% were sluggish. Motility was best in Ae. aegypti SS midguts. In the case of An. stephensi midguts, 12% of the microfilariae were dead or immobile whereas in Ae. aegypti SS midguts and in the control experiments, 1% of the microfilariae were immobile.

Data in Table 63 shows motility of microfilariae B. pahangi incubated in bloodfed midguts of Ae. aegypti SS and An. stephensi. As in the previous experiment motility was poorest in the control, 96% of the microfilariae being sluggish 3 hours post incubation and 4% being immobile. In this experiment there was a reduction in the percentage of dead or immobile microfilariae incubated in An. stephensi midguts. Only 6% fell in this category, a greater proportion, 69%, were sluggish while 25% were moderately active. Incubation of microfilariae in blood filled midguts of Ae. aegypti SS appear to have prolonged the motility of the microfilariae the longest, 89% being moderately active after 3 hours incubation.

Results in Table 64 show no significant differences in motility of microfilariae B. pahangi in midguts of unfed C. quinquefasciatus (DAR) and Ae. aegypti SS. The results appear to be very similar, with 54% of the microfilariae being moderately active in both cases, 44% being sluggish and 10% dead or immobile. In the control experiment 99% of the microfilariae were sluggish. The most significant observation was that motility of B. pahangi was poor in the control experiment using only saline.

Motility of microfilariae was prolonged when Weymouths MB271/1 medium was used for incubation (see Table 65). Motility of microfilariae in C. quinquefasciatus (DAR) and An. stephensi midguts was very similar while in the case of Ae. aegypti, all the microfilariae were moderately active. In the control experiment microfilariae were less motile, 39% being immobile and 61% moderately motile.

Data in Table 66 show that in vivo, a greater number of microfilariae remained active in the gut of Ae. aegypti SS than C. quinquefasciatus (DAR). This, indicates the presence of some factor(s) in the midgut of C. quinquefasciatus (DAR) which has some adverse effect on B. pahangi microfilariae, causing rapid loss of motility.

Migration of microfilariae was observed in vitro (see Table 67) from the midguts of Ae. aegypti SS fed on an infective bloodmeal, to the surrounding medium. However, no migration of microfilariae took place from midguts of C. quinquefasciatus (DAR) fed on the same infective bloodmeal.

Results in Tables 68 and 69 indicate that increasing CO_2 content in the incubation chamber does not facilitate exsheathment of microfilariae B. pahangi in vitro.

Data in Table 70 show that exsheathment of microfilariae B. pahangi occurred in the presence of slit midguts of Ae. aegypti SS and C. quinquefasciatus (DAR) with and without blood and blood clots. Exsheathment also took place in the presence of thorax tissue, but not in the presence of salivary glands. The percentage exsheathment was very variable and not reproducible for each type of tissue used and between the different tissues. All exsheathed microfilariae were found in the vicinity of the slit midguts, unexsheathed microfilariae occurring in the medium. The addition of blood or blood clots to slit midguts in the

hanging drop appears to have increased the percentage of microfilariae that exsheathed. Microfilariae incubated in MK/VP12 + 20% F.B.S. medium appeared to remain motile for a longer time than those in Hayes saline or Weymouths medium.

11.3.4 Discussion

The greater percentage of microfilariae B. pahangi found to be dead or immobile when incubated in unfed An. stephensi midgut compared to Ae. aegypti SS unfed midguts suggests the presence of factors in the midgut of An. stephensi which affect the viability of the microfilariae. When microfilariae were incubated in blood fed midguts of the 2 species of mosquitoes, although there was a decrease in the proportion of microfilariae dead or immobile in An. stephensi, motility was still better in microfilariae incubated in Ae. aegypti SS midguts, whereas in the case of An. stephensi, the greatest proportion remained sluggish. This reduced motility of microfilariae of B. pahangi incubated in An.stephensi midguts could be an explanation for the lack of migration of exsheathed B. pahangi found in the midgut of An. stephensi following ingestion of an infective blood meal reported in Chapter 9.

It is surprising that no differences in motility were observed between microfilariae of B. pahangi incubated in unfed C. quinquefasciatus (DAR), and Ae. aegypti SS midguts, and even more puzzling when motility of B. pahangi with midguts of unfed C. quinquefasciatus (DAR) , An. stephensi and Ae. aegypti SS incubated in Weymouths MB721/1 medium, were found to be almost similar. It is possible that the previous variation in motility observed was due to the age and state of microfilariae used in these experiments since they were obtained from different jirds which had been infected at different times.

The results consistently show poor motility in controls lacking midguts of any mosquito species, showing that in some way, the presence of midguts of mosquitoes in the media enhances the viability of microfilariae. The reason for this remains unexplained but may relate to nutrient substances leaching from the gut. Motility of microfilariae was better in the presence of Weymouths MB721/1 medium probably because this medium contains a wider range of nutrients which maintain the microfilariae in a better condition and provide them with energy. Hayes saline is not a rich culture medium.

In vivo observations on motility of microfilariae *B. pahangi* ingested by *C. quinquefasciatus* (DAR) and *Ae. aegypti* SS showed that motility was better in *Ae. aegypti* than in *C.quinquefasciatus* (DAR). This suggests the presence of factors in the midgut of *C. quinquefasciatus* (DAR) effecting the viability of *B. pahangi* and inhibiting migration of *B. pahangi*. What these factors are and how they function remains unknown.

The results of the experiments on the *in vitro* migration of *B. pahangi* from midguts of *Ae. aegypti* SS following an infective blood meal show that the stimulus which facilitates migration to the thorax is not located in the abdominal haemocoel, or thorax, but within the midgut itself. This stimulus is absent in *C. quinquefasciatus* (DAR) mosquitoes.

The exsheathment of the microfilariae of *B. pahangi* observed in the presence of slit midguts, with and without blood or blood clots and in thoracic tissue was probably brought about by the microfilariae adhering to the matrix provided by these tissues and then freeing themselves from the sheath, rather than it being due to some enzymes or other substances oozing from the slit midguts. In these experiments

exsheathment appeared to be primarily due to a physical process, which is unlikely to be the case in the midgut of a good host mosquito, such as Ae. aegypti SS. In previous experiments in which Ae. aegypti SS and C. quinquefasciatus (DAR) were inoculated with unexsheathed B. pahangi microfilariae, some developed successfully. Probably in this case, these microfilariae were able to loose their sheaths by adhering to the thoracic tissues, further implying that to some extent exsheathment is determined by the availability of a suitable adhering surface or substance found within the midgut of susceptible mosquitoes.

An incubation period of approximately 1½-3 hours was required before exsheathed microfilariae were observed. Since the process of exsheathment in Ae. aegypti SS midgut in vivo starts almost as soon as microfilariae arrive in the midgut, the longer time of exsheathment observed in vitro indicates that the conditions simulated in the hanging drops were not the optimal conditions necessary for exsheathment.

During the examination of motility of ingested microfilariae of B. pahangi in midguts of C. quinquefasciatus (DAR) and Ae. aegypti SS, it was noticed that clotting of the bloodmeal occured at a quicker rate in C. quinquefasciatus (DAR) than in Ae. aegypti SS and it was easier to tease apart bloodmeals from Ae. aegypti SS. The quicker rate of blood clotting in C. quinquefasciatus (DAR) could partly be responsible for the decreased motility and lack of migration of microfilariae observed, whereas in Ae. aegypti SS microfilariae remained motile for a longer period and migrate from the bloodmeal.

There are several possibilities which can be considered in trying to interpret the results of experiments on the motility and exsheathment of microfilariae B. pahangi in vitro in the presence of midguts of

C. quinquefasciatus (DAR), Ae. aegypti SS and An. stephensi. Firstly, there maybe differences between the secretions from midgut cells of these mosquito species, which affect the motility of microfilariae. Owen (1977) came to the same conclusion in his studies of the migration of B. pahangi in refractory and susceptible mosquitoes of the Aedes scutellaris complex. Secondly, the migration of B. pahangi in Ae. aegypti SS, could be a response to some stimulating substance secreted around the periphery of the bloodmeal in the process of digestion. Since the central part of the bloodmeal is the last part to be affected by digestive action and therefore the digestive enzymes are unlikely to affect that part of the meal for several hours (Gooding, 1972), the response is thought to be very sensitive. Those microfilariae which do not respond quickly get trapped in the central portion of the blood meal and do not succeed in migrating towards the epithelium. In addition to a sensitive response to some secretion, there could be a chemotactic response towards the gut epithelium prior to the clotting of the blood meal. Thirdly, it is possible that the microfilariae just wander about in the bloodmeal and finally reach the epithelium by chance. This suggestion is the least likely explanation since in susceptible mosquitoes, almost 100% of the microfilariae migrate successfully while in refractory species less than half of the microfilariae migrate to the thorax.

Erythrocytes were not observed to play any role in the process of exsheathment in these studies. However, Owen (1977) postulated that attachment of erythrocytes to the sheathed microfilariae, leads to the building up of a substantial "plaque", which makes the sheath sufficiently rigid for the swimming action of the microfilariae to free itself of its sheath. Erythrocytes did not attach to exsheatned worms or cast sheaths in the experiments.

Wade (1974) noted attachment of erythrocytes to sheathed B. pahangi in blood that had beenchilled for transport and Devaney (1979) noted erythrocyte attachment in vitro during studies on the culture of filarial worms.

Investigations on the effect of CO_2 on exsheathment of microfilariae showed that no exsheathment took place at higher CO_2 levels. Similar studies by other workers on the effects of CO_2 on the development of gametocytes of Haemoproteus and Plasmodium falciparum species have been reported. Bishop et al. (1956) carried out investigations on the factors affecting the emergence of the gametocytes of P. gallinaceum from the erythrocytes and exflagellation of the male gametocytes. They found that 5% CO_2 completely inhibited the development of both female and male gametocytes, but the gametocytes of both sexes emerged from the erythrocytes within the first few minutes of the 5% CO_2 being replaced by air. Whereas the gametocytes appeared to be undamaged by the exposure to 5% CO_2, recovery from exposure to undiluted CO_2 was very uncertain. These results confirmed those of Marchoux and Chlorine (1932), quoted by Bishop et al. (1956), upon the inhibitory effects of CO_2 on exflagellation in Haemoproteus and P. falciparum. It is unlikely that high CO_2 levels also inhibit exsheathment of microfilariae.

The conclusions that can be drawn from these experiments are that although exsheathment did occur to some degree in vitro, it is unlikely to be occurring by the same mechanism in vivo, in midguts of susceptible mosquitoes. One should be cautious in comparing the observations made in vitro with those made in vivo, since the conditions simulated in vitro are unlike those present in vivo. More information is required of the condition of the midgut of refractory and susceptible hosts prior to and after an infective bloodmeal.

Table 62: Motility of *B. pahangi* incubated with midguts of unfed *Ae. aegypti* SS (AMSS) and *An. stephensi* (STEPH).

Mosquito species	Incubation period (hrs)	Nos of mff examined	Motility Scores of mff (%)			
			0	+	++	+++
STEPH	3	344	42 (12)	230 (38)	172 (50)	0 0
AMSS	3	377	6 (1)	70 (19)	264 (70)	37 (10)
CONTROL	3	473	4 (1)	394 (83)	75 (16)	0 0

Table 63: Motility of *B. pahangi* incubated with midguts of bloodfed *Ae. aegypti* SS (AMSS) and *An. stephensi* (STEPH)

Mosquito species	Incubation period (hrs)	Nos of mff examined	Motility Scores of mff(%)			
			0	+	++	+++
STEP	3	142	9 (6)	98 (69)	35 (25)	0 0
AMSS	3	141	5 (4)	10 (7)	126 (89)	0 0
CONTROL	3	143	6 (4)	137 (96)	0 0	0 0

Table 64: Motility of *B. pahangi* incubated with midguts of unfed *C. quinquefasciatus* (DAR) and *Ae. aegypti* SS (AMSS)

Mosquito species	Incubation period (hrs)	Nos of mff examined	Motility Scores of mff(%)			
			0	+	++	+++
DAR	3	287	4 (1)	127 (44)	256 (54)	0 0
AMSS	3	264	2 (0.8)	120 (45)	142 (54)	0 0
CONTROL	3	174	1 (0.6)	173 (99)	0 0	0 0

Table 65: Motility of *B. pahangi* incubated with unfed midguts of *C. quinquefasciatus* (DAR), *Ae. aegypti* SS (AMSS) and *An. stephensi* (STEPH) in the presence of Weymouths MB721/1 medium.

Mosquito species	Incubation period (hrs)	Nos of mff examined	Motility Scores of mff(%)			
			0	+	++	+++
DAR	3	69	0	0	38 (55)	31 (45)
AMSS	3	100	0	0	100 (100)	0 0
STEPH	3	98	0	0	56 (47)	42 (43)
CONTROL	3	98	0	38 (39)	60 (61)	0 0

Table 66 : Motility of *B. pahangi* ingested by *C. quinquefasciatus* (DAR) and *Ae. aegypti* SS (AMSS) (examined in Hayes saline)

Mosquito Species	Incubation period (hrs)	Nos of mosquito examined	Motility scores of mff (%)			
			0	+	++	+++
AMSS	2	11	5 (4)	82 (62)	35 (27)	10 (8)
DAR	2	10	87 (60)	56 (38)	2 (1)	0 0

Table 67: *In vitro* migration of *B. pahangi* from bloodfed midguts of *C. quinquefasciatus* (DAR) and *Ae. aegypti* SS (AMSS)

Mosqutio species	Nos mosquitoes examined	Incubation time	Total nos of mff (range per mosquito)
AMSS	10	2	70 (3-10)
DAR	10	2	0

Table 68: Showing the effect of CO_2 on exsheathment of B. pahangi incubated in midguts of unfed C. quinquefasciatus (DAR), Ae. aegypti SS and An. stephensi (STEPH).

Mosquito species	% Exsheathment of Microfilariae at 3% CO_2		at 8% CO_2	
	Nos mff examined	% exsheathed	Nos mff examined	% exsheathed
DAR	140	0	193	0
AMSS	158	3	190	0
STEPH	189	0	203	0
CONTROL	159	0	199	0

Table 69: Showing the effect of CO_2 on exsheathment of B. pahangi incubated in midguts of bloodfed C. quinquefasciatus (DAR), Ae. aegypti SS (AMSS) and An. stephensi (STEPH).

Mosquito species	% Exsheathment of Microfilariae at 3% CO_2		at 8% CO_2	
	Nos mff examined	% exsheathed	Nos mff examined	% exsheathed
DAR	84	0	108	0
AMSS	60	0	97	0
STEPH	70	0	124	0
CONTROL	82	0	98	0

Table 70: *In vitro* exsheathment of *B. pahangi* incubated in midguts of *Ae. aegypti* SS and *C. quinquefasciatus* (DAR) in various media. (50 microfilariae were scored for exsheathment in each experiment)

Mosquito species	Tissue Used	Volume of medium (µl)	Incubation time (hrs)	% Exsheathment in Hayes	Weymouths	MK/VP12+20% F.B.S.
AMSS	slit midgut	6	3	4, 2, 10*	0, 0, 2	2, 0, 7
	thorax	6	3	6, 6, 3	20, 0, 2	-, 15, 0
	slit midgut + blood	6	3	74, 21, 14	15, 0, 0	41, 0, 13
	slit midgut + blood clots	6	3	13, 0	11, 5	35, 44
	salivary glands + blood	6	3	0, 0	0, 0	0, 0
DAR	slit midgut	6	3	0, 0, 0	10, 0, 0, 3, 2	2, 2, 0, 6
	thorax	6	3	0, 20, 4	0 15, 2, 0, 5	0, 0, 19, 15
	slit midgut + blood	6	3	23, 0, 3	11, 15, 23	3, 13, 15
	slit midgut + blood clots	6	3	15, 0	20, 11	18, 0
	salivary glands + blood	6	3	0, 0	0, 0	0, 0

* figures separated by commas represent different replicates

11.4 The *in vitro* exsheathment of *B. pahangi* incubated in mosquito cells.

11.4.1 Introduction

Results of the previous experiments in section 11.3, showed that exsheathment did occur *in vitro*, in hanging drops, in the presence of various media and tissues. Exsheathment was thought to be primarily a 'physical' process not involving any secreted substances. Due to the variability in % exsheathment observed, despite using the same volume of medium, an attempt was made to improve reproducibility by using medium which had been observed to prolong motility of microfilariae and to provide cells from mosquitoes, to the surface of which microfilariae may adhere.

11.4.2 Materials and Methods

(i) Maintenance of cell cultures

Cells used were either established from eggs or larvae of *Ae. aegypti* and designated as MOS 20A cells. For primary culture, cells were established in MK/VP 12 + 20% FBS then this was reduced gradually to 5% F.B.S. (Foetal bovine serum). MOS 20A cells grew very rapidly once established and needed to be passed usually every 7-10 days as follows:-

a) the old medium was taken off either by pouring off using the vacuum pump or suction with a pipette attached.

b) a small amount of fresh medium was added, then the monolayer scraped off using a rubber policeman, which consists of a pipette with a small rubber bulb attached. There were autoclaved prior to use.

c) after scra ping off, cells were separated using a Pasteur pipette as they tend to clump together and then a count taken. A haemocytometer was used to estimate the number of cells per

ml and from this an estimate was made of how many cells to pass into each tube or flask. With a Falcon flask ($25cm^2$), at least 750,000 cells were passed whilst with a Leighton tube 25,000 cells were passed. Cells could be observed using an inverted microscope.

Once passed, medium was added to the tubes and flasks then they were returned into an incubator at 25-27°C. They were checked for contamination after 24 hours. When contaminated they appeared cloudy. To avoid contamination all bottles and pipettes were washed properly and rinsed in double distilled water, then autoclaved. Medium which could not be autoclaved was filtered using a Millipore filter bell 0.22µms.

(ii) Incubation of B. pahangi in MOS 20 A cells in Hanging drops.

MOS 20A cells were obtained from cell cultures which had just been passed. Passed cells suspended in Lockes solution were spun down for 5 minutes at 1,000g. The supernatant was discarded and the pellet was resuspended in 0.5ml of MK/VP 12 + 20% FBS. Hanging drops were prepared by placing known volumes of cell suspension on a siliconized cover slip, then adding the sheathed microfilariae and inverting this over a cavity slide. Incubating time varied from 1-4 hours after which the microfilariae were examined for exsheathment, under a phase contrast microscope. This experiment was repeated several times.

(iii) Incubation of B. pahangi on Monolayers of MOS 20A cells

Overgrown detached and floating cells in culture tubes were removed, leaving only a continuous monolayer of cells at the bottom of a tube. A concentrated suspension of sheathed microfilariae (1000mff/$20mm^3$) was distributed over the monolayer so that the microfilariae were in close contact with the cells. These tubes were incubated for

3 hours then examined under a phase contrast microscope for exsheathment.

11.4.3 Results

Results in Table 71 show that exsheathment was very variable. Despite setting up several hanging drops and varying the amount of medium used, the volume of cells used and the incubation time, exsheathment was not reproducible. However, there was an increase in % exsheathment with an increase in the incubation time. (see Fig. 23). Microfilariae were not observed to be attaching to mosquito cells, neither were they located in any particular region of the hanging drop. Data in Table 72 show that there was variability in % exsheathment of B. pahangi incubated in Monolayers of MOS 20A cells. X^2 tests showed there was a significant difference in % exsheathment between experiments ($P < 0.001$). In experiment 1 and 2, X^2 tests showed no significant differences in % exsheathment between tubes ($P > 0.05$), however in experiment 3, there were significant differences in % exsheathment between tubes. Microfilariae of B. pahangi remained very active during the 3 hours of incubation. They could be seen moving rapidly among the cells of the monolayer, unattached to other microfilariae or to the cells. A few empty sheaths could be seen among the cells. Greater numbers of microfilariae were observed at the edges of the monolayer then in the middle of the culture tube.

11.4.4 Discussion

The variability in % exsheathment observed when microfilariae B. pahangi were inoculated in suspensions of MOS 20A cells appears to be unrelated to the treatment schedule. Since the microfilariae used were obtained from peritoneal washings of different jirds, it is possible that differences in the age of the microfilariae could play a role in their ability to exsheath, hence affect the % exsheathment.

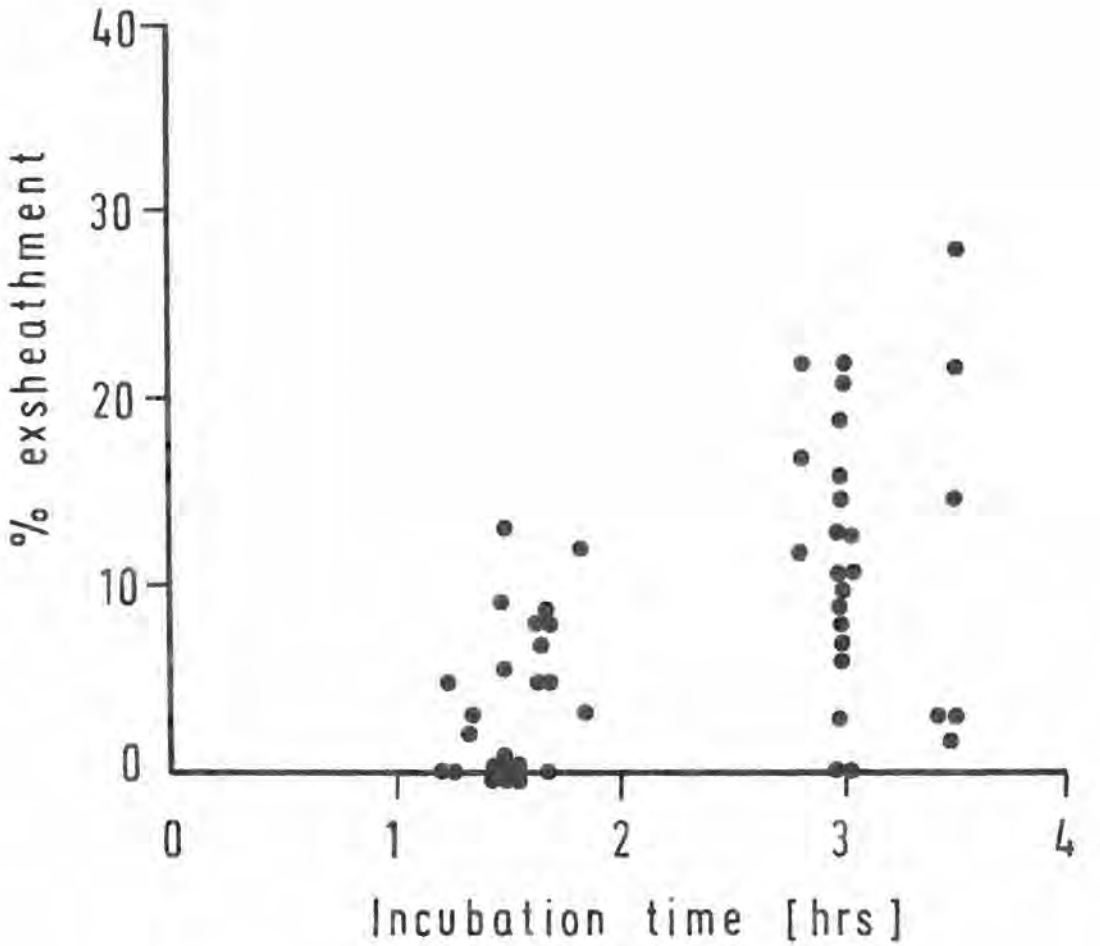


Fig. 23: The Exsheathment of B. pahangi microfilariae incubated in suspensions of MOS 20A cells.

The increase in exsheathment rate with time observed, could be due to some substance leaching from the cells and increasing in concentration with time, to the extent that it facilitates exsheathment to a certain degree.

The variability in exsheathment observed also indicates that a suspension of MOS 20A cells does not provide a stable surface for microfilariae to adhere to before they proceed to exsheath. As a result Monolayers of MOS 20A cells were used for incubation. It was thought that they would provide a mechanically stable and immobile surface for attachment, which would lead to a greater number of microfilariae exsheathing in a consistent manner. However the results show that exsheathment continued to be variable. Again, one source of variability could be the state of microfilariae used. The percentage exsheathment within the first two experiments was not significantly different suggesting that the microfilariae used were homogeneous. It also suggests that the conditions prevailing within the tubes in each experiment were similar and the scoring of exsheathment was reliable, since there were no great deviations. However, the differences in exsheathment rates between experiment 1 and 2 suggest either differences in the microfilariae used or in the experimental set up. In experiment 3, the significant differences in % exsheathment among tubes, shown by a X^2 test ($P < 0.001$), suggests differences in the conditions prevailing within the tubes. Since the microfilariae used in all three tubes were the same, they can be ruled out as being the source of variation. The conditions within the tubes which could account for the differences in exsheathment rate are:-

(a) the density of the monolayer cells

(b) differences in the age of the cells

(c) the volume of medium remaining over the monolayer of cells.

(d) the numbers of microfilariae added to each tube.

Some of the exsheathment rates observed in experiment 2 (e.g. 32%) and experiment 3 (30%, 40%) are surprisingly high, compared to those observed *in vivo* in midguts of *C. quinquefasciatus* (DAR) and do suggest that the conditions simulated in the monolayer cells, to some degree resemble those prevailing *in vivo* in the midgut of a good host mosquito. However, it remains unclear how this *in vitro* exsheathment is taking place. It could be that the cells are secreting some factors which facilitate exsheathment or that the cells provide a good adherent surface for attachment of the sheaths.

What can be concluded from these experiments is that although *in vitro* exsheathment in this case does appear to some extent to be a physical process, in order for it to occur at a higher rate and be reproducible, as in the midgut of susceptible mosquitoes, some other stimulus is needed to facilitate exsheathment of microfilariae, which is not manifested readily *in vitro*.

Table 71: Showing in vitro exsheathment of B. pahangi in MOS 20A cells

Expt. No.	Vol. of MK/VP 12+20% FBS + MOS 20A (µl)	Vol. of HBSS (µl)	Incubation time (hrs) (mins)	Nos mff examined	% Exsheathment
1	1	2	1.30	21, 14, 23*	0, 0, 0*
	2	2	1.40	27	0
	3	1	1.15	37, 27	0, 0
	3	2	1.40	15, 26	8, 7
	3	2	1.15	39, 31	5, 0
	4	1	1.20	59, 47	3, 2
	5	1	1.30	35. 46	0, 0
2	2	1	1.30	37, 16	0, 6
	3	1	1.30	32, 45	13, 9
	4	1	1.40	58, 26	5, 8
	5	1	1.50	34	12
	10	1	1.50	58	3
3	3	1	2.50	66, 27, 18	2, 22, 17
	4	1	3.30	15, 31, 24	15, 28, 22
	5	1	3.30	35, 59, 39	3, 2, 3
4	3	1	3.00	51, 34, 52, 12, 57, 36	0, 21, 19, 8, 9, 0
	4	1	3.00	43, 19, 54, 51, 40	16, 11, 7, 10, 13
	5	1	3.00	55, 63, 62, 11, 35, 30	15, 6, 11, 22, 3, 13

* Figures separated by commas represent different replicates.

Table 72: In vitro exsheathment of B. pahangi in monolayers of MOS 20A cells.

Expt. No.	Incubation time (hrs)	Nos mff examined	% Exsheathment
1			
Tube 1	3	154	5
	3	107	4
Tube 2	3	127	5
	3	112	4
Tube 3	3	76	3
	3	110	6
2			
Tube 1	3	185	22
Tube 2	3	114	32
Tube 3	3	110	32
3			
Tube 1	3	126	18
	3	106	16
Tube 2	3	75	27
	3	89	21
Tube 3	3	91	40
	3	105	30

12. GENERAL DISCUSSION

The results of each section have already been discussed in great detail and have also been related to similar work by other workers, hence will not be repeated here. It is intended in this section to draw together some general conclusions from this study, to comment on any difficulties encountered and to suggest some possible further investigations related to this study.

Studies on the development of W. bancrofti in normal and aposymbiotic strains of C. quinquefasciatus, showed conclusively that the rickettsial like organism Wolbachia pipientis did not play any apparent role in the successful infection of C. quinquefasciatus by W. bancrofti. This investigation had been initiated by a suggestion of rickettsial involvement, in the development of filariae in a member of the Aedes scutellaris group by Duhrkopf and Trpis (1981). These findings were however not substantiated by the work of Meek and Macdonald (1982), whose results showed that nuclear genes were involved in the inheritance of susceptibility to filarial infection. Further studies on the crossing relationships among seven members of the Ae. scutellaris group carried out by Meek and Macdonald (1984) indicate that the effect of the rickettsiae like organisms in the Ae. scutellaris group varies from that observed in the Culex pipiens complex. These observations indicate that it is possible that there are differences in the type of rickettsiae found within Culex and Aedes mosquitoes, which would account for the differences observed in their effect on susceptibility to filariae and compatibility in the two mosquito species. Several types of variation in compatibility have been observed in wild and laboratory populations, and their genetic analysis, exhibit the complicated genetic nature of this phenomenon. In addition, Subbarao et al. (1977) observed 'segragation' in a

laboratory 'mutant', ISB 20, and Subbarao (1982) suggested that Wolbachia may not be the sole causative agent of the phenomena of cytoplasmic incompatibility. Furthermore, Meek (1981) produced evidence of nuclear inherited differences in susceptibility involving apparently aposymbiotic strains. All these variations on the effects of Wolbachia reported by various workers show the complex nature and mode of action of Wolbachia and call for further investigations on their structure and function. In theory, it would be useful to be able to isolate and culture these rickettsiae, then inoculate them into Culex and Aedes mosquitoes, to study their effect on cytoplasmic incompatibility and their role, if any, on filarial susceptibility. However, in practice, it has been difficult to establish rickettsiae in ovaries of adult mosquitoes by inoculation, (Yen, 1972) and neither are there any reports in literature of success in these type of studies.

Various geographical strains of C. quinquefasciatus all had low frequencies of the gene sb (filarial susceptibility to B. pahangi) (Obiamiwe and Macdonald, 1973) and had low susceptibility rates to B. pahangi. Failure in attempts to select for susceptible and refractory stocks of C. quinquefasciatus suggest that in this mosquito there is no major gene responsible for controlling susceptibility to B. pahangi. Rather, control is polygenic and probably affected by modifier genes, making it difficult to select for any one gene. Macdonald (1962a,b) was able to select a major gene f^m, controlling susceptibility not only to B. malayi, but to other thoracic dwelling filariae these being B. pahangi and W. bancrofti, but not to D. immitis and D. repens, which develop in the Malphigian tubules (Macdonald and Ramachandran, 1965). Subsequently, Sulaiman and Townson (1980) and Townson et al. (1981) showed that separate genes designated fi and fr were responsible for controlling

susceptibility to Dirofilaria immitis and D. repens in Ae. aegypti. The frequency with which the gene f^m occurs in various geographical strains of Aedes aegypti is variable and in most natural populations, it is relatively low (Rodriguez and Craig, 1973; Paige and Craig., 1975).

The higher susceptibility of C.p. molestus to B. pahangi observed raised hopes of the possibility of selecting for susceptible and refractory stocks, but problems encountered in feeding them on infective bloodmeals, since they were autogenous, led to termination of these attempts. In trying to overcome this problem, C.p. molestus females were crossed with a bloodfeeding stock of C. quinquefasciatus males, following Obiamiwe's (1970) method. This cross yielded very few progeny which did not feed.

The susceptibility of C. quinquefasciatus (DAR) and An.stephensi to B. pahangi inoculated directly into the thorax, increased significantly, showing that by by passing the midgut some of the factors responsible for killing B. pahangi could be avoided. However, since not all microfilariae inoculated into the thorax developed, this indicated that there were other factors in the thorax which inhibit development. Previously, it had been suggested that the 'midgut barrier' was the most important in determining the fate of ingested B. pahangi in C. quinquefasciatus (Obiamiwe, 1970). It appears that the gene(s) controlling susceptibility to B. pahangi in C. quinquefasciatus (DAR) and in An. stephensi does not limit its activity to the midgut, rather it affects the whole mosquito body. The normal development of sheathed microfilariae B. pahangi inoculated directly into the thorax showed that exsheathment occurs within the thorax, but it is not clear whether the mechanism of

exsheathment within the thorax is similar to that within the midgut. It is possible that exsheathment within the thorax is mainly a physical process, the thoracic tissues providing a suitable matrix for attachment preceding exsheathment, while in the midgut, secretion of yet unknown factors by the midgut cells or the effects of certain components of the blood, could play a role in exsheathment.

Attempts were made in preliminary experiments to infect mosquitoes using the enema technique. This technique would have been advantegeous since any factors anterior to the midgut would have been bypassed, enabling me to determine whether secretions from the salivary glands and other anterior parts of the alimentary canal play any role in limiting the development of microfilariae in these refractory mosquitoes. The main problem encountered was smooth entry and withdrawal of the micropipette, into the posterior midgut, without fatal injury to the mosquitoes.

Studies on exsheathment and migration of B. pahangi in C. quinquefasciatus (DAR), C.p. molestus and An. stephensi, with Ae. aegypti SS acting as a control, yielded some interesting results. In the three refractory species of mosquitoes, although some microfilariae did exsheath, migration to the thorax was almost negligible and was not enhanced by increasing the anticoagulant heparin, whose function it was to increase the clotting time of blood. It was originally thought that the rapid clotting of blood prevented migration of exsheathed microfilariae. In fact, high heparin concentrations had a detrimental effect, in that the mosquitoes became reluctant to feed. Those that fed became moribund and the migration rate decreased. These results are very different from those of Kartman (1953), who succeeded in increasing the migration of D. immitis to the Malphigian tubules of An. quadrimaculatus. He suggested that lack of anticoagulant in the salivary glands of mosquitoes

like C. quinquefasciatus were responsible for their refractoriness to D. immitis. However, the lack of an increase in the susceptibility of C. quinquefasciatus (DAR), C.p. molestus and An. stephensi to B. pahangi, as a result of adding heparin to an infective bloodmeal, suggests that absence of anticoagulants in the secretions of the salivary gland of these mosquitoes does not play any important role in determining their susceptibility to B.pahangi. In fact the evidence for the absence of anticoagulant is not conclusive, although it was reported by Metcalf(1945).

Other results also indicated that lack of exsheathment of microfilariae was not the sole reason for the lack of migration. This point was demonstrated more clearly when in C. quinquefasciatus (DAR) and An.stephensi fed on endopeptidase exsheathed B. pahangi suspended in blood did not migrate to the thorax. It was interesting to note that in susceptible Ae. aegypti SS, although migration occurred, it took place at a lower rate, compared to when it is fed on sheathed B. pahangi. This would suggest that the presence of the sheath in some way, plays a role in triggering the mechanisms which facilitate exsheathment and migration. On the other hand it could be due to reduced viability of in vitro exsheathed parasites. Furman and Ash (1983), using fluorescienated lectins, showed that the sheaths of mature microfilariae of B. pahangi have certain carbohydrates which bind to these lectins, which are not present in mature or immature exsheathed microfilariae. They suggested that it is possible that sheath carbohydrates are a component of the molecular trigger initiating exsheathment and development, once the mosquito ingests its microfilariae laden bloodmeal. They speculated on the effects of interaction between mosquito lectins and the sheath carbohydrates in the determination of successful maturation of the microfilariae to infective third stage larvae. Devaney and Howells (1979)

suggested that endopeptidase facilitates exsheathment by exerting a digestive action on at least part of the sheath. In the light of the studies of Furman and Ash (1983), it is possible that endopeptidase digests part of the sheath away, leading to the exposure of certain carbohydrates, which bind to mosquito lectins, thus facilitating exsheathment. A better understanding of the surface carbodhydrates of the sheath and cuticle of microfilariae is necessary, as it would facilitate development of immunological and chemical measures to disrupt their normal biological function.

The increase in the exsheathment and migration rates of B. pahangi in C. quinquefasciatus (DAR) as a result of feeding them on mixed infective bloodmeals of W. bancrofti and B. pahangi, led to the speculation that whatever factor(s) facilitates exsheathment and migration of these microfilariae, differs in its nature or that these two filariae have different thresholds for this factor(s). It can be hypothesized that when C. quinquefasciatus (DAR) ingested a mixed infective blood meal of W. bancrofti and B. pahangi, some factor(s) was secreted which readily facilitated the exsheathment and migration of W. bancrofti microfilariae. This factor(s) also enabled a small proportion of B. pahangi to exsheath and migrate but was not sufficient to allow a large number of B. pahangi microfilariae to exsheath and migrate since this factor had been used up by W. bancrofti microfilariae which are more sensitive to this factor. Although the results of these experiments were not very conclusive, being affected by the use of cryopreserved microfilariae W. bancrofti, which appeared to be less motile and viable than microfilariae from human donors, they clearly provide a basis for further experimentation on the interaction of these two microfilariae in C. quinquefasciatus, particularly to the types of changes they elicit within the midgut, when fed to

mosquitoes individually and in combination. Other interesting investigations which could be performed, would be to feed C. quinquefasciatus (DAR) on exsheathed W. bancrofti and sheathed B. pahangi to observe whether there would be any changes in the exsheathment and migration pattern of B. pahangi in this mosquito, and also to see whether the migration of W. bancrofti would in any way be altered by the absence of a sheath. In addition, C. quinquefasciatus could be fed on mixed infective blood meals containing both exsheathed W. bancrofti and B. pahangi, and the migration rates observed. Such studies would throw more light on the importance, if any, of the sheath of microfilariae in initiating the important processes of exsheathment and migration of microfilariae in mosquitoes, which are important prerequisites for the normal development of these microfilariae in susceptible mosquito hosts.

The pattern of exsheathment and migration of W. bancrofti in C. quinquefasciatus (DAR) and Ae. aegypti SS was not clearly defined. This was largely thought to be due to the adverse effects of cryopreservation and thawing of microfilariae and it would have been better to use blood taken directly from donors or allowed mosquitoes to feed directly on donors, had these been available.

Another aspect of migration that was investigated was the role, if any, of red blood cells. Investigations by other workers have shown that various components of the blood are involved in determining the development of parasites in their vectors. Rosenberg et al (1984) provided evidence that an erythrocyte substance, released by mosquito digestion, was needed for ookinete invasion of the gut epithelium and Maudlin (1983) reported that certain serum factors determined whether midgut infections of T. congolense were established and whether they matured in Glossina. There was no evidence of the role of red blood cells

in the migration of exsheathed B. pahangi in C. quinquefasciatus (DAR). Probably other factors in the midgut play a major role in inhibiting migration of these microfilariae. Neither were these factors systemic or present in the abdominal haemocoel since microfilariae inoculated into the abdominal haemocoel of C. quinquefasciatus (DAR) migrated to the thorax. However, there was no further development of most of the microfilariae which reached the thorax, again showing the presence of factors in the thorax which kill microfilariae.

Providing C. quinquefasciatus (DAR) with various metabolites, which might be missing and trying to alter the prevailing physiological state within the mosquito midgut, had no effect either in altering the refractory nature of these mosquitoes to B. pahangi. In Ae. aegypti SS cations had no effect on exsheathment and migration of B. pahangi, but they did slow down the rate of blood digestion and development of larvae, indicating some interference with the normal physiological state of the mosquitoes. This interference however did not alter the pattern of susceptibility in Ae. aegypti SS.

Sauerman and Nayar (in press) in their investigations in trying to characterize the refractoriness in Ae. aegypti (Vero beach strain, Florida) to infection by Dirofilaria immitis, put forward 3 suggestions as to how the gene controlling refractoriness, manifested itself physiologically. These were:-

(a) the presence or absence of a particular metabolite or antimetabolite.

(b) a particular host physiological balance that determines the host-parasite equilibrium.

(c) a particular morphological component at the ultra-structural level, such as membranes that surround intracellular parasites.

However, their investigations did not prove any of their suggestions and there is recent evidence provided by Bradley et al. (1984), that there are no membranes surrounding the intracellular parasites. The causes of refractoriness hence remain unknown.

Investigations on the effects of abdomen homogenates of C.quinquefasciatus (DAR),An. stephensi and Ae. aegypti SS on endopeptidase induced exsheathment of B. pahangi showed the presence of factors which inhibit exsheathment in homogenates of the former two mosquito species but not in Ae. aegypti SS homogenate. Heating the homogenates removed the inhibitory activity observed previously, indicating that these factors had been deactivated. The supernatants of these abdomen homogenates after centrifugation also showed a decrease in the inhibitory activity observed in whole abdomen homogenates. Whatever these inhibitory factors present in C. quinquefasciatus (DAR) and An. stephensi abdomen homogenates are, remain unknown and the way in which they inhibit endopeptidase induced exsheathment of B. pahangi microfilariae remains unclear. The inhibitory action observed was thought to be partly due to protein content of these homogenates binding to endopeptidase and preventing its activity, but since in Ae. aegypti SS abdomen homogenate, which also had protein, the exsheathment rate was high, strongly suggests the presence of other factors which inhibit exsheathment in C. quinquefasciatus (DAR) and An. stephensi abdomen homogenates.

The lack of manifestation of this inhibitory activity in midgut homogenates of the 3 species did not rule out its presence in C. quinquefasciatus (DAR) and An. stephensi, but suggested the dilution of these factors to concentrations that were unable to initiate any inhibitory activity. These studies did not however reveal the exact location or origin of these inhibitory factors.

Although various workers have succeeded in inducing exsheathment of various microfilariae *in vitro*, by using various techniques, they have only been able to speculate as to how exsheathment occurs. It is of primary importance for studies to be conducted, which would elucidate the mechanisms of *in vitro* exsheathment and how this can be inhibited *in vitro*. Once such studies have been carried out, it would be possible to relate them to the *in vivo* situation in a good host mosquito, and to see whether the causes of *in vivo* exsheathment of microfilariae, in a mosquito midgut, can be explained. This would be a stepping stone to understanding how genes controlling susceptibility or refractoriness of mosquitoes to filariae, translate their coded messages into physiological processes.

Further investigations on the *in vitro* exsheathment, motility and migration of *B. pahangi*, incubated with midguts of the 3 species of mosquitoes, showed that exsheathment did occur at a very low rate. In this case, exsheathment was thought to be primarily a physical process, the midgut providing an adhering surface for the microfilariae. Although CO_2 concentration was observed to have no effect on exsheathment of microfilariae in *in vitro* experiments, it does not rule out its participation *in vivo*, in the mosquito midgut. The CO_2 content in mosquito midgut is most likely much higher than that used *in vitro* and it changes with ingestion and digestion of the bloodmeal, which could in some way affect the behaviour of the microfilariae ingested in the bloodmeal. It would be worthwhile to experiment further on the effects of CO_2 on microfialriae.

The lack of migration of microfilariae from *C. quinquefasciatus* (DAR) midgut *in vitro* and its occurrence from midguts of *Ae. aegypti* SS, showed definitely that the stimulus which facilitates migration, originates

within the midgut of mosquitoes and not in the abdominal haemocoel or thorax. The motility of B. pahangi microfialriae was prolonged by the presence of midguts in the incubating medium, their absence causing a rapid decrease in motility. Probably they provided nutrient substances.

In an attempt to increase the in vitro exsheathment rate of B. pahangi microfialriae, mosquito cells either in suspension or monolayers were provided for incubation purposes, on the basis that they would provide a more mechanically stable and adherent surface. In the case where monolayers were used for incubation, surprisingly high exsheathment rates were obtained in some experiments. It is possible that exsheathment was probably due to the cells providing a better surface for adherence prior to exsheathment, or in addition, the mosquito cells secreted substances which facilitate exsheathment. Where cells in suspension were used for incubation, the percentage exsheathment of microfilariae although variable increased with time. This could suggest the gradual accumulation of some factors secreted by the cells, which aided the exsheathment process. It would be interesting to assay the medium in which the cells and microfilariae were incubated in, at various time intervals after incubation, to see whether there were any changes in its constitution, which might be related to an increase in the exsheathment rate with time. Although these in vitro exsheathment experiments indicate that the exsheathment process is a physical one, it is possible that in fact in the in vivo situation, in the mosquito midgut, exsheathment of microfilariae occurs in a similar manner. It is possible that following the ingestion of an infective bloodmeal, certain factors are secreted by the midgut cells of the mosquito, which create a fine matrix to which microfilariae attach and exsheath. The peritrophic membrane which is formed around the blood following the ingestion of a

bloodmeal is an example of such a matrix, and perhaps it would be worthwhile in future to investigate if this membrane could in any way be participating in the exsheathment of microfilariae, in the mosquito gut.

These studies on the factors affecting the establishment and development of microfilariae in various mosquito species, particularly B. pahangi microfilariae in C. quinquefasciatus (DAR), show the complexity of the factors involved and the manner in which they interact remains difficult to elucidate. The'midgut barrier' no doubt remains the most important in determining the further development of B. pahangi microfilariae in refractory mosquito species, although the thorax too has been shown to play an important role as well. It is apparent from the results of these studies that further investigations are necessary particularly on the factors affecting exsheathment and migration of microfilariae, as they play a major role in determining the vectorial capacity of mosquito species. Once these process are understood, it would be a step nearer to understanding the nature of the genes controlling susceptibility to filarial parasites and how they work.

13 SUMMARY

1. Literature pertaining to important vectors of Brugia and Wuchereria infections, genetic variation of the vectorial capacity of mosquitoes to various pathogens and factors affecting the exsheathment, migration and development of filariae in mosquitoes was reviewed.

2. Investigations on the development of W. bancrofti in normal and aposymbiotic strains of C. quinquefasciatus showed that Wolbachia is not a requirment for successful infection of C. quinquefasciatus by W. bancrofti. The growth and development of W. bancrofti proceeded at the same rate and infective larvae attained the same size in aposymbiotic and normal strains.

3. Four strains of C. quinquefasciatus from Dar-es-salaam, Monrovia, Bangkok and Suva had low susceptibility to B. pahangi and the frequencies of the gene sb (filarial susceptibility to B. pahangi) calculated were 0.20, 0.16, 0.28 and 0.15 respectively .

4. A strain of C.p. molestus from Dagenham, London was also found to have a low susceptibility to B. pahangi but it was slightly higher than that of C. quinquefasciatus (DAR) to B. pahangi.

5. C. quinquefasciatus (DAR) did not respond to selection for refractoriness and susceptibility to B. pahangi. This was thought to be due to there being more than one gene controlling susceptibility to this filarial parasite. Neither did C.p. molestus respond to selection for bloodfeeding refractory and susceptible stocks to B. pahangi. Furthermore in the latter case, the autogenous nature of this mosquito species made blood feeding and selection difficult.

6. The susceptibility of C. quinquefasciatus (DAR), C.p. molestus, An. stephensi and Ae. aegypti SS to B. pahangi, determined by inoculation with exsheathed and unexsheathed B. pahangi showed that C. quinquefasciatus

(DAR) inoculated with both types of B. pahangi, had susceptibility rates ranging from 10-39%, which was higher than those rates obtained when C. quinquefasciatus (DAR) was fed on infective blood via a membrane feeder. Similarly, the susceptibility rate of An. stephensi to B. pahangi increased from 5% to 23% when the filariae were inoculated into the thorax rather than being fed to them via a membrane. There was no significant increase in the susceptibility rate of C.p. molestus inoculated with B. pahangi, while Ae. aegypti SS were found to be more susceptible to unexsheathed B. pahangi (88%) then to in vitro exsheathed B. pahangi (36%). This low susceptibility rate may reflect damage during in vitro exsheathment of the microfilariae.

7. A heparin concentration of 10 iu per ml of the infecting blood, slightly increased the migration of B. pahangi from the midgut of C. quinquefasciatus to the thorax, compared to those mosquitoes fed directly on a cat (no-anticoagulant). However, high heparin concentrations of 300 or more international units per ml of blood decreased the microfilariae migration, led to a reluctance of mosquitoes to ingest a bloodmeal and caused a decrease in the volume of blood ingested.

8. Exsheathment and migration of B. pahangi were shown to occur in Ae. aegypti SS and by 4 hours post ingestion there was 100% exsheathment and 49% migration of B. pahangi to the thorax. However in C. quinquefasciatus (DAR) the exsheathment was only 35% 4 hours post feeding and no migration took place. Similarly, in C.p. molestus although some exsheathment took place (13-64%), the migration rate was very low (0.3-2%). In An. stephensi, there was 7-62% exsheathment of B. pahangi but migration to the thorax was in the range of 3.4-11%.

9. It was shown that there was no increase in the migration rate of endopeptidase exsheathed B. pahangi fed to C. quinquefasciatus (DAR) and

An. stephensi, at heparin concentrations of 10-50 i.u. per ml of blood, compared to those rates observed when these 2 mosquito species were fed on unexsheathed B. pahangi in the bloodmeal. In Ae. aegypti SS, the migration rate of exsheathed B. pahangi was less than that of unexsheathed B. pahangi, these rates being 13-51% and 90% respectively at 24 hours post feeding.

10. The exsheathment and migration rates of B. pahangi in C. quinquefasciatus (DAR) fed on mixed infective bloodmeals of W. bancrofti and B. pahangi increased significantly compared to those rates observed when C. quinquefasciatus (DAR) had been offered infective bloodmeals of B. pahangi only. These two processes did not show any trend with time and there was alot of variability in the percentage exsheathment over a 24 hour period. In Ae. aegypti SS, the exsheathment and migration rates of both B. pahangi and W. bancrofti were very variable, due to the small numbers of microfilariae ingested and the variability in the distribution of microfilariae among the individual mosquitoes, making it difficult to determine the exsheathment and migration pattern of these 2 filariae. In considering the variability in exsheathment and migration rates of W. bancrofti in both C. quinquefasciatus (DAR) and Ae. aegypti, the adverse effects of cryopreservation and thawing of these microfilariae were thought to have played an important role.

11. On dissection day 12 and 14 post infection, 27% of C. quinquefasciatus (DAR) fed on mixed infective bloodmeals were found to be infected with an average of 2 larvae per mosquito. 78% of the total numbers of larvae found were W. bancrofti while 22% were B. pahangi. Both types of infective larvae had mean anal ratios of 3.8:1. 41% and 54% of Ae. aegypti SS from two similar experiments, dissected on the same days as C. quinquefasciatus (DAR), were found to be infected, each infected mosquito having an

average of 2-4 larvae. 54% of the total infective larvae recovered were W. bancrofti, whilst 46% were B. pahangi in the first experiment, and 57% in the second experiment were W. bancrofti while 43% were B. pahangi. The mean anal ratios of the W. bancrofti and B. pahangi in experiment 1 were 4:1 and 3:1 whilst in the second experiment this was 4:1 for both infective larvae.

12. The exsheathment and migration of W. bancrofti in C. quinquefasciatus (DAR) and Ae. aegypti SS were examined in two similar experiments performed on separate days. In the first experiment, the exsheathment rate in C. quinquefasciatus (DAR) increased with time up to 3 hours post feed but in Ae. aegypti SS there was no apparent trend with time. However in experiment 2, the exsheathment rate of W. bancrofti in both mosquito species increased with time. Percentage exsheathment within and between C. quinquefasciatus and Ae. aegypti was found to be significantly different. A comparison between the exsheathment rate of W. bancrofti in C. quinquefasciatus (DAR) and Ae. aegypti SS fed only W. bancrofti and those fed on a mixed infective bloodmeal, was found to be significantly different ($P < 0.001$). The % migration of W. bancrofti in both C. quinquefasciatus (DAR) and Ae. aegypti SS increased with time but there were significant differences in the migration rate. Due to the variability in the rates of exsheathment and migration observed in C. quinquefasciatus and Ae. aegypti, as a result of using cryopreserved W. bancrofti, it was not possible to get a clear picture of the exsheathment and migration patterns of W. bancrofti in these two species of mosquitoes.

13. C. quinquefasciatus (DAR) and Ae. aegypti SS mosquitoes did not feed readily on suspensions of exsheathed B. pahangi in human and turkey serum and ingested small volumes of this suspension. There was

no migration of exsheathed microfilariae in serum from the midgut of C. quinquefasciatus to the thorax whereas in Ae. aegypti, migration occurred. None of the C. quinquefasciatus (DAR) mosquitoes which ingested exsheathed B. pahangi suspended in serum became infected. However 71-100% of Ae. aegypti were found to be infected with B. pahangi on dissection day 10 postfeed. Egg development took place in those mosquitoes of both C. quinquefasciatus (DAR) and Ae. aegypti SS which ingested a serum meal.

14. Cations were found to decrease the rate of blood digestion in Ae. aegypti SS, but did not affect the normal exsheathment and migration of B. pahangi. They caused a decrease in the rate of larval development in the thorax of Ae. aegypti SS.

15. It was shown that sulphadiazine, penicillin, ascorbic acid and whole body extracts of mosquitoes had no effect on the innate insusceptibility of C. quinquefasciatus (DAR) to B. pahangi or the susceptibility of Ae. aegypti SS to B. pahangi.

16. The migration of exsheathed B. pahangi from the abdominal haemocoel of C. quinquefasciatus (DAR) to the thorax indicated there were no systemic factors preventing migration in C. quinquefasciatus mosquitoes. Very few of the microfilariae that migrated successfully to the thorax developed to the infective larval stage. In control Ae. aegypti SS, migration to the thorax occurred and the microfilariae developed normally to the third larval stage.

17. Studies on the in vitro exsheathment of B. pahangi showed that abdomen homogenates of C. quinquefasciatus (DAR) and An. stephensi were capable of inhibiting endopeptidase induced exsheathment of these microfilariae, whereas abdomen homogenate of Ae. aegypti SS did not inhibit exsheathment. Midgut homogenates of the 3 species of mosquitoes did not inhibit exsheathment.

18. Exsheathment occurred in B. pahangi microfilariae incubated with midgut and thoracic tissue, in hanging drop preparations, in the absence of endopeptidase. The rate of exsheathment was low and variable. Exsheathment also occurred when microfilariae of B.pahangi were incubated in hanging drops made from mosquito cells. The percentage exsheathment was low and variable, but increased with time. The exsheathment rate increased when microfilariae were incubated in monolayers of mosquito cells and in some experiments the exsheathment rate was higher than that observed in vivo in C. quinquefasciatus (DAR) midgut. CO_2 concentration was shown to have no effect on in vitro exsheathment of B.pahangi microfilariae.

19. In vitro migration of B. pahangi was observed from Ae. aegypti SS midguts but not from C. quinquefasciatus (DAR) midguts, indicating that the stimulus for migration originated from the midgut and not the haemocoel or thorax. Motility of microfilariae B. pahangi was prolonged by the presence of midguts in the incubating medium, motility being noticeably better in the presence of Ae. aegypti SS midguts than in C. quinquefasciatus (DAR) or An. stephensi midguts. MK/VP12 + 20% FBS medium was the most suitable for incubating microfilariae and prolonged their motility compared to Hayes saline and Weymouths medium.

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