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FACTORS AFFECTING THE ROTATIONAL USE OF INSECTICIDES
FOR THE MANAGEMENT OF RESISTANCE IN MOSQUITOES

by

SITI HAWA KASIM

December 1991

Thesis submitted for the degree of Doctor of Philosophy
in the University of London

....dedicated to my beloved husband

and three children,

Irwan Johari

Imran Johari

Nurrul Hana Johari

ABSTRACT

The rotation of the use of chemically unrelated insecticides has been advocated to delay the build up of resistance. To examine this concept in the laboratory, Anopheles albimanus and Culex quinquefasciatus were subjected to two kinds of rotational selection which may be referred to as short term pre-planned rotation and "opportunistic" rotation. No difference was observed between these two methods in term of the time for each resistance level to reach 50%.

The effectiveness of selection for resistance depends on the protection conferred by resistance genes and this was tested by laboratory releases of the DDT resistant and susceptible homozygotes and heterozygotes of An. gambiae into DDT sprayed and unsprayed miniature huts. All the genotypes were killed with freshly applied DDT but survivors were observed from month 2, 3 and 5 onwards for RR, RS and SS genotypes, respectively.

Persistence of DDT on the sprayed wall and roof of the mud hut was studied by bioassays and biochemical assays on scrapings from the wall and roof.

Linkage between resistance genes could effect the rotational use of insecticides. No linkage was found

between propoxur and dieldrin resistance genes by combining bioassay and biochemical methods.

As a preparation for a field studies in Malaysian field collected Aedes aegypti and Culex quinquefasciatus larvae were selected with temephos and Bti. Cx quinquefasciatus responded to temephos selection but Ae. aegypti did not respond to temephos and neither species responded to Bti selection.

Caged adults were exposed to thermal fogging in the field. The partially resistant Cx quinquefasciatus strain hardly survived any better than susceptible strains.

Resistant and susceptible larvae were exposed to water samples from containers which had been treated with temephos sand granules. 100% mortality was obtained for all the strains up to week 6. Resistant Culex started to survive at week 7 but susceptibles did not do so until week ten.

The prospects for the various proposed strategies for resistance management are discussed.

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CHAPTER 1

1.1 GENERAL INTRODUCTION

Resistance means the inherited ability to survive more of a toxicant than normal for the species (Brown and Pal 1971). The emergence of insecticide resistance is not a new event - being first reported by Melander (1914) when he recognised that resistance to lime sulphur in the San José scale (Aspidiotus perniciosus) had evolved by natural selection. He also recognised that incomplete coverage and genetic recessiveness of the resistance were relevant to the rate of evolution of resistance. Since then resistance has continued to increase, slowly at the beginning, but becoming more rapid with the introduction of DDT during and after the Second World War. Resistance has now become a major problem, with at least 447 species of arthropods being resistant to at least one compound (Georghiou 1986). This problem has continued to spread and to affect disease control programmes, as well as those in the agricultural sector. It is thus of great importance both in agriculture and in public health to try to safeguard the available chemicals against the development of resistance.

Since Melander's first observation on resistance, relatively little progress has been achieved in taking counter-measures for retarding or forestalling its

evolution. Since the genes for resistance are favoured by Darwinian selection when pesticides are used, it has seemed that its evolution is inevitable and is just a matter of time. However, there do appear to be theoretically possible ways to preserve the resource of susceptibility and the question is now whether good management of pesticide usage can at least delay the process of evolution of resistance. Resistance management has been a great concern to many people from various sectors, the chemical companies for example, are very concerned about the length of usable "life" of their products as the cost to discover, develop, register and manufacture a new compound may exceed \$30 million (Metcalf 1980, Jackson 1987).

Management of insecticide resistance has been discussed by numerous authors over the past few years (e.g. Comins 1977a,b, 1979a,b, 1986; Curtis 1981, 1985, 1987; Curtis and Lines 1986; Curtis et al. 1978, 1991; Denholm et al. 1985, 1987, 1990; Daly 1987; Daly and McKenzie 1987; Georghiou 1980, 1983, 1986; Georghiou and Taylor 1986; Hammock and Soderlund 1986; Leeper et al. 1986; Roush and Miller 1986; Roush and Daly 1990; Roush 1989; Sawicki 1975; Sawicki and Denholm 1987; Tabashnik 1986, 1989, 1990a,b; 1991). One aspect of the problem is ways of using insecticides to forestall the appearance of resistance, and the other is how to manage insecticide use once resistance to the compound concerned has been detected or when there is a control failure in the field. This problem has been dealt with

extensively in the literature (Brown 1977; Curtis 1985, 1987; Daly and McKenzie 1987; Denholm et al. 1983, 1985, 1990; Forrester 1988; Forrester and Cahill 1987; Georghiou 1983; Hemingway and Lines 1985; Hammock and Soderlund 1986; Leeper et al. 1986; MacDonald et al. 1983a,b; Ozaki 1983; Roush 1989; Sawicki and Denholm 1986,1987; Tabashnik 1989, 1991;). However little has yet been done in the field or in the laboratory to test the applicability of the ideas which have been proposed. It is the aim of this study to investigate some factors in the laboratory that are relevant to methods intended to delay the evolution of insecticide resistance.

Resistance genes raise the tolerance to one or more insecticides but in general do not give absolute protection. To understand and begin to quantify selection pressures for resistance it is necessary to determine whether and to what extent examples of resistance detected in the laboratory would protect the insects in the field.

1.2 FACTORS INFLUENCING THE EVOLUTION OF INSECTICIDE RESISTANCE.

Before manipulating or devising any management strategies for delaying or forestalling the evolution of pesticide resistance, a thorough understanding of the

factors that influence the selection process is desirable. These factors have been reviewed by Georghiou and Taylor (1977a,b) and Georghiou (1980, 1983). They broadly divided these factors into three categories, i.e. biological, genetical and operational. Table 1.1 is based on their list, but has been updated and made applicable specifically to mosquitoes. Most factors in the biological and genetical categories are beyond human control as these factors are the inherent qualities of the species or the population, but their assessment is essential in determining the "risk" of resistance appearing in a particular population. The operational factors, on the other hand, are man-made and are thus within our control. These factors could be altered as part of a resistance management programme, but not to the extent that adequate control of the pest is lost. Certain of these factors have received special attention in the search for practical approaches to management of resistance.

1.3 RESISTANCE MANAGEMENT THEORY

Many studies have contributed information which provides a background for modelling of resistance management systems. Based on these studies Georghiou (1980, 1983) formulated his views of resistance management. He divided it into three categories, i.e. management by moderation, management by saturation and management by multiple attack.

Table 1.1

SUGGESTED FACTORS INFLUENCING THE SELECTION OF RESISTANCE TO INSECTICIDES IN THE FIELD POPULATIONS OF MOSQUITOES (based on Georghiou and Taylor [1977] with modifications)

A. Genetics

- 1-Frequency of R alleles (mutation / selection equilibrium or balanced polymorphism)
- 2-Number of R alleles (monogenic/polygenic)
- 3-Dominance of R alleles under field conditions
- 4-Penetrance and expressivity (i.e the extent to which genes confer effective protection)
- 5-Past selection by other chemicals
- 6-Effect of R alleles on fitness
- 7-Extent of integration of R genes with fitness modifying factors
- 8-Linkage of different R alleles /linkage equilibrium.
- 9-Gene duplication or amplification

B. Biological factors

a.Biotic

- 1-Generation turn over (but see Tabashnik 1990b)
- 2-Offspring per generation
- 3-Seasonal fluctuations in density

b.Behavioural

- 1-Isolation, mobility, migration
- 2-Anthropophily /zoophily
- 3-Fortuitous survival, refugia
- 4-Endophily/exophily

C. Operational factors

a.The chemical

- 1-Chemical nature of pesticide
- 2-Relationship to earlier used chemicals (cross resistance)
- 3-Persistence of residues of particular formulations

b. The application.

- 1-Application according to a regular schedule or in response to disease outbreaks
- 2-Dosages sufficient to kill heterozygotes and/or homozygotes
- 3-Life stage(s) selected (larvae /adults)
- 4-Selection on both sexes or on mated females only
- 5-Mode of application (house spaying/fogging/net impregnation etc)
- 6-Space-limited selection (% of houses covered/mosaic coverage etc)
- 7-Alternating selection (rotation over time)
- 8-Selection by mixtures.

1.3.1 Management by moderation

The aim of this form of management is to maintain the frequency of the resistance genes at a low level by either reducing the selection pressure or by allowing the opportunity for immigration of susceptible individuals. The importance of the latter has been demonstrated in laboratory experiments with the housefly, Musca domestica, (Taylor et al. 1983) and Anopheles gambiae mosquitoes (Prasittisuk and Curtis 1982).

Selection pressure could be reduced by either :

a. decreasing the dose of insecticides to that which allows the survival of some susceptibles.

Georghiou and Taylor (1977b) have studied the effect of several variables in delaying the evolution of insecticide resistance using computer models and concluded that lower insecticide dose levels and the presence of refugia are potential methods of preventing or delaying the evolution of resistance. Comins (1977a) also recommended the low pesticide use strategy as a method to delay resistance and he favoured it in comparison with the high dose strategy (see below) which requires high immigration by susceptibles and functional recessiveness of the resistance genes, which would be difficult to achieve in the field. However, Curtis (1985, 1987) has argued that low dosage of insecticide is more likely to cause a rapid evolution of insecticide

resistance, as low dosages may only kill susceptible individuals but not resistance heterozygotes. Furthermore, the low dosage method has the serious danger that it may defeat the purpose of pest control. In a malarious area, for example, the purpose of spraying is to reduce the mosquito survival to a sufficient extent to reduce or stop the transmission of the disease, and in highly endemic areas very high mosquito mortality is required to make any progress against the disease.

b. using non-residual chemicals and avoidance of slow release formulations, e.g. by using space-spraying.

This method of management has been studied by Denholm et al. (1983) with regard to the development of pyrethroid resistance by M. domestica. In their study they treated the buildings on one farm with permethrin, which is a persistent pyrethroid, and on another farm they used bioresmethrin as a non-persistent space-spray. Adequate control without any resistance was obtained on the farm treated with bioresmethrin throughout the experiment, but not on the farm where permethrin was used, because resistance developed after 3 weeks. The authors explained this result by pointing out that with the persistent insecticide the insects were in frequent or continuous contact with it, whereas the space sprays killed only adult flies present at the time of treatment but not those emerging or immigrating

subsequently. This method of management may give adequate control in developed countries or in urban areas where people can afford to use space sprays. But this is not practical against rural malaria vectors as the people in the areas concerned could not afford the frequent use of such sprays.

Computer simulations by Taylor and Georghiou (1982) supported the view that short-lived insecticides would tend to delay or forestall the evolution of resistance. This was more pronounced when both larvae and adults were assumed to be exposed to the insecticides and the doses applied were sufficiently high to kill the heterozygotes (see below). The presence of susceptible immigrants at optimal rates also enhanced the process. Essentially the same conclusions were also reached from the computer simulations of Taylor et al. (1983) and Mani and Wood (1984) on the use of space-sprays and short-lived insecticides.

c. decreasing the frequency of use of pesticides by developing alternative control measures in an integrated pest management (IPM) programme.

This has been advocated by Weidhaas and Haile (1985) among many others. The use of biological control agents such as carp or tilapia fish which, when young, eat mosquito larvae and when older are of nutritional value to the people, may well allow the decreasing use of pesticides. For example

the successful use of the larvivorous fish Oreochromis spilurus spilurus against An. arabiensis and malaria transmission in northern Somalia proved the effectiveness of the use of fish as a biological control agent for controlling the breeding of mosquito vectors in certain types of man-made water sources (Alio et al. 1985). Reuben et al. (1990) reviewed other examples of use of metazoa for biological control of mosquitoes and de Barjac and Sutherland (1990) reviewed the use of bacteria for the same purpose. Smith et al. (1991) suggested that Spiroplasma taiwanense (a prokaryote) or toxins produced from it, are potentially useful for use in integrated mosquito control programmes. However, biological control is probably more applicable to the control of domestic breeding of mosquitoes such as Aedes aegypti and Ae. albopictus than malaria mosquitoes.

Rajagopalan et al. (1990) reviewed environmental and water management for Culex and Anopheles control. Environmental control by cleaning up the breeding sites of Aedes mosquitoes is another approach which can be effective (Chan et al. 1990) and floating layers of expanded polystyrene beads suppress mosquito breeding in tanks and pits (Curtis et al. 1990).

Sharma (1987) described the joint use of environmental management and biological control against malaria in Gujerat, India. However, although IPM is attractive in

theory, in practice relatively little has yet been done in its application to mosquitoes and it may not be practical in controlling malaria in highly endemic rural areas.

As mentioned above, migration of susceptible individuals may reduce the frequency of the resistance genes to a low level (Georghiou and Taylor 1977a,b; 1986; Comins 1986; Curtis et al. 1978; Taylor and Georghiou 1979; Wood and Mani 1981; Taylor et al. 1983; Curtis 1985, 1987; Roush and McKenzie 1987; Roush 1989; Tabashnik 1990a,b). Migration of susceptible individuals will "dilute" the frequency of resistant individuals that have survived the insecticide exposure in the treated areas. This is particularly beneficial in the use of mixtures or where resistance is effectively recessive (see below) and in these cases few selected individuals would survive so long as resistance was rare and therefore the breeding population would come mainly from refugia (Curtis 1985, 1987; Comins 1977a and 1986). However immigration of susceptible individuals and its impact on the treated population is difficult to measure in the field (Rawlings and Davidson 1982, Denholm et al. 1985). The models of Georghiou and Taylor (1977b) and Taylor and Georghiou (1979) assumed that the pool of immigrants would remain unaffected by outward migration, but Comins (1977a) argued that the immigrant pools would eventually be contaminated by outward migration from treated areas. Comins (1977a) also demonstrated that

there exists a critical migration rate above which the evolution of resistance would be strongly retarded.

The leaving of some areas as untreated refugia (Georghiou and Taylor 1977b) would increase the likelihood of the immigration of susceptible individuals into the treated areas. They studied the importance of refugia and gave an example where, with 20% of the population treated as refugia, it would take more than 20 generations for a resistance gene to reach a level of 50%, compared with one generation in the absence of refugia. Curtis and Lines (1986) and Curtis (1987) also emphasised the importance of refugia in retarding the evolution of insecticide resistance. If some areas were deliberately left untreated this could be considered as a form of "management by moderation". In malaria control programmes, this method of management has been unintentionally practised, as there are almost inevitably many huts, houses or animal sheds which are left unsprayed either due to refusal by the occupants to admit the spray teams or the absence of the occupants, leaving the houses locked, when the spray teams arrive. However, for this situation of incomplete coverage to be beneficial it is important that in the treated houses the dose applied is high enough to kill not only all susceptibles (SS) but also heterozygous (RS) resistant individuals. This is explained in the next section.

1.3.2 Management by saturation

The effectiveness of this type of management depends on the effective recessiveness of the resistance genes. When the resistance genes are recessive almost all the exposed susceptible homozygous and heterozygous individuals would be killed. It may be possible to ensure that this situation applies by manipulating the dosage of insecticide applied or the frequency of re-application. As shown in fig. 1.1 if the dose A is applied, only the SS (susceptible) individuals would be killed, i.e. resistance is said to be effectively dominant, whereas a dose B would kill all SS and RS individuals, in which case resistance is effectively recessive. However in many cases the RR and RS regression lines are close together (Fig.1.2). In this case, if a dose B is applied only a certain percentage of the RS individuals would be killed. In this case resistance is said to be incompletely dominant.

It would be extremely advantageous to kill all the heterozygotes when (a) the resistance gene is still rare and (b) part of the population escapes exposure. Curtis (1987) showed numerical models supporting the high-dose strategy to ensure the killing of resistance heterozygotes on condition that a proportion of the population is unexposed to the insecticides, resistance genes are initially rare, there is uniform exposure and the residues are not allowed to decay

Fig: 1.1 The dose-response lines for the three genotypes where resistance is determined by a single gene and dominance is intermediate. RS and RR can be separated by a discriminating dose.

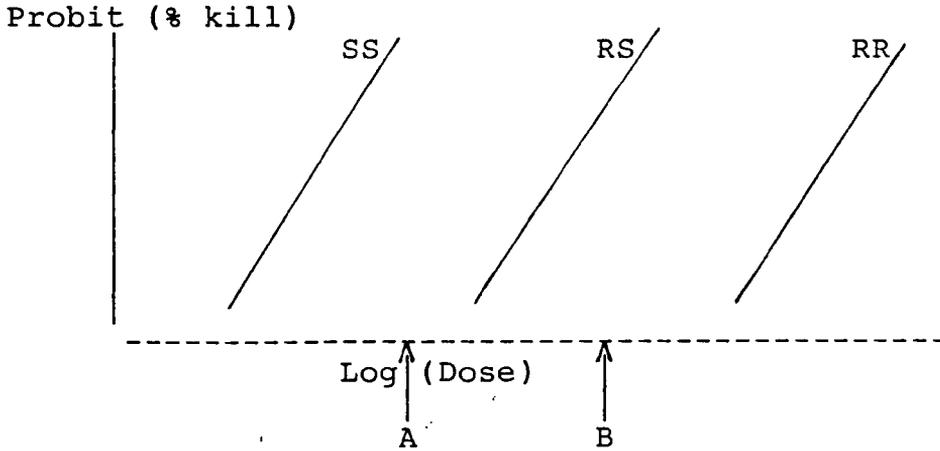
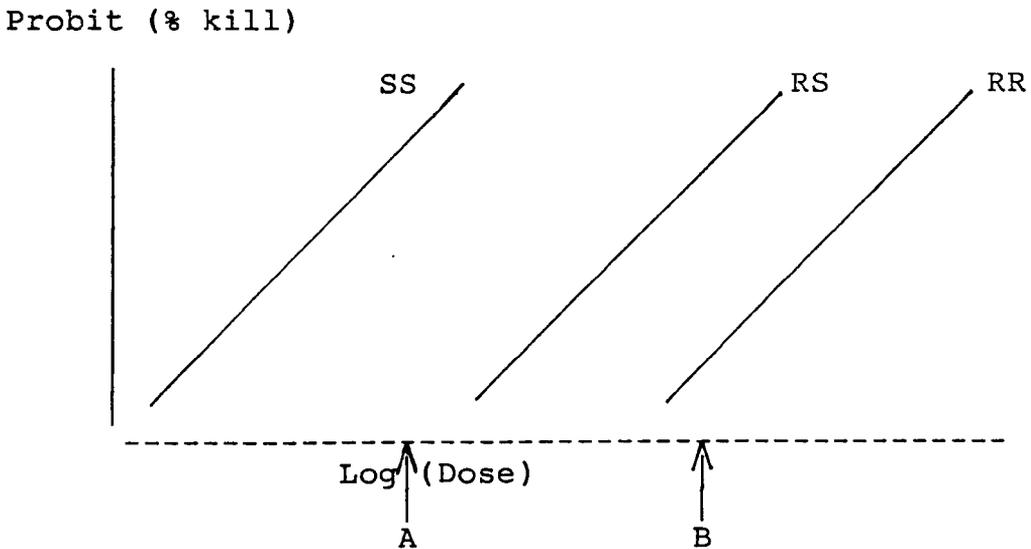


Fig : 1.2 The dose-response lines for the three genotypes where resistance is determined by a single gene and resistance is incompletely dominant. RS and RR cannot be separated by a discriminating dose.



to a level at which heterozygotes would survive. Table 1.2 shows further numerical models supporting this concept.

In a natural environment where no pesticide has been used, resistance alleles are expected to be rare and the homozygotes for them would therefore be extremely rare (the square of the resistance gene frequency according to the Hardy-Weinberg ratio). Thus in the example shown in table 1.2(i) when the resistance allele is still rare, the use of a high dosage which kills all $A^R A^S$ and $A^S A^S$ individuals causes the resistance gene frequency to change very little. It is assumed that in this table that 20% of the population are unexposed to the insecticide and therefore unselected. As already discussed unexposed individuals could either be due to immigration from untreated areas, or incomplete coverage within the treated area, either purposely by leaving a few houses unsprayed or due to refusal by the people to the spraying of their houses. They could also be due to the behaviour of the mosquitoes themselves which may refuse to enter the sprayed houses either because of naturally exophilic behaviour or the repellent effect of the insecticide itself. Another way of introducing unexposed individuals into treated areas could be by releasing susceptible individuals, especially non-biting males (Curtis et al. 1978, Taylor and Georghiou 1979, Curtis 1981). Under the conditions assumed in table 1.2 (i) the number of $A^R A^R$ individuals that survived the high dose would be dwarfed by the 20% of the population that escape exposure. If however the dose applied only kills $A^S A^S$ individuals

[Table 1.2(iii)] the frequency of the resistance gene increases by about five fold in one generation. Only a slightly better result is observed in table 1.2(ii) where the dosage used is high enough to kill all $A A^R S$ individuals but only 50% of $A A^R$.

Wood and Mani (1981) argued that when resistance genes are rare, refugia should be left unsprayed whenever vector control is not essential, and a dosage sufficient to kill all $R S$ and $S S$ genotypes should be applied in sites that would only be encountered by females after mating (i.e. adulticides in houses, which male mosquitoes may not enter, were preferable to larvicides). They also suggested that, when resistance reaches a detectable level, the dosage applied should be reduced and more insects should be allowed to escape, if practical, but if the heterozygote is becoming more resistant (due to the evolution of dominance modifying genes) the dose applied should be increased in order to ensure that the R gene remains effectively recessive.

Whether any of these forms of resistance management could be used depends on the feasibility of making the resistance genes effectively recessive. This will depend on the properties of the resistance and susceptibility alleles and on the dosage and persistence of insecticide applied to a particular population.

Tabashnik and Croft (1982) and Tabashnik (1990a,b) argued that the high dose strategy to kill all the

Table: 1.2 One-generation comparison of using pesticides at high or low dose

	No. of individuals				Total	Freq. R of A
	R R A A	R S A A	S S A A			
No. in populations at Hardy-Weinberg equil.	90	59,820	9,940,090		10,000,000	0.30%
If 20% escape exposure:	18	11,964	1,988,018			
& (i) if 80% are exposed to a dose which kills S S R S A A and A A : then survivors as parents:	72	-	-			
or (ii) if 80% exposed S S to dose which kills A A R S but only 50% of A A : then survivors as parents:	90	11,964	1,988,018		2,000,054	0.3036%
	72	23,928	-			
or (iii) if 80% exposed S S to dose which kills only A A : then survivors as parents:	90	35,892	1,988,018		2,024,000	0.8911%
	72	47,856	-			
	90	59,820	1,988,018		2,047,928	1.465%

heterozygotes would be unachievable because a variety of different R alleles probably arise by mutation and/or gene duplication and any which was effectively dominant would be rapidly selected. Furthermore, there are of course limits to the use of high doses in the field imposed by safety and economic criteria. Studies of effective dominance in field (or simulated field) conditions, particularly as pesticide residues decay, are needed to determine whether there is any real prospect of implementation of a high-dose strategy. Only a few studies of this question have been carried out in mosquitoes (Rawlings et al. 1981; Curtis et al. 1984; Roush et al. 1986; Mpofo et al. 1988). It was therefore part of this study to investigate this question more fully under controlled conditions.

1.3.3. Management by multiple attack

This type of management is aimed at achieving control through the action of two or more independently acting insecticides, e.g. by the use of mixtures of insecticides, or different insecticides in rotation. Mixtures and rotations as counter-measures for resistance have been studied either by experiment (Cutright 1959; MacDonald et al. 1983a,b; Georghiou et al. 1983; Denholm et al. 1983, 1985) or theoretically (Kable and Jeffery 1980; Skylakakis 1981; Georghiou 1980, 1983; Knipling and Klassen 1984; Mani 1985, 1989; Mellon and Georghiou 1985; Curtis 1985, 1987;

Roush 1989, 1992; Curtis et al. in press; Curtis and Otoo 1986; Tabashnik 1986, 1989, 1990a,b).

1.3.3.1 Mixtures

This method has recently received renewed attention as a possible insecticide resistance-delaying tactic, although the concept was introduced for tuberculosis chemotherapy many years ago, and it has been strongly advocated by Peters (1990) for use with anti-malarials. However, surprisingly little has been done to define the requirements for its application. The concept of using mixtures assumes that the mechanisms for resistance to each component of the mixture are different and that they occur together in any single individual within a given population only rarely or not at all. The assumption underlying this is that the different resistance genes affecting each component of the mixture are rare and distributed at random, so the probability of a double resistant individual is the product of the frequencies of each resistance gene (Table 1.3). Thus all or most of the insects that survive one of the chemicals in the mixture would be killed by the other. If a proportion unexposed to insecticides is assumed, these would be expected to greatly outnumber any rare surviving double resistants (Table 1.3).

Table: 1.3 Numerical model of one generation of use of a pesticide mixture

	No. of individuals of each genotype								Frequency of R A and B	
	R S A B	S R A B	R S A B	R S A B	R S A B	S R A B	S R A B	S S A B		Total
Initial population at linkage equilibrium	40	40	160	39,760	39,760	39,760	39,760	9,920,240	10,000,000	0.2%
If 20% escape exposure: and if 80% exposed to mixture which kills S S A A and B B :	8	8	32	7,952	7,952	7,952	7,952	1,984,048	2,000,128	0.2032%
then survivors as parents:	8	8	160	7,952	7,952	7,952	7,952	1,984,048	2,000,128	0.2032%

R R R S R R S R R R R R
 N.B. : The A B / A B , A B / A B and A B / A B types would be so rare under the above conditions that none of them would be expected to exist in a population of 10,000,000 individuals.

Recently more detailed theoretical studies on the use of mixtures have been done. Curtis (1985, 1987) for example concluded that the use of mixtures of unrelated insecticides could be very efficient in delaying resistance provided that both resistance genes are initially rare, the insecticides decay at equal rates and the resistance genes are not closely linked. He also stressed the importance of refugia in insect populations if a mixture of two unrelated insecticides is to be successfully used.

Other factors which may influence the use of mixtures as a method to delay the build up of resistance have been discussed by Mani (1985). He emphasised that they would only be successful if both resistances were not fully dominant. Curtis (1985) explained this by pointing out that very effective selection for double resistance which occurs when resistance is dominant can cause the build up of linkage disequilibrium, i.e. non-random distribution of the resistance genes, faster than the disequilibrium would be broken down by sexual reproduction with recombination. This also explains the importance of linkage because this would inhibit the recombination process and enhance the risk that linkage disequilibrium would build up.

Knipling and Klassen (1984) suggested the practical advantages of using a half-strength mixture, i.e. one in which both components are used at half their standard concentration. However, Curtis (1987) argued that such a

half-strength mixture might not be so effective in delaying resistance as a full-strength one if the dominance of the resistance gene was more pronounced when the insecticides were at half the normal strength. MacDonald et al. (1983a) have reported from their laboratory studies that the use of 1:1 mixtures of permethrin and dichlorvos on a strain of M. domestica showed a substantial suppression of the development of resistance to permethrin and dichlorvos. However, it is very unlikely that this mixture would have a potential use in the field, since the persistence of permethrin and the short residual life of dichlorvos could result in flies emerging from puparia three to four days after treatment being selected by permethrin alone. Thus for the use of mixtures to be successful the components of the mixture must have approximately similar decay rates, or preferably only persist for a short time in the environment (Georghiou 1980).

Roush (in press) emphasised the likely failure of mixtures because of unequal decay rates or incomplete mortality of genetically susceptible types despite exposure to the mixture. Curtis et al. (in press) have illustrated his argument with numerical models and discussed means of determining whether it would be a total refutation the idea of using mixtures.

Another important point when considering mixtures is the possibility of synergistic action between the chemicals which would greatly complicate prediction of the outcome.

As already stated it is necessary that if one intends to use a mixture for resistance management one should start early before resistance to one of the components has been selected and while both resistance genes are rare (Georghiou 1983). Some of the practical studies which report failure of the mixture method (e.g. Immaraju et al. 1990) have ignored this point and are therefore not valid arguments against this concept. The requirement for both resistances to be rare means that one would have to take the decision that there is likely to be the long term risk of resistance and that it is worthwhile to invest the extra cost of using a mixture in the expectation that one could avoid or delay the long term costs of double resistance which would be inevitable if the compounds were used one by one.

1.3.3.2 Rotation

As mentioned earlier, the use of insecticides inevitably selects for genetic mutations that confer resistance. In considering strategies involving the use of two insecticides, one generally cannot afford to ignore the risk of the target population developing resistance to both compounds. It has been suggested that this risk may be reduced by switching between two or more unrelated

insecticides and this has been designated "rotation" (Georghiou 1980). Rotations of two or more insecticides are considered by Roush (1989) to be a more hopeful strategy than the use of mixtures of insecticides or mosaics (i.e. use of different compounds in nearby areas). There has been much discussion recently about rotations of two or more insecticides in time and space, but the experimental and field work has been rather unconvincing. It was therefore a major aim of this study to investigate some of the factors which affect the rotational use of insecticides for the management of insecticide resistance in mosquitoes.

The concept of rotations has been examined experimentally and theoretically in only a small number of cases (Cutright 1959; Ozaki 1969; Ozaki et al 1973) and recently has received increasing attention (Skylakakis 1981; Knipling and Klassen 1984; Curtis 1985, 1987; Curtis and Lines 1986; Comins 1986; Sawicki and Denholm 1986; Tabashnik 1989, 1990a,b; Denholm et al. 1990, Curtis et al. in press). The concept of using a rotation of chemicals generally assumes that individuals that are resistant to one chemical have substantially lower biotic fitness than susceptible individuals, so that the resistance frequency declines during the intervals between application of the chemical concerned (Georghiou 1983).

The chemicals used should also be from different groups with different killing mechanisms which therefore do not exhibit cross-resistance (Georghiou 1980). If cross-resistance does exist, there would be a tendency for resistance to both chemicals to develop during both phases of the rotation. As with the use of a single insecticide, in order to understand and plan a rotational response to the resistance problem, data are needed on the degree of protection conferred by resistance genes to each of the compounds which it is intended to use, in the heterozygous and homozygous states under field conditions.

In trying to consider the method of rotation in more detail Curtis (1985, 1987) has distinguished three distinct methods of rotation, namely (1) - switching when the level of resistance to a pesticide reaches an intolerable level or when forced to do so and switching back if the resistance level regresses sufficiently, (2) - switching when resistance to a pesticide is first detected, (3) - switching according to a pre-planned schedule. The first method is effectively what occurs in any vector control programme without an explicit resistance management strategy but with the capacity to monitor and respond to resistance. Type (1) and (2) rotations may be called "reactive" or "opportunistic" because switches are made in response to data on resistance, not to a pre-arranged timetable. Some entomologists consider that pre-planned rotations are the

best methods of resistance management. However Curtis (1987) and Curtis et al. (in press) suggest that there may not be much difference between the end results (i.e. time to reach double resistance) with "reactive"/ "opportunistic" as compared with pre-planned rotations and this question is further discussed in chapter 10 of this thesis.

An early example of a rotation has been reported by Cutright (1959) in the case of the two-spotted spider mite [Tetranychus telarius (L)] and the European red-mite [Panonychus ulmi (Koch)].

The most recent and successful major insecticidal programme against a disease vector is the Onchocerciasis Control Programme in West Africa. This uses a complex rotation, the change of chemicals being necessitated by the presence of temephos resistance in members of the Simulium damnosum complex and/or when the seasonal river flow is unsuitable for a particular chemical (Kurtak 1986; Kurtak et al. 1987; Curtis et al. in press). In this programme temephos, chlorphoxim, permethrin and carbosulfan are rotated with the bacterial toxin Bacillus thuringiensis israelensis (Bti or Bt H-14) the risk of resistance to which is discounted. Also 100% larval kills are aimed at, so that Bti apparently wipes out organophosphate resistance populations, leaving immigrants to re-establish a susceptible population. Bti is only usable during the dry season when the river flow in the Simulium breeding places is low, and it is considered too environmentally dangerous

to use permethrin or carbosulfan when the river flows are low. The synthetic chemicals are used during the wet season when river flow is high. It used to be the policy to use temephos until resistance was detected but recently it has been suggested that temephos should not be used for more than 15 weeks at a stretch (Agoua et al. 1991). This could be taken as a change from a "reactive" or "opportunistic" to a pre-planned rotation (Curtis et al. in press).

MacDonald et al. (1983b) investigated the value of rotations in a field study of houseflies. They alternated the use of permethrin and dichlorvos on a series of farms in Ontario. They observed that resistance to permethrin developed more rapidly under continuous permethrin pressure than when permethrin and dichlorvos were rotated. The same result was obtained by Georghiou et al. (1983) in laboratory experiments with larvae of the mosquito Culex quinquefasciatus. They noticed that temephos application alone caused rapid resistance development, but in alternation with permethrin did not cause substantial resistance. The effectiveness of rotations for temephos is consistent with data suggesting strong fitness disadvantages associated with temephos resistance (Al-Khatib and Georghiou 1985b). However, they also observed that rotations had limited effect on resistance to permethrin and propoxur which evolved very rapidly.

In Australia and Egypt pre-planned rotations have been reported to be successfully used to prevent pyrethroid resistance in cotton pests (Sawicki and Denholm 1987). In Egypt synthetic pyrethroids were rotated with other compounds against the cotton leafworm, Spodoptera littoralis, with the intention of preventing or delaying pyrethroid resistance. The application of pyrethroids was restricted to once a year when S. littoralis was reaching its peak on cotton and the government forbade their use on any other crops. Since this programme has been introduced no new cases of resistance to these insecticides have been reported (Sawicki and Denholm 1987).

Field failures of pyrethroids to control the cotton pest Heliothis armigera at Emerald, Queensland, Australia, (Gunning et al. 1984), have prompted the government to formulate a strategy in an attempt to prevent further resistance to pyrethroids and other insecticides. This strategy restricts the annual use of pyrethroids to three applications over a 42-day period against one generation of H. armigera. Other insecticides such as endosulfan, chlordimeform or B. thuringiensis (Bt) are used before and after the 42-day pyrethroid period. This strategy has been claimed to be successful (Daly and McKenzie 1987; Forrester and Cahill 1987) in view of the fact that after 3 years (i.e. a total of 124 days of pyrethroid usage) pyrethroid resistance had only risen from 14% to 42%. More recent data (supplied by courtesy of N. Forrester and G.P. Fitt

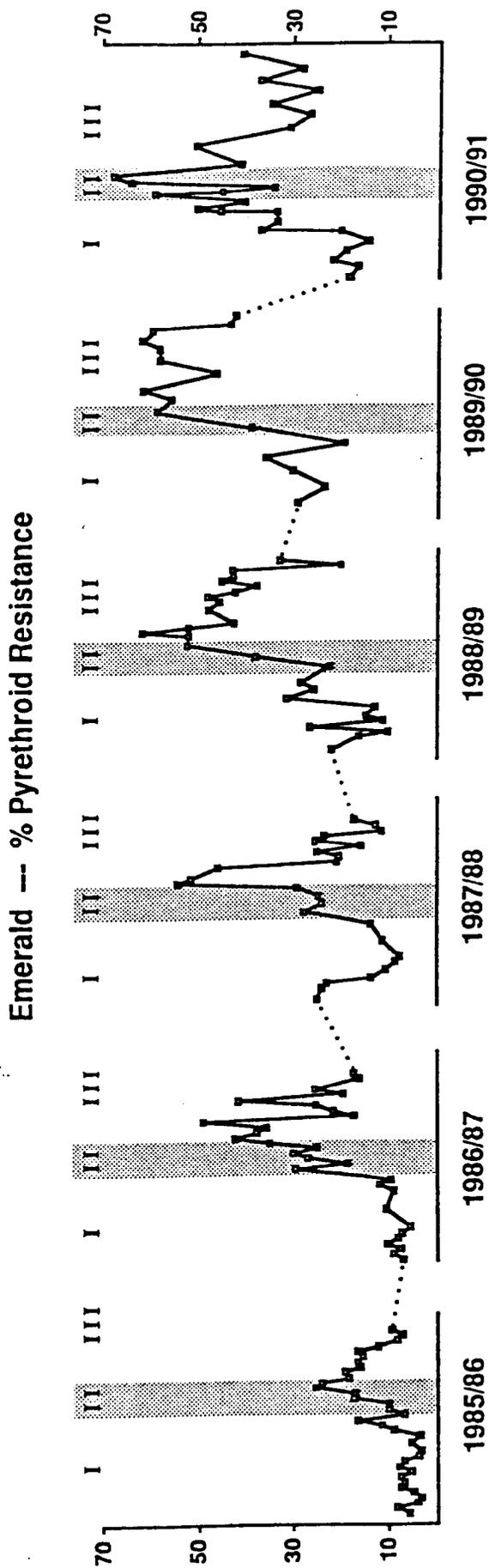
unpublished) are reproduced in fig.1.3. These show a continued "saw tooth" upward trend in pyrethroid resistance.

The same principle as used in Egypt and Australia was adopted in the control of Heliothis zea and tobacco budworm H. virescens, the important pests of cotton in the United States, where pyrethroids are rotated with chlordimeform. Pyrethroids are restricted to the critical mid-season period only. This seems to be working very well (Plapp et al. 1989, 1990) with no increase in resistance for three consecutive years.

In Zimbabwe, a pre-planned rotation was introduced to prevent further resistance to dimethoate in the mites Tetranychus cinnabarinus and T. lombardini which have become major pests of cotton. The country was divided into approximately three equal regions. Each of the regions used acaricides of different chemical groups for two consecutive years and then changed to the next type of chemical for the following two years, and so on. Thus each chemical can only be re-used in a given region after about a four year interval. This period of non-exposure to the same compound is intended to allow reversion, should any resistance have arisen. Since this strategy has been introduced more than 10 years ago, no new cases of resistance have been reported.

In another case, to combat resistance to pyrethroids in H. armigera, the Zimbabwe government has banned the use of

Fig :1.3
Response of *H. armigera* to rotational use of pyrethroids and other insecticides in Australia



N.B. Data of N. Forrester and G.P. Flitt (pers. comm.). Shaded areas indicate the weeks during which pyrethroids are used.

pyrethroids for three months during winter. Pyrethroids can only be used for not more than the nine weeks that coincide with maximum flowering of the cotton when bollworms occur in damaging numbers on the crop. Outside this period the bollworms are controlled with either endosulfan or carbaryl and thiodicarb (Sawicki and Denholm 1987).

In each of these cases it is reported that the pre-planned rotation has been successful, but there is little evidence about what would have happened if another policy had been adopted. In particular there is almost no data on resistance to the alternatives of pyrethroids (endosulfan etc.) whose expanded use is enforced by the limitation on pyrethroid use.

In parallel with the national programmes for the use of chemicals in rotations, there have been theoretical discussions on this subject (Leeper et al. 1986; Comins 1986; Curtis 1987; Roush and McKenzie 1987; Roush 1989; Tabashnik 1989; Mani 1989; Roush and Daly 1990; Curtis et al. in press). Curtis and Lines (1986), Curtis (1987) and Curtis et al. (in press) compared two methods of rotations, i.e. a pre-planned rotation (type 3 in the classification) and switching when forced to do so (type 1), and concluded that the time before double resistance was reached would be very similar in each case unless very strong influence of fitness modifying genes is assumed. The same conclusion was also reached by Mani (1989) from a much more general

mathematical treatment. Immaraju et al. (1990), on the other hand, considered that a pre-planned rotation of fluvalinate and formetanate had been shown to be advantageous in retarding the development of resistance in citrus thrips [Scirtothrips citri (Moulton)]. However, the comparison was made between a few generations of rotation with the same number of generations of use of one compound only, not to both compounds used successively. It is not surprising that a larger total number of generations of selection by one compound produced more resistance to it.

In most control programmes, insecticide resistance is allowed to rise to a relatively high frequency before there is a switch to a new insecticide, i.e. no action is taken when resistance is first detected and only when it reaches a level at which it is affecting disease transmission or agricultural productivity is a switch to a new insecticide advised (i.e. a type 1 rotation is used, according to the above classification). There is, however, a risk in this kind of programme that it may improve the general fitness of the resistance genes by selection for fitness modifiers and prevent the population from reverting to susceptibility on withdrawing the insecticide concerned. Keiding (1967) pointed out that in the field populations generally do not revert to a state of full susceptibility similar to that of an untreated population unlike laboratory selected strains.

Curtis (1987) simulated the behaviour of a modifier gene which could eliminate the fitness disadvantage of a resistance allele and found that it would only have an appreciable influence if the resistance gene frequency is allowed to become very high before switching insecticides. Curtis et al. (in press) made further simulations of a modifier gene but in these cases it started at a relatively high frequency because the modifier was at mutation/selection equilibrium, with only very slight fitness disadvantage associated with it. They found that only when the use of one insecticide continued for a long time (24 generations in this case) did the modifier have any appreciable effect in preventing the resistance gene from declining in frequency when the insecticide concerned ceased to be used.

Roush and McKenzie (1987) and Roush (in press) have reviewed the evidence about whether insecticide resistance genes commonly shows co-adaptation, i.e. reduction in fitness which is removed by the selection of modifiers. They concluded that, contrary to what is generally believed, this has not been commonly observed except in the case of the Australian sheep blow fly, Lucilia cuprina, where a fitness modifying gene for the diazinon resistance gene has been identified. In this case diazinon usage continued in the field for years after high levels of resistance were reached.

Due to a lack of reports of properly controlled experiments in the literature about rotations and the lack of information to confidently choose which resistance management tactics to use, it was a major purpose of the present study to carry out a laboratory test of whether there is any advantage of using a pre-planned rotation over switching of insecticides when forced to do so. These tests were made with two species of mosquitoes, i.e. An. albimanus from El Salvador, which is resistant to propoxur and dieldrin and a Malaysian strain of Cx quinquefasciatus, which is resistant to malathion and permethrin. Other aims of the study were to test an improved method of studying linkage of resistance genes because of the potential importance of linkage to any method of management by multiple attack, i.e. the rotation method as well as the method of mixtures. It was also an aim of this study to determine the effective dominance of certain resistance genes in field conditions simulated in the laboratory and in an actual field experiment. This could have an important influence on rotational use of insecticides by determining the rate of increase of resistance during the phase of a rotation in which these compounds were used. For one resistance gene a laboratory study was carried out of its fitness in the absence of use of the relevant chemical. The overall intention was to attempt to clarify some of the current ideas for the management of insecticide resistance in mosquitoes.

CHAPTER 2

SELECTION OF MALAYSIAN Aedes aegypti LARVAE WITH TEMEPHOS
AND Bti AND SCREENING OF THE ADULTS WITH CERTAIN
INSECTICIDES

2.1 INTRODUCTION

Aedes aegypti has been incriminated as the vector of virus borne diseases infecting man such as yellow fever, dengue and chikungunya on the basis of epidemiological, virus isolation and virus transmission studies. Yellow fever is confined to Africa and Latin America and dengue fever (DF) and dengue haemorrhagic fever (DHF) have been reported mainly from South East Asia and the Caribbean. The haemorrhagic form of dengue (DHF) has caused many deaths in these areas. In the Caribbean, about 30 countries have reported dengue outbreaks between 1977 and 1982 (Knudsen 1983). In Cuba, DHF claimed 158 lives and there were 344,203 cases in 1981 (Tonn et al. 1982) and in South East Asian countries the disease was estimated to have caused death of almost 17,000 people and the hospitalisation of 350,000 between 1956 and 1980 (Halstead 1980).

Ae. aegypti is an urban mosquito living in close association with man and is highly anthropophilic. It breeds mainly in natural or artificial water containers both

indoors and outdoors. In some parts of the world Ae. aegypti were found breeding in the same containers as Ae. albopictus. In Malaysia there has been found to be an extensive sharing of natural habitats by Ae. aegypti and Ae. albopictus (Yap 1975). Ae. albopictus also breeds in clear stagnant water but prefers the open-air, i.e outside houses in man-made water containers such as tyres, tins, bottles etc. It also breeds in tree holes, mud-holes, discarded coconut shells etc. The vectors bite during the day time especially between 06.00-10.00 and 16.00-18.00 hrs.

The Malaysian Ae. aegypti population is thought to have originated from Tropical Africa (Rudnick and Lim 1986). In Malaysia, this species has been identified as a major vector of DF and DHF. Dengue fever was first described in Malaysia in 1902 (Skae 1902) and in 1973, there was a major outbreak of the disease in the country. DF and DHF remain serious public health problems, especially in the urban areas and a number of outbreaks are reported every year and appear to reach an epidemic situation every two years. Since there is no specific treatment for dengue and as yet there is no generally available vaccine against it (Brandt 1988), the control of the vector mosquitoes is the only possible method of disease control. This has been the main task of the Anti-dengue Control Division under the Malaysian Ministry of Health. Their work includes health education, collection of water holding garbage, law enforcement against allowing breeding in privately owned premises, insecticidal fogging

etc. Aedes control in Malaysia began in the 1960's. It was directed to areas around the international airport with residual spraying of dieldrin to obtain a 400-yard-wide Aedes free belt around the airport (Gratz 1967). This was successful in just the area mentioned but not elsewhere. The control of the vectors nationwide only commenced in the late 70's but this only had limited scope and aimed only to respond to epidemics. The principal insecticides in use were temephos as a larvicide and malathion as an adulticide (Rudnick and Lim 1986).

Ae. aegypti has been reported to have developed resistance to organochlorines, especially DDT, in many countries, including Malaysia (MacDonald 1958; Abedi and Brown 1960; Abedi et al. 1963; Kimura and Brown 1964; Brown 1964; WHO 1980a). Therefore malathion has been used to replace it since 1976 and only a few cases of organophosphate resistance have been reported. A strain collected from Penang island, Malaysia, showed a natural larval tolerance to DDT of 5 to 10 times the level in Ae. aegypti from regions outside Malaysia, but this is not unusual for Malaysian strains (Busvine and Coker 1958). Selection of this strain with DDT increased the resistance level to 500-fold within 10 generations (Abedi and Brown 1960). This is in sharp contrast to two other laboratory strains which remained unchanged under DDT selection: the Tübingen strain for 5 generations (Craig 1957) and the London strain for 15 generations (Shidrawi 1957).

As for organophosphate (OP) insecticides, not until the last decade has resistance been widely reported in Ae. aegypti to these compounds, despite the fact that they had been used for more than 15 years successfully in routine control of Ae. aegypti and during epidemics. Earlier reports (Mouchet et al. 1969 and 1972 a, b), indicated that organophosphorus resistance was absent in all parts of the world except in Malaysia and Vietnam where malathion resistance had been reported. However, in 1980, the WHO Expert Committee on Vector Biology and Control (WHO 1980a) cited the presence of OP resistance in the following countries or areas :- Caribbean islands and neighbouring countries : malathion, fenitrothion, temephos, fenthion; India, Malaysia, Thailand and Vietnam: malathion; New Caledonia : temephos. Attempts to induce high resistance to OP's through selection pressure in the laboratory have been unsuccessful (Brown and Abedi 1960; Madhukar and Pillai 1970). Selection of the Penang strain with temephos for 25 generations caused only 6-fold increase in LC50 (Ziv et al. 1969). Field et al. (1984) studied a strain of Ae. aegypti collected in Puerto Rico in 1978 that had been exposed to malathion in the field. By means of subsequent strong selection in the laboratory for 12 generations, malathion resistance of adults rose by 10-fold. More recently, Georghiou et al. (1987) reported the results of bioassays of temephos with Ae. aegypti larvae from 28 sites in the Caribbean islands and neighbouring countries during the period 1983-85. They found that nearly all wild populations

of Ae. aegypti displayed elevated LC95 values towards temephos. In two sites, Tortola and Antigua, the LC95 value of temephos was 47-fold higher than the normal laboratory strain, indicating the presence of significant resistance. Temephos has been used as a larvicide in the Caribbean area for more than 15 years for Aedes control as it has in Malaysia.

The reports of development of resistance to conventional insecticides have generated great interest in biopesticides as alternative pest control agents. There are three types of biopesticides which are of considerable current interest for various pest species namely : toxin proteins produced by bacteria e.g. Bacillus thuringiensis (Bt) and Bacillus sphaericus, macrocyclic lactones produced by the actinomycete Streptomyces avermetilis and baculoviruses e.g. nuclear polyhedrosis and granulosis viruses. To date, the spore-crystal protein complex of Bt has been the most successful microbial insecticide. One such example is the agent of Bacillus thuringiensis israelensis (Bti) which is gaining great attention. Bti is only toxic to mosquito and Simulium larvae. As mentioned above it is used in the Onchocerciasis Control Programme alternately with temephos or other synthetic insecticides (Kurtak 1986, Curtis et al. in press). Both temephos and Bti are non-toxic to humans (WHO 1984a) but people do not like the taste of temephos in drinking water, whereas Bti is tasteless. However, the effect of sand granules impregnated

with temephos (Abate S.G.) persists longer than the best slow release formulations of Bti (WHO 1984a). Bt products have been very effective in controlling agricultural Lepidopteran pests. Many studies have been carried out to investigate the potential use of Bti (de Barjac and Sutherland 1990).

Selection experiments with Bti and other strains, of B. thuringiensis which have other insect 'targets', have been reported in a number of different insect species and fortunately very few cases of resistance have been reported. Usually Bti selection for resistance has been unsuccessful (Yamvrias 1962; Burges 1971; Sneh and Schuster 1983). However, McGaughey (1985) found a significant resistance to a strain of Bt in the stored grain pest, Plodia interpunctella. In this experiment a 100-fold increase in LD50 was obtained after 15 generations of selection. In another experiment by Harvey and Howell (1965) a 14-fold resistance was observed after 50 generations of selection with B. thuringiensis in houseflies. Calberg and Lindstrom (1987) also reported a 10-fold resistance developed after 30 generations in the fruit-fly Drosophila melanogaster reared in medium containing 0.3 - 1% of the agent. Very recently, Tabashnik et al. (1990) reported a development of resistance to B. thuringiensis subspecies kurstaki in field populations of a major lepidopteran pest of vegetables, diamondback moth, Plutella xylostella (L). However, no Bti resistance has yet been reported in Ae. aegypti.

The study described in this section had two principal objectives : (i) to survey various populations of Malaysian Ae. aegypti for resistance to the organophosphates temephos and malathion, and to DDT and Bti, (ii) to determine the resistance potential of the species under vigorous selection pressure by temephos and Bti in the laboratory. Had responses to selection been found, the wild collected strains would have been used to carry out a further test of whether a pre-planned rotation of temephos and Bti every generation was different in its long term effects from sequential use of the compounds.

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

2.2.1.1. Mosquitoes used

2.2.1.1.1 Aedes aegypti :

Larvae of this mosquito were collected from the field and brought to the insectarium in the Institute of Medical Research, Kuala Lumpur, Malaysia. The collection method will be discussed later.

2.2.1.1.2 Aedes albopictus :

The larvae were collected from the same breeding place as Ae. aegypti.

2.2.1.2 Insecticides used.

Temephos : various concentrations of temephos in ethanol solution provided by WHO.

Malathion: Standard concentrations of malathion solution and 5% malathion papers obtained from WHO.

DDT: 74% Technical grade (T.G.) DDT provided by Wellcome Foundation and 4% DDT papers provided by WHO.

Bti : The Bacillus thuringiensis israelensis (Bti) used in the study was the standard wettable powder IPS-82 prepared by the Pasteur Institute, Paris. It has 15000 International Toxic Units against Ae.aegypti per mg (15000 ITU/mg) (Barjac and Thiery 1984). All the tests were conducted under Malaysian ambient conditions, (temperature 29 +/- 1° and relative humidity of 80 +/- 5% in the laboratory.

2.2.2. METHODS

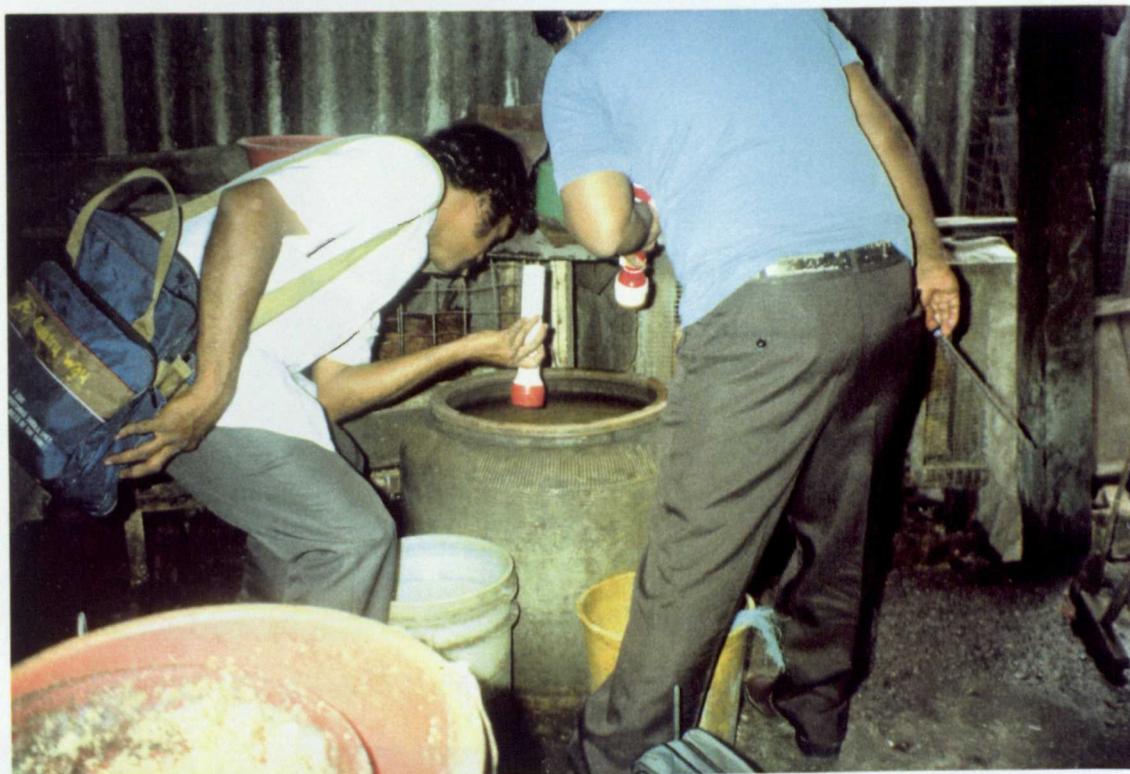
2.2.2.1. Collection of larvae

Mosquito larvae of different stages were collected from Jinjang Utara near Kuala Lumpur. They were collected from earthenware jars, tins, cement-lined wells, ant-traps etc inside and outside houses (see photographs). The larvae were pooled and then reared to adulthood. The adults were then identified and classified as Ae. aegypti, Ae. albopictus, Cx quinquefasciatus etc. The pooled batch of Ae. aegypti larvae were designated the AT strain. All Aedes adults were fed with sucrose and vitamin B complex solution and allowed to feed on white mice when eggs were required. The eggs were collected on pieces of white Whatman No. 1 filter paper in white plastic cups. Eggs were hatched and the larvae were reared to late 3rd or early 4th instar which were then used for the bioassay.

A second batch of larvae was collected from Jinjang Utara for comparison and was designated the JU strain. A third batch of larvae was collected from 6 km to the south of Jinjang Utara, at Jinjang Selatan, from which larvae were previously reported to be resistant to temephos (Lee and Wutiponge 1989). As before, larvae were collected from a variety of containers brought to the laboratory and reared. The strain was designated T-T.

Photograph: 2.1 The collection sites of *Ae. aegypti* and *Cx quinquefasciatus* in Kuala Lumpur

earthenware jar



cement-lined well



ant-trap



plastic container



2.2.2.2. Larval susceptibility test.

Late 3rd or early 4th instar larvae were used for the bioassay with temephos. Temephos solutions were prepared by pipetting the appropriate amount of the standard solution provided in the WHO test-kit into 250 ml cups and almost filling the cups with distilled water. 25 - 30 larvae were then added and the contents of the cup topped up to the 250ml mark with distilled water. The range of concentrations was first determined from preliminary trials. Each concentration was replicated at least 3 times and twice for the control. The controls were prepared by the addition of 1 ml of ethanol to the water in each cup. The larvae were exposed for 24 hours and the mortality rates of the larvae were scored as the proportion dead or moribund. When required for breeding, the surviving larvae were transferred to clean water in a rearing bowl and reared to the adult stage.

Temephos tests were conducted against all the strains mentioned above and a laboratory strain which had not been exposed to any insecticide for a long period of time. The AT strain was also tested for its susceptibility status towards malathion and DDT.

The same procedure as above was carried out on Ae. albopictus.

2.2.2.3 Selection of T-T strain with temephos

Late 3rd or 4th instar larvae of the T-T strain were subjected to 10 generations of selection with temephos. The selection dosage used in every generation was expected to cause 80-90% mortality. Those which survived the test at each generation were kept to be the parents of the next generation. The first five generations were selected with concentrations of temephos increasing from 0.006 mg/l to 0.02 mg/l. The WHO diagnostic dosage of 0.02 mg/l was used from generation F5 to F10.

2.2.2.4 Adult susceptibility test

The following strains or species were used for this test : AT, JU, T-T and laboratory strains of Ae. aegypti and Ae. albopictus. Insecticide susceptibility tests were conducted with papers impregnated with 5% malathion using the standard WHO adult test kit (WHO 1975). All tests were performed on 3-day-old adults. Mortality was scored 24 hours after the exposure to the insecticide. During this period the mosquitoes were allowed access to water from dampened cotton-wool pads.

This procedure was also used with 4% DDT-impregnated papers and 0.25% permethrin papers.

2.2.2.5 Preparation of Bti (Bactimos) mixture for bioassay of Ae. aegypti larvae

50 mg of Bactimos powder was weighed and poured into a 20 ml penicillin flask and 10 ml of de-ionised water and 15 glass balls (6 mm diameter) were added. This suspension was agitated for 10 mins at 700 strokes/min on a vortex machine. 1 ml of this suspension was immediately added to 9 ml of de-ionised water. Further dilution was carried out when necessary.

Using micropipettes, appropriate amounts of this mixture were added to distilled water in 200 ml plastic cups to produce the required range of concentrations. Four cups were used for each concentration and for the control. The control cups contained distilled water alone, 25 late 3rd or early 4th instar larvae were added to each cup. These cups were then topped up to the 150 ml level with distilled water. Mortality was recorded at 24 hours.

2.2.2.6 Selection of Ae. aegypti larvae with Bti

Batches of larvae were exposed to several concentrations of Bti. The survivors from a concentration which gave 80-90% mortality were kept to be the parents of the next generation. Several hundred larvae were exposed to this concentration in order to obtain enough survivors to start the next generation. This procedure was repeated for 10 generations.

2.3 RESULTS

Table 2.1 shows the susceptibility of various strains of Ae. aegypti to the WHO recommended diagnostic dosage of 0.02 mg/l temephos. By this criterion no resistance was detected in any of the strains. The LC50 values of these strains towards temephos and Bti are shown in table 2.2. The estimated resistance ratio of the wild Ae. aegypti strains was about 1.5 to 1.8 relative to the laboratory strain, but only in the case of T-T did the 95% confidence limits of the LC50 values not show considerable overlap (Table 2.2). The Ae. albopictus strain gave similar results to the Ae. aegypti laboratory strain. The AT strain was also exposed to malathion and DDT (Table 2.2). The susceptibility tests of the adults are shown in table 2.3. All the wild collected Ae. aegypti strains were highly resistant to DDT, completely susceptible to malathion and partially resistant to the WHO recommended discriminating dosage of permethrin.

Figure 2.1 shows the results of selecting the T-T strain with Bti and temephos for 10 generations. No indication of the development of Bti resistance was observed in this strain. However, a low level of tolerance/resistance seems to have been selected to temephos - the LC50 at generation 10 was 0.0048mg/l as compared with 0.0030mg/l at the beginning.

Table: 2.1

Exposure of various strains of Ae. aegypti and Ae. albopictus larvae to the WHO recommended diagnostic dosage of 0.02 mg/l temephos.

Species/ Strain	Dead	Total	% Mortality
AT	215	215	100
JU	203	203	100
T-T	205	205	100
Laboratory	219	219	100
<u>Ae.</u> <u>albopictus</u>	209	209	100
Control	0	211	0

Table: 2.2

LC50 values observed in the different Malaysian strains of Ae. aegypti and Ae. albopictus towards temephos, malathion, DDT and Bti (95% confidence limits in parentheses).

Species/ Strain	LC50 (mg/l)			
	Temephos	DDT	Malathion	Bti
AT	0.0028 (0.0017-0.0037)	0.736 (0.7101-0.7646)	0.3065 (0.2797-0.3381)	0.0066 (0.0061-0.0069)
JU	0.0026 (0.0018-0.0032)	-	-	-
T-T	0.0030 (0.0025-0.0035)	-	-	0.0144 (0.0121-0.0163)
Laboratory <u>Ae. aegypti</u>	0.0017 (0.0010-0.0026)	-	-	0.0117 (0.0086-0.0136)
<u>Ae. albopictus</u>	0.0016 (0.0009-0.0023)	-	-	-

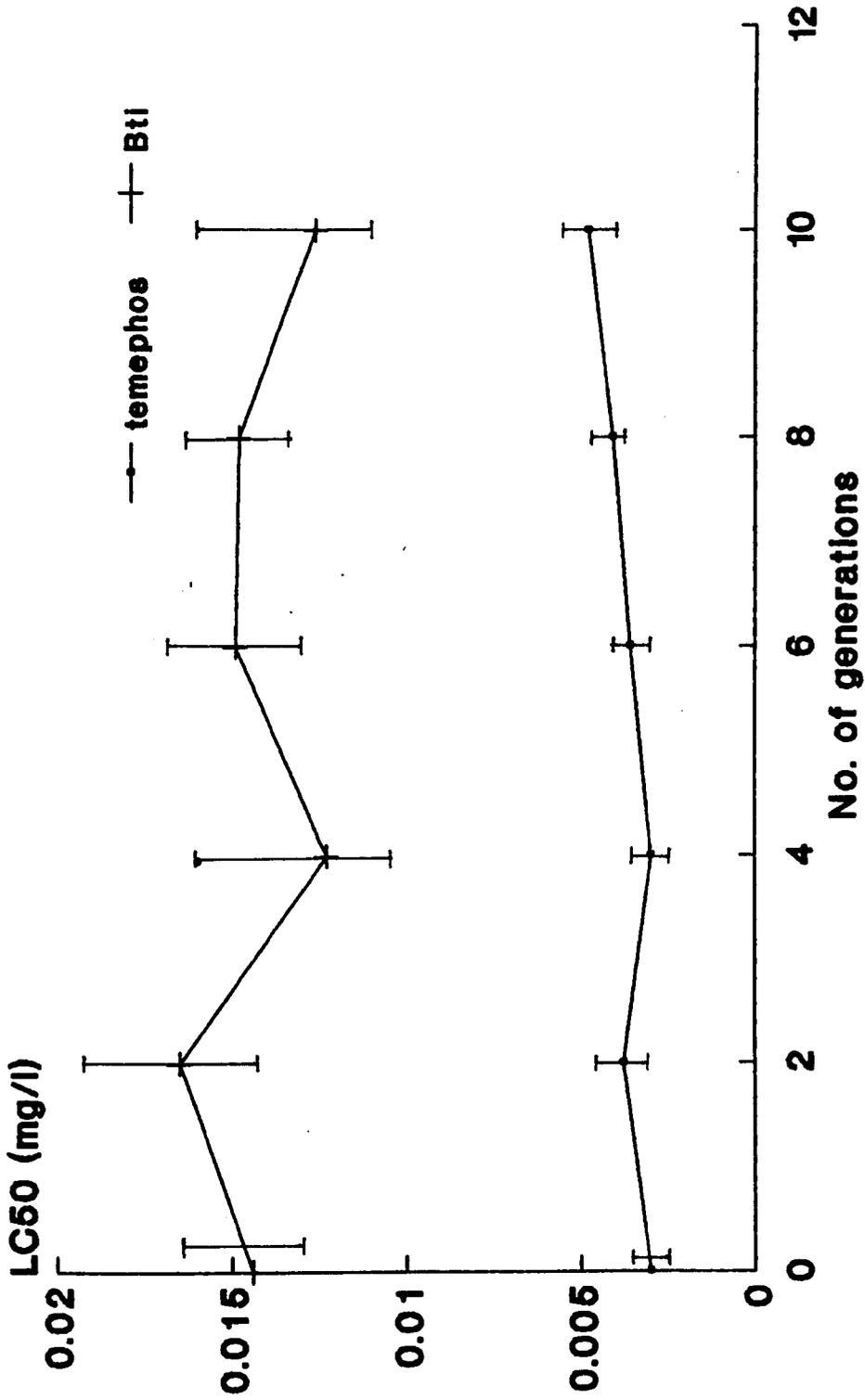
Note : The LC50 values and the confidence limits were computed using the Nanostat program.

Table: 2.3

Exposure of adults of various strains of Ae. aegypti and Ae. albopictus to 4% DDT, 0.25% permethrin and 5% malathion for one hour.

Species/ Strain	Insecticide	Dead	Total	% Mortality
AT	4% DDT	0	225	0.0
	0.25% Permethrin	125	225	55.6
	5% Malathion	225	225	100.0
JU	4% DDT	0	249	0.0
	0.25% Permethrin	109	225	48.4
	5% Malathion	225	225	100.0
T-T	4% DDT	0	250	0.0
	0.25% Permethrin	113	225	50.2
	5% Malathion	250	250	100.0
Laboratory Strain	4% DDT	215	225	95.6
	0.25% Permethrin	218	225	96.9
	5% Malathion	225	225	100.0
<u>Ae.</u> <u>albopictus</u>	4% DDT	24	250	9.6
	0.25% Permethrin	170	250	68.0
	5% Malathion	225	225	100.0

Fig : 2.1
Selection of *Ae. aegypti* larvae (T-T)
strain with temephos and Btl



N.B. The LC50 values and the 95% confidence limits were computed using the Nanostat program

2.4 DISCUSSION

The diagnostic dosage for larval susceptibility of Ae. aegypti towards temephos has been set by WHO at 0.02 mg/l. This is by definition twice the lowest concentration that will kill 99.9% of a susceptible population (WHO 1980b). Survivors on this dosage would strongly suggest that resistance exists in the population (Davidson and Zahar 1973). This dosage was used to determine the susceptibility status of different strains of Ae. aegypti collected from the field. As can be seen from table 2.1 none of the strains survived this concentration which indicates that, by this criterion, the strains are still susceptible to temephos. However, when compared to the susceptibility baseline LC50 value of a laboratory strain, the T-T strain displayed just statistically significant evidence of tolerance to temephos. The LC50 value of the T-T strain in this study was very much lower than that reported by Lee and Wutipongse (1989) and Georghiou et al. (1987) from the Caribbean (Table 2.4) and slightly higher than that reported by Lee et al. (1984) from Kepong, Malaysia.

Selection of the T-T strain with temephos for 10 generations induced only about a 1.6 fold increase in tolerance/resistance (Fig 2.1) to temephos even though the original strain was detectably tolerant to this insecticide. This finding is comparable to the results of the laboratory selection of Indian strains of Ae. aegypti for 20

generations with temephos which induced only a 2.4 fold increase in tolerance (Madhukar and Pillai 1969). In another example, selection of the Penang strain with temephos for 25 generations increased the LC50 levels more or less steadily up to 0.0128 ppm, which was over 6 times the starting level of 0.0021 ppm (Ziv et al. 1969) and significantly higher than the maximum in the present study. The Bangkok strain of Ae. aegypti, on the other hand, responded very little to selection with only a 20% increase in the LC50 level after 7 generations of selection with temephos (Ziv et al. 1969). In contrast, Georghiou et al. (1987) found that selection of a synthetic population of Ae. aegypti from various countries in the Caribbean area by temephos pressure in the laboratory produced a 104-fold increase in resistance within 19 generations.

The low level of tolerance found in the present study may perhaps be attributed to the fact that many people in Malaysia refuse to use temephos in their water containers, thus providing numerous "refugia" and thus preventing high selection pressure against the whole field population. Alternatively there may be a lack of mutational potential for resistance in the Ae. aegypti in Malaysia in contrast to the Caribbean.

Selection of the Penang strain increased the malathion resistance level 5-times over 8 successive generations (Brown and Abedi 1960). However, in the present study

adult tests showed no malathion resistance (Table 2.3) and the the AT strain had a larval LC50 value of 0.3065 mg/l which is in the lower range of the strains tested by Ziv et al. (1969, Table 2.4) and by Lee et al.(1987). The low level of tolerance or resistance could be due to the fact that though malathion has been used in Malaysia for fogging over a 15 year period, this has only been done during epidemics and when cases of DF/DHF are reported, not in nationwide routine fogging. The fact that the fogs are non-persistent probably reduce their likelihood of selecting for resistance, by analogy with results on pyrethroid resistance in house flies (Denholm et al. 1983).

In the Caribbean islands and the neighbouring countries, malathion resistance was low and present only in 10 sites out of 28 sampled (Georghiou et al. 1987). There were, however, early reports of malathion resistance in Malaysia (Thomas 1970, 1976; Lee et al. 1987)

The adult mosquitoes of all the strains tested were 100% resistant to DDT. This is not surprising as DDT resistance is widespread world-wide and the Malaysian strain of Ae. aegypti was characterised by having a natural DDT-tolerance in the larval stage (Busvine and Coker 1958). Ae. albopictus adults were as resistant to DDT as Ae. aegypti. The reason for this could be that Ae. albopictus and Ae. aegypti share the same breeding places.

Table: 2.4 Published LC50 values of strains of *Ae. aegypti* larvae to temephos and/or malathion

Year	Place of origin	LC50 (mg/l)		References
		Temephos	Malathion	
1978	Malaysia, Kuala Lumpur	0.0026	-	Chen and Sudderuddin
1984	Malaysia, Kuala Lumpur, (Kepong)	0.0027	-	Lee et al.
1988	Malaysia, Kuala Lumpur, (Jinjang Selatan)	0.0122	-	Lee and Wutiponge
1990	Malaysia, Kuala Lumpur, (Jinjang Selatan)	0.0030	0.3065	this report
1979	India, Pondicherry	0.0016	-	Das and Rajagopalan
1981	Singapore	0.0018	-	Ong et al.
1982	Thailand, Bangkok	0.065	-	Upatham et al.
1987	Caribbean (Antigua & Tortola)	0.996 & 0.982 (LC95)	0.52 & 0.91 (LC95)	Georghiou et al.
	Kongolikan, Upper Volta susceptible	0.0023	0.0930	cited from Ziv et al. (1969)
	Trinidad, susceptible	0.0024	0.1050	
	Kongolikan, malathion-selected.	0.0039	1.050	
	Montego Bay, Jamaica, field malathion-tolerant.	0.0045	0.360	
	Penang, Malaysia DDT-resistant	0.0044	0.220	
	Trinidad DDT-resistant	0.0045	0.250	-

Even though pyrethroids has been scarcely used in Malaysia, this study showed that both Ae. albopictus and Ae. aegypti were partially resistant to permethrin. The kdr gene was reported in Ae. aegypti in Bangkok by Chadwick et al. (1977) and Prasittisuk and Busvine (1977) to cause DDT resistance, with cross-resistance to permethrin. The results in table 2.3 suggest that this gene may also exist in Malaysia. Further investigations involving selection with one compound and testing with the other should be carried out to test this hypothesis.

The results obtained from the present study also showed the high sensitivity of Ae. aegypti to Bti: the LC50 value was about 0.0144 mg/l. No indication of the development of resistance was observed on the selection of a field collected strain of Ae. aegypti. However, a resistance level of < 2-times was observed in Ae. aegypti selection with Bti after 14 generations of exposure (Goldman 1987, cited in Georghiou 1990). The present lack of resistance to Bti in Ae. aegypti in Malaysia, indicates that it could be used as an alternative to conventional insecticides or in a pre-planned rotation with them for control with resistance management of dengue (DF) and dengue haemorrhagic fever (DHF).

CHAPTER 3

SELECTION OF A MALAYSIAN STRAIN OF Culex quinquefasciatus.

3.1 INTRODUCTION

Culex quinquefasciatus is an important biting pest of man and vector of several diseases in various parts of the world (Harwood and James 1979; WHO 1980a). It is a vector of chikungunya virus (White 1971), West Nile virus (Rao 1975) and in the Americas it can transmit St Louis encephalitis virus. In India, Sri Lanka, Burma, Brazil and urbanised areas in East Africa, it is the main vector of bancroftian filariasis. In South East Asian countries, it is a vector of the urban strain of Wuchereria bancrofti (Ramachandran et al. 1964; Subra 1980). In Malaysia this species is not of great medical importance, but two strains of Wuchereria bancrofti occur in Kuala Lumpur and Penang Island which are transmitted by this mosquito (Wharton 1960; Ramachandran et al. 1964; Thomas and Ramachandran 1970). Cx quinquefasciatus is very difficult to control and the larvae can develop in virtually all types of breeding places found in the environment associated with man (Khatib and Georghiou 1985a), most abundantly in highly polluted stagnant water rich in organic matter (Kurihara 1963), especially sullage and sewage (Hamon et al. 1967; Gratz 1973; Curtis and Feachem 1981), in agricultural as well as urban environments.

The Tenth Report of the World Health Organisation Expert Committee on Vector Biology and Control stated that 41 species of culicine mosquitoes have developed resistance to DDT. Cx quinquefasciatus is not an exception. It was found to be naturally tolerant to DDT and has developed resistance to DDT in many parts of the world (WHO 1976, 1980a, 1986) and in many areas resistance was found to be still high despite the elimination of use of this insecticide from most forms of pest control for the past several years. In California, resistance to DDT was first reported in Orange County (Lewallen 1960) and in Singapore Cx quinquefasciatus adults were found to be resistant not only to DDT, but also to dieldrin, malathion, fenthion, fenitrothion and propoxur, even though the last three insecticides were not used in the region for mosquito control (Ong et al. 1981). Other workers also reported that this species has developed resistance to organophosphates, for example in the Americas (Barr 1962; Priester et al. 1981; Georghiou et al. 1975, 1985; Georghiou and Pasteur 1978, 1980; Villani et al. 1983), Africa (Hamon and Mouchet 1967) and Asia (Pennington 1968; Thomas 1970; Yasutomi 1970). Ranasinghe and Georghiou (1978) reported that selection of this species with temephos increased the resistance level about 600 fold.

There is evidence that organophosphate resistance in Cx quinquefasciatus is due to high levels of esterase. Laboratory selection studies have shown that this species

has the potential to evolve higher and higher levels of organophosphate resistance, due to the amplification of genes coding for the esterase (Mouches et al. 1986; Hyrien and Buttin 1986). In Dar es Salaam, a high level of organophosphate resistance was detected 10 years after chlorpyrifos (Dursban) was first used in the control programme (Curtis and Pasteur 1981). The impact of Dursban resistance on the persistence of control has been studied by Curtis et al. (1984). They showed that the resistance did not prevent the larvae from being killed with freshly applied insecticide. However, the resistant larvae started to survive 2 weeks after spraying whereas the susceptible larvae continued to be killed for at least 9 weeks after spraying.

Because the development of resistance to organophosphates, carbamates and organochlorines has become a serious obstacle to the control of these mosquitoes (Brown and Pal 1971; WHO 1980a; Curtis and Pasteur 1981), more attention is now devoted to the use of other more promising insecticides such as the synthetic pyrethroids. However, the continued presence of DDT resistance at relatively high levels in most parts of the world suggests that any consideration of using pyrethroids in mosquito control must be approached with caution in view of the cross resistance interrelationship of DDT and pyrethroids due to the kdr gene in this species (Priester and Georghiou 1980; Halliday and Georghiou 1985). Attention has thus been shifted towards

environmental management integrated with insecticide use (Rajagopalan et al. 1990), juvenile hormone mimics such as methoprene, biopesticides etc.

It was the aim of this part of the present study to carry out an up-to-date survey of the susceptibility status of a Malaysian population of Cx quinquefasciatus using DDT, malathion, permethrin, temephos and Bti and to select for temephos resistance.

3.2. MATERIALS AND METHODS

3.2.1 MATERIALS

3.2.1.1 Mosquitoes

Culex quinquefasciatus

The larvae of Cx quinquefasciatus were first separated from those of Ae. aegypti collected as described in chapter 2, from Jinjang Utara, 10 km north west of Kuala Lumpur city in Malaysia. They were found breeding in the same places as Ae. aegypti in exposed, partially polluted, containers found around houses. The types of containers included tyres, tins, flower pots etc. The larvae were then reared to adulthood. The adults were fed on white mice and allowed to lay eggs. The late third and early fourth instar larvae were used in this study.

3.2.1.2 Insecticides

As described in chapter 2.

3.2.2 METHODS

3.2.2.1 Larval susceptibility test.

The larvae were exposed to various insecticides as described in chapter 2.

3.2.2.2 Adult susceptibility test

About 200 adults were exposed to diagnostic concentrations of each of the following insecticides : DDT, malathion and permethrin. The WHO (1975) adult testing procedure were adopted. Another batch of adults were exposed to 4% DDT papers for a series of times from 1 - 48 hours.

3.2.2.3 Selection of Culex quinquefasciatus with temephos

The F1 larvae of field collected Cx quinquefasciatus were subjected to a temephos susceptibility test. Another batch of larvae were selected for temephos resistance for four generations. The larvae were first exposed to a low (0.01 mg/l) concentration of temephos. After 24 hours, the numbers of dead and live larvae were counted. The survivors were collected and reared to adulthood. The females were

then blood fed on white mice, twice a week. On the second day after feeding egg-laying cups containing small pieces of mouse chow were provided. Egg rafts were collected the next day and allowed to hatch in trays. Four or five rafts being placed in each tray.

The larvae were fed with small pieces of mouse chow and the fourth instar larvae were tested with temephos at a concentration that gave about 80% mortality. The survivors were again collected and reared to adulthood. This procedure was repeated for three more generations with increasing concentrations of temephos at each generation. They were then selected with the W.H.O. discriminating dosage (0.02 mg/l) of temephos for four generations. At this stage they were allowed to breed for four generations without further selection. After four generations of such relaxation of selection they were again exposed to the WHO recommended discriminating concentration of temephos to check the temephos resistance level. This was done for four generations without further selection.

3.2.2.4 Preparation of Bti solution for bioassay of

Cx quinquefasciatus

The procedure was described in chapter 2.

3.2.2.5 Selection of Culex quinquefasciatus larvae with Bti

The same procedure as described above for temephos selection was repeated for Bti selection of Cx quinquefasciatus, but only one concentration was used throughout the selection process over 9 generations.

3.3 RESULTS

The adult susceptibility tests on Cx quinquefasciatus are shown in table 3.1. Resistance was found to the WHO diagnostic dosages of DDT, malathion and permethrin. The highest level of resistance was observed towards DDT with lower levels towards malathion and permethrin. A proportion of the population could even survive 48 hours of exposure to 4% DDT (Table 3.2).

The larvae were also found to survive a high dosage of DDT but not to survive the W.H.O. discriminating dosage of malathion (Table 3.3). Table 3.4 shows the susceptibility test with temephos. This strain was still fully susceptible to the WHO recommended discriminating dosage of 0.02mg/l but there was survival at slightly lower doses, suggesting incipient resistance (Georghiou 1977). Table 3.5 shows the selection results on Cx quinquefasciatus with increasing concentrations of temephos. They responded strongly to

Table:3.1

Exposure of adult Cx quinquefasciatus from Jinjang Utara, Malaysia, to 4% DDT, 5% malathion and 0.25% permethrin papers for one hour (WHO diagnostic dosages)

Insecticide	Dead	Total	% Mortality
DDT	32	235	13.6
Malathion	198	215	92.1
Permethrin	235	250	94.0
Control	0	100	0

Table : 3.2

Exposure of Cx quinquefasciatus to 4% DDT for a series of times.

No. of hours	Dead	Total	% Mortality
1	32	235	13.6
2	51	328	15.5
5	92	300	30.7
6	153	375	40.8
24	212	350	60.6
48	328	375	87.5

Table: 3.3

LC50 values observed in the present study on Malaysian Cx quinquefasciatus larvae towards DDT, malathion, temephos and Bti.

Insecticide	LC50 (mg/l)	95% confidence limits	% survival on discriminating dosage
DDT	0.313	0.280 - 0.344	*
Malathion	0.085	0.069 - 0.0997	0
Temephos	0.0038	0.0034 - 0.0042	0
Bti	0.013	0.009 - 0.015	-

Notes : The LC50 values and the confidence limits were computed using the Nanostat program.

* No discriminating dosage for Cx quinquefasciatus on DDT has been designated by WHO

Table: 3.4 Susceptibility tests of the field collected Malaysian strain of Cx quinquefasciatus larvae with temephos.

Concentration (mg/l)	Dead	Total	Percent mortality
0.006	212	280	75.7
0.008	174	250	69.6
0.01	221	265	83.4
0.015	239	245	97.6
0.02	200	200	100

Table: 3.5 Selection of Cx quinquefasciatus with temephos

Generation	Concentration (mg/l)	Dead	Total	Percent mortality
P	0.01	296	340	87.1
F1	0.015	274	325	84.3
F2	0.018	292	330	88.5
F3	0.02	380	420	90.5
F4	0.02	480	520	92.3
F5	0.02	369	420	82.0
F6	0.02	558	1076	51.9
F7	0.02	218	566	38.5

There followed four generations of relaxation of selection (F8 - F11), followed by checking of the temephos resistance level without selection:-

F12	0.02	406	612	66.3
F13	0.02	474	689	68.8
F14	0.02	629	800	78.6
F15	0.02	698	1008	69.2

N.B. This strain was referred to as JUT strain.

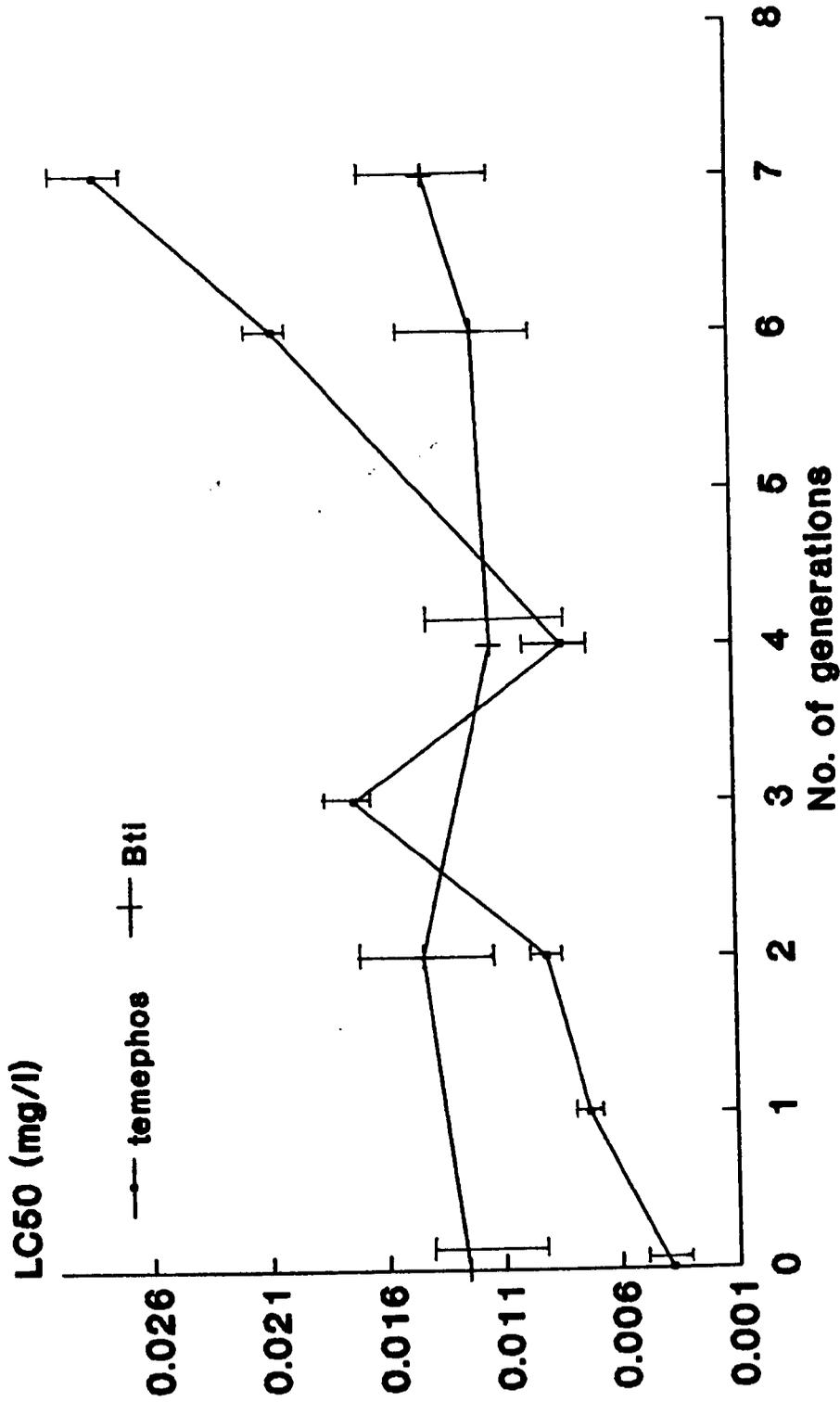
selection, with mortality declining to 91% on exposure to the WHO discriminating dosage of 0.02mg/l at F3. Further selection with the discriminating dosage yielded a strain with 39% mortality at F7. However, the resistance level in this strain dropped from 62% to 34% after four generations of relaxation but remained at this level for several more generations without selection. Fig. 3.1 shows the results of the 7 generations of selection in terms of the LC50.

The results of Bti selection are also shown in fig. 3.1. There is no evidence of a build up of tolerance in this species towards Bti as is indicated by the overlapping of the 95% confidence limits of the LC50 at each generation tested.

3.4 DISCUSSION

The findings in this study add to the existing list of the number of strains of Cx quinquefasciatus resistant to various insecticides such as DDT, malathion and permethrin. The adults of this strain were found to be highly resistant to DDT (Table 3.1) and about 13% of the population survived 48 hours exposure to this compound (Table 3.2). The adults also displayed a low level of resistance to both malathion and permethrin (Table 3.1) even though these compounds are not being deliberately used against Culex in Malaysia. As reported in chapter 2, Ae. aegypti was found to be

Fig 3.1
Selection of Culex quinquefasciatus with
temephos and Bti



Note: The LC50 values and the confidence limits were computed using the Nanostat program.

susceptible to malathion, (the compound used in fogging against adults) and to temephos (the larviciding compound used in the Ae. aegypti control programme). As noted in the materials and methods section, Aedes and Culex shared the same breeding containers and hence experienced the same kind of treatment. Thus it seems likely that the malathion resistance observed in Culex was due to selection by fogging against adults. The other possibility is that the malathion resistance gene in the larval stage is not "turned on" and thus does not express itself in this stage.

As for permethrin, the resistance observed in this species might be due to the well-known cross-resistance inter-relationship of DDT and pyrethroids as described in chapter 2. The permethrin resistance observed here could also have been induced by the restricted use of Resigen (which is a pyrethroid) in thermal fogging for Aedes control.

The larvae of a strain of Cx quinquefasciatus from California showed a high resistance to permethrin after artificial selection in the laboratory (Priester and Georghiou 1978). This strain was selected with d-trans permethrin and developed more than 4000-fold resistance to this isomer by generation F18 and a high cross-resistance towards the cis-isomer and various other pyrethroids. But only about 40-fold resistance was developed to either isomer when this strain was selected with the cis-isomer up to generation F22.

The present study also showed that Cx quinquefasciatus larvae were resistant to DDT but were still susceptible to malathion and temephos although slightly higher tolerance levels were obtained in comparison to previous Malaysian reports (Thomas 1976; Lee et al. 1987). Georghiou and Calman (1969) reported that Cx quinquefasciatus showed a 4-fold increase in the LC50 value to DDT and 30-fold increase to fenitrothion compared with their laboratory standard strain. In another report by Georghiou et al. (1975) the "Camara" and "Knudsen" - strains showed 6-fold increase in LC50 values to DDT compared with a laboratory susceptible strain, even though DDT had not been used for a long time. A more recent report by Georghiou et al. (1985) indicated that resistance to DDT was still high even after another 11 years without usage of this insecticide (Table 3.6). In the present report an LC50 value of 0.313 mg/l to DDT was observed which is comparable to the resistant strain of Georghiou et al. (1985) despite the fact that DDT has not been used for the past 15 years in Malaysia.

The LC50 to malathion was found to be 0.085mg/l which is only very slightly higher than that reported earlier by Thomas (1970). Pennington (1968), however, reported that Cx quinquefasciatus larvae from Okinawa developed 33.5-times resistance to malathion after about 8 years of use. A moderate level of resistance to malathion was also reported by Georghiou et al. (1985) from two regions in California (Table 3.6). Early cases of resistance to malathion in

Table: 3.6 Published LC50 values of 4th-instar larvae of Cx quinquefasciatus (field collected strains) to DDT, malathion and temephos.

Country/State	Strain/place	Year	LC50 (mg/l)		
			DDT	Malathion	Temephos
California	Laboratory susceptible	1974	0.065	0.11	0.0018
California	"Camara"	1974	0.390	1.80	0.210
California	"Knudsen"	1974	0.360	1.00	0.067
California	Laboratory susceptible	1985	0.022	0.076	0.0026
California	Los Angeles	1985	0.310	0.547	0.04
California	Coachella Valley	1985	0.200	0.040	0.007
California	Northern San Joaquin Valley	1985	0.299	0.403	0.090
Singapore		1981	0.165	0.138	0.00085
Malaysia	Kuala Lumpur	1970	0.500	0.080	
Malaysia (present report)	Kuala Lumpur	1990	0.313	0.085	0.0038

Cameroon in 1959 (Mouchet 1960) and Sierra Leone in 1963 (Hamon and Mouchet 1967) reverted to normal shortly thereafter.

Temephos resistance was not immediately apparent in the Malaysian strain used in the present study, as indicated by the absence of survivors when exposed to the WHO recommended diagnostic dosage of 0.02 mg/l. The apparent lack of larval resistance to temephos could be attributed to the dilution of the population from less heavily treated areas in which the species also breeds. However, selection with temephos produced a marked increase in tolerance/resistance (Table 3.5, Fig. 3.1). Upon suspension of selection pressure, for 5 generations, temephos resistance in this strain was found to decline rapidly (Table 3.5) presumably due to lower biotic fitness in the resistance genotypes (Khatib and Georghiou 1985b). However, the resistance level seemed to stabilize thereafter with 30 - 40% resistance remaining in the population in the absence of insecticide pressure. This suggests that the resistance homozygotes may have reduced fitness but the heterozygotes have normal or even above normal fitness. Ferrari and Georghiou (1981) also reported a reduced fitness in a temephos-resistant strain of Cx quinquefasciatus. The development rate in the temephos-resistant homozygotes was longer than the susceptible counterparts, and that of the heterozygotes was intermediate between the two. Although in certain cases it has been found that biotic fitness re-attained its normal

level following extensive selection (Abedi and Brown 1960; McEnroe and Neagle 1968), it is commonly assumed that resistance genotypes have a lower adaptive value than do their susceptible counterparts. In California, Georghiou et al. (1985) reported that there was resistance to temephos in all the three areas sampled (Table 3.6). The population from northern San Joaquin Valley showed higher resistance in comparison to populations from southeast Los Angeles and Coachella Valley, i.e. 34.6-fold at the LC50 and 86.8-fold at the LC95 values. A 117-times increase in the LC95 was also reported by Georghiou et al. (1975) in the "Camara" strain. This high level of resistance was attributed mainly to cross-resistance from other organophosphates heavily used in agriculture in that area. The findings in the present report suggest that it is only a question of time before cases of resistance to temephos will be selected in Cx quinquefasciatus in areas of heavy breeding and intensive chemical control. Raymond et al. (1991) suggested that the amplified gene for organophosphate resistance has been accidentally transported around the world and has not arisen in each area due to independent mutational events.

The high level of resistance to temephos obtained in this study after a few generations of selection could be due to cross-resistance pressure from other organophosphates and possibly from temephos which is known to have been used at the collection site.

The LC50 value of Cx quinquefasciatus for Bti was found to be 0.013 mg/l. This value is similar to that obtained for Ae. aegypti as reported in chapter 2. Upon selection with Bti Cx quinquefasciatus did not show any response. The lack of tolerance/resistance observed in this study could be due to the fact that the selection pressure or duration of selection were not sufficient. The only selection experiments with Bti in mosquitoes have been those of Georghiou (1990) on Cx quinquefasciatus and on Ae. aegypti by Goldman (1987). In both cases only low levels of resistance were observed and they evolved far more slowly than with conventional insecticides: resistance was only noticed after 10 generations of Bti selection (Georghiou 1990). Maximal resistance was obtained in the F46, beyond which it maintained an oscillating plateau. This suggests that under the right conditions with strong and prolonged selection pressure, resistance to Bti could arise. Similar results were also obtained by Stone et al. (1989) on selection of tobacco budworm, Heliothis virescens, with genetically engineered Pseudomonas flourescens. In a study by Sun et al. (1980) involving two strains of An. albimanus and five strains of Cx quinquefasciatus none showed cross-resistance to Bti, despite the presence of high levels of resistance to organophosphates, carbamates and pyrethroids. This agrees with the findings in the present study.

The low levels of resistance reported earlier and the

absence of tolerance obtained in the present study on Bti selection in Cx quinquefasciatus may be due to the fact that Bti has several separate toxin proteins and it seems unlikely that a mosquito could become resistant to all of them in one step (Georghiou 1990); this is comparable to the strategic use of an insecticide mixture for resistance management (see e.g. Curtis et al. in press). This encourages optimism for the use of this biological control agent in mosquito control programmes.

CHAPTER 4

MORTALITY OF MALATHION SUSCEPTIBLE Aedes aegypti AND
SUSCEPTIBLE AND RESISTANT STRAINS OF Culex quinquefasciatus
WHEN EXPOSED TO MALATHION OR RESIGEN THERMAL FOGGING

4.1 INTRODUCTION

In Malaysia, malathion has been used since 1976 to control Ae. aegypti and Ae. albopictus, the vectors of dengue and dengue haemorrhagic fever. The Malaysian Ministry of Health has recommended malathion 90% technical grade (T.G.) for Ultra-Low-Volume (ULV) treatment, in which the liquid insecticide is dispersed undiluted and in a very small quantities over large areas, or thermal fogging in which the insecticide is mixed with oil or water and introduced into a hot blast of air. As malathion thermal fogging is often objected to by the public due to its odour and its unsightly oil deposits on floors, furniture etc., Resilin 10/10 (bioresmethrin 10% w/v with piperonyl butoxide 10% w/v as synergist) has been the insecticide of choice in thermal fogging instead of malathion. These insecticides have been used all over the country following a set of guidelines recommended by WHO for Aedes control (WHO 1980b). These two insecticides are good adulticides with low mammalian toxicity but high toxicity to mosquitoes (Kenaga and Morgan 1978). Resilin thermal fogging is usually carried

out when there are reported cases of DF/DHF or when larvae were found breeding in the inspected houses. Usually all the houses within 220 yards radius from a house with a DF/DHF case are sprayed. This is usually followed by ULV malathion application to cover a larger area. This might be considered as a kind of mosaic pattern of spraying.

As mentioned in chapter 2, at an earlier date in some areas in Malaysia, Ae. aegypti adults were reported to have developed resistance to malathion (WHO 1980a) and the larvae of this species were reported to have developed resistance to malathion by Thomas (1970). Despite these reports of resistance malathion has continued to be used.

It was the original intention of this study to assess the protection conferred by this resistance factor against malathion fogging, bearing in mind that fogging is a very different way of presenting the insecticide than is tarsal contact with a residue or larval immersion which are the standard WHO resistance testing methods. As reported in chapter 2 malathion resistance could not now be found in Malaysia in Ae. aegypti but it was found in Cx quinquefasciatus (Chapter 3). Therefore the study was carried out with resistant and susceptible Cx quinquesfasciatus and susceptible Ae. aegypti adults.

A few trials have been carried out in Malaysia, to evaluate the effectiveness of a ULV formulation of malathion

96% T.G., but there have been very few reported field tests of malathion thermal fogging using the swing-fog machine. Fogging with bioresmethrin failed to control Ae. aegypti in Bangkok, and laboratory studies showed that Ae. aegypti from Thailand, Indonesia and Guyana were strongly resistant to DDT with a moderate cross-resistance to pyrethroids (Chadwick et al. 1977; Prasittisuk and Busvine 1977). Similar results were also reported by a number of field investigators on thermal and ULV application of Reslin 10/10 (R) (Panthumachinda et al. 1976; Lo et al. 1981; Vythilingam and Li 1982). These have prompted the Malaysian Government to look for new insecticides. These included cypermethrin, lambda-cyhalothrin and Resigen (a mixture of synergized pyrethroids). Lambda-cyhalothrin and cypermethrin seemed to be very cost-effective against Ae. aegypti and houseflies (Lim and Visvalingam 1990). Resigen is an extremely safe insecticide and is suitable for use as a ULV space spray or thermal fog. It can be diluted with water or diesel (the former being preferred because it avoids unsightly oil deposits indoors). This insecticide is readily acceptable as it has no odour problem. It is also cost-effective and economic in use. Several trials have been carried out in Malaysia using this formulation and it is said to give 100% kill of Ae. aegypti and Cx quinquefasciatus mosquitoes after indoor fogging, diluted with either diesel or water.

Resigen might be adopted as the other component of a rotation with malathion. Therefore its impact on malathion

susceptible and resistant mosquitoes needs to be assessed in the field and in the present study a re-examination was included of the claim for the high field effectiveness of Resigen against susceptible Ae. aegypti and malathion susceptible and resistant strains of Cx quinquefasciatus.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

4.2.1.1 Mosquitoes

4.2.1.1.1 Aedes aegypti

The A-T strain described in chapter 2 was used in this experiment. The larvae were reared to adulthood and maintained in the laboratory for a few generations. The adult mosquitoes were found susceptible to 5% malathion for one hour using the WHO test kit.

4.2.1.1.2 Culex quinquefasciatus.

The origin of this strain was described in chapter 3. The adults were then selected with 5% malathion until the resistance level had reached about 50%. Another field

strain was collected about 2 km from the site of collection of the resistance strain. A sample of about 200 showed zero survival of the diagnostic dosage and this strain was taken as being susceptible and used in the subsequent experiments.

4.2.1.2 Study areas

The study was conducted in Jinjang Utara, an urban slum about 10 km north west of Kuala Lumpur city. The houses consist of rows of wooden or half brick houses without proper sewage or drainage systems. DF/DHF cases are reported in these areas from time to time, sometimes reaching epidemic proportions. Consequently parts of this area are frequently subjected to intensive fogging with malathion.

4.2.1.3 Insecticides

4.2.1.3.1 Resigen

A mixture of synergized pyrethroids containing S-bioallethrin {(+) trans chrysanthemic acid ester of (+) allethrolona} as an active ingredient, at 0.75% w/v, permethrin {3-phenoxybenzyl (+) - cis, trans - 2, 2 - dimethyl - 3 - (2,2 dichlorovinyl) - cyclo - propane - 1 - carboxylate} at 17% w/v and a synergist piperonyl butoxide {(3,4-methylenedioxy-6-propylbenzyl)-butyl diethyleneglycol ether at 17% w/v. The Resigen formulation was diluted with diesel at a ratio of 1:16.

4.2.1.3.2 Malathion 96%

A technical grade malathion (diethyl mercapto succinate S-ester with O, O-dimethyl phosphorodithioate) was used. This formulation was diluted with diesel oil at a ratio of 40 ml insecticide/litre diesel.

4.2.2 METHODS

Tests were carried out with Malathion 96% T.G and Resigen, applied as a thermal fog, and caged susceptible Ae. aegypti and malathion susceptible and resistant strains of Cx quinquefasciatus. Three to five day old adult mosquitoes were blood fed and then transported to the test site in paper cups, about 35 per cup. The cups were covered with muslin cloth and provide with cotton wool moistened with sugar solution. Clean empty cages made of fine cotton mesh cloth on wire frames measuring about 30 x 30 x 30 cms were also transported to the test site. The size of the cotton mesh was about 1mm. At the test site, house owners were approached and permission was sought to spray their houses. In obtaining permission to enter private property, the field personnel acquainted the householders with the objective of the programme. These houses were then marked and the house numbers were recorded. Five houses were randomly chosen on this basis at a distance of about 100 meters from each

other. The occupants were asked to leave their houses prior to fogging and not to return until 20 minutes after fogging.

Mosquitoes were transferred to the empty cages by releasing them from each cup, one cup per cage. These cages were then placed in each house. One cage each of Aedes, the Culex susceptible strain and the Culex resistant strain, were placed in the living room, kitchen and bedroom respectively of each house. The cages were placed at approximately 0.5 - 1.0 meters from the ground, half an hour before fogging.

As in the normal practice of fogging in Malaysia, the sprayman stood at the front door of each house with the thermal fog generator pointing towards the interior of the house. The machine was then switched on for approximately five minutes at a discharge rate of 15 litres per hour. Spraying of insecticides was conducted between 0900 and 1100 hours. The cages were removed from the houses one hour after the fogging at which time the numbers knocked down were noted. The mosquitoes were brought back to the laboratory and were transferred back to the paper cups and held for twenty-four hours after which the percentage mortality was recorded. Four trials (with five houses each time) were conducted at different sites at weekly intervals, two with Resigen and two with malathion 96% T.G. In each trial, one cage was placed well away from the test site as a control.

4.3 RESULTS

The data from the fogging trials with malathion on caged Ae. aegypti adults and Cx quinquefasciatus (susceptible and resistant strains) are shown in tables 4.1 and 4.2 and the mortalities and percentage knock down with 95% confidence limits are shown as histograms in figs. 4.1 and 4.2. In general there was no significant difference in mortality between the three strains placed in the same room. However, a significant difference in mortality was observed between living rooms and kitchen and living rooms and bedrooms respectively, but no significant difference (overlap of 95% confidence limits) in mortality between kitchens and bedrooms.

Resigen gave 100% knock down of all three strains placed in the living rooms. There were a few not knocked down in the inner rooms of the houses with no significant difference in the knock down between kitchens and bedrooms (Table 4.3). Almost the same results were obtained after the 24 hour holding period (Table 4.4).

4.4 DISCUSSION

The Cx quinquefasciatus resistant strain used in this study showed no significant difference in mortality from the other two susceptible strains. Thus it seemed that the

Table : 4.1

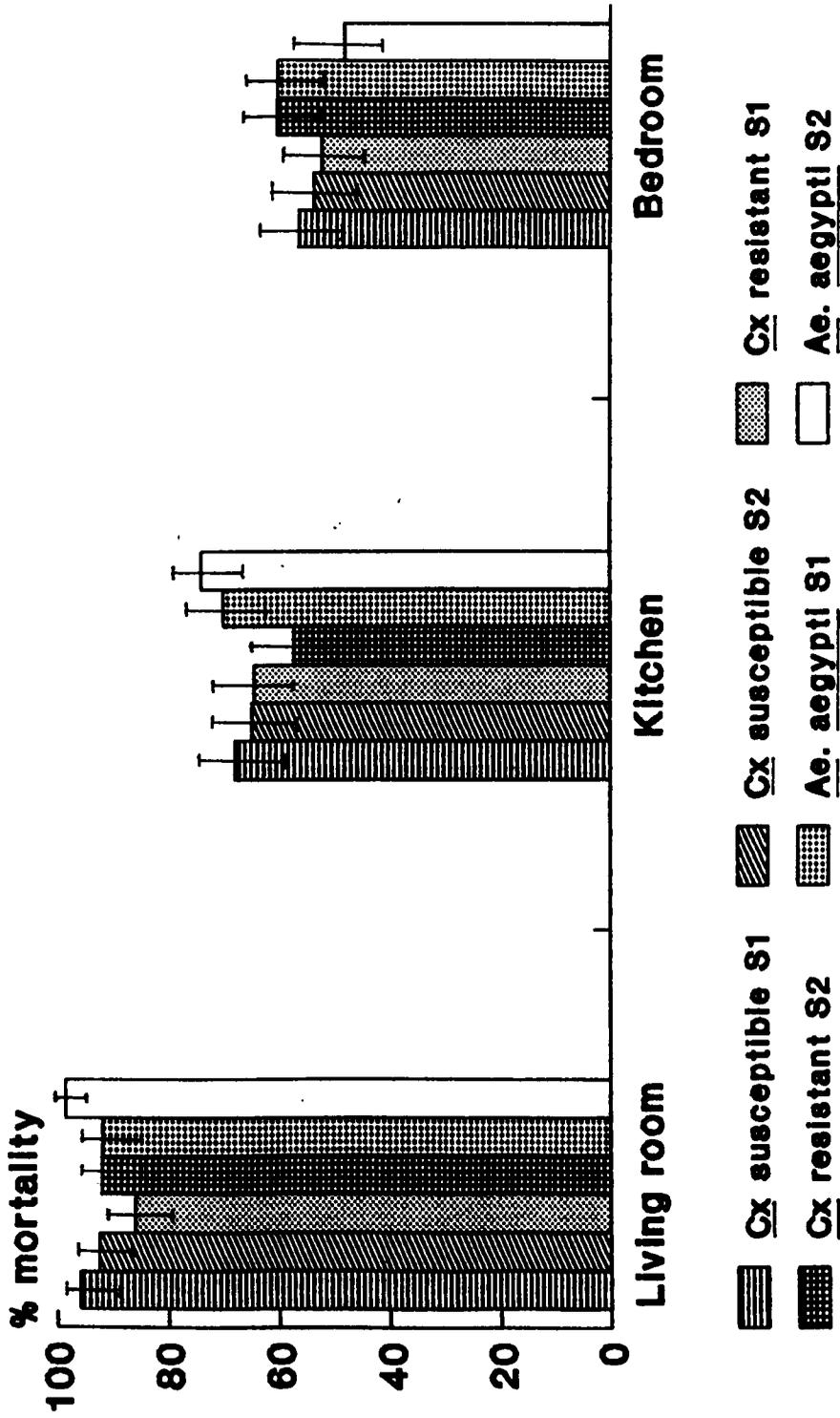
Mortalities of *Ae. aegypti* (susceptible) and *Cx quinquefasciatus* (malathion resistant and susceptible strains) in different parts of houses subjected to malathion thermal fogging (95% confidence limits from tables based on the binomial distribution)

Species/ Strain	Living room		Kitchen		Bedroom		Control		
	1st fogging	2nd fogging	1st fogging	2nd fogging	1st fogging	2nd fogging	1st fogging	2nd fogging	
<u>Culex</u> susceptible	Dead	154	159	108	105	88	86	0	0
	Total	161	172	159	162	157	161	30	31
	% Mortality	95.7	92.4	67.9	64.8	56.1	53.4	0	0
95 % confidence limits	91.2-98.2	87.3-95.9	60.3-75.2	56.4-71.7	48.8-64.6	45.1-61.0	-	-	-
<u>Culex</u> resistant	Dead	128	139	96	91	83	95	0	0
	Total	149	151	149	159	160	158	30	29
	% Mortality	85.9	92.0	64.4	57.3	51.9	60.1	0	0
95 % confidence limits	79.4-91.1	86.4-95.8	56.4-72.2	49.4-65.2	43.8-59.8	52.6-68.2	-	-	-
<u>Ae.</u> <u>aegypti</u> susceptible	Dead	136	146	104	119	91	75	0	0
	Total	148	148	149	161	152	156	25	26
	% Mortality	91.9	98.6	70.0	73.9	60.0	48.1	0	0
95 % confidence limits	86.4-95.8	95.3-99.8	62.0-77.2	66.2-80.4	51.0-67.4	41.5-57.4	-	-	-

Table : 4.2 Percentage knocked down in malathion thermal fogging using the swing-fog machine, on Ae. aegypti (susceptible) and Cx quinquefasciatus (resistant and susceptible strains).

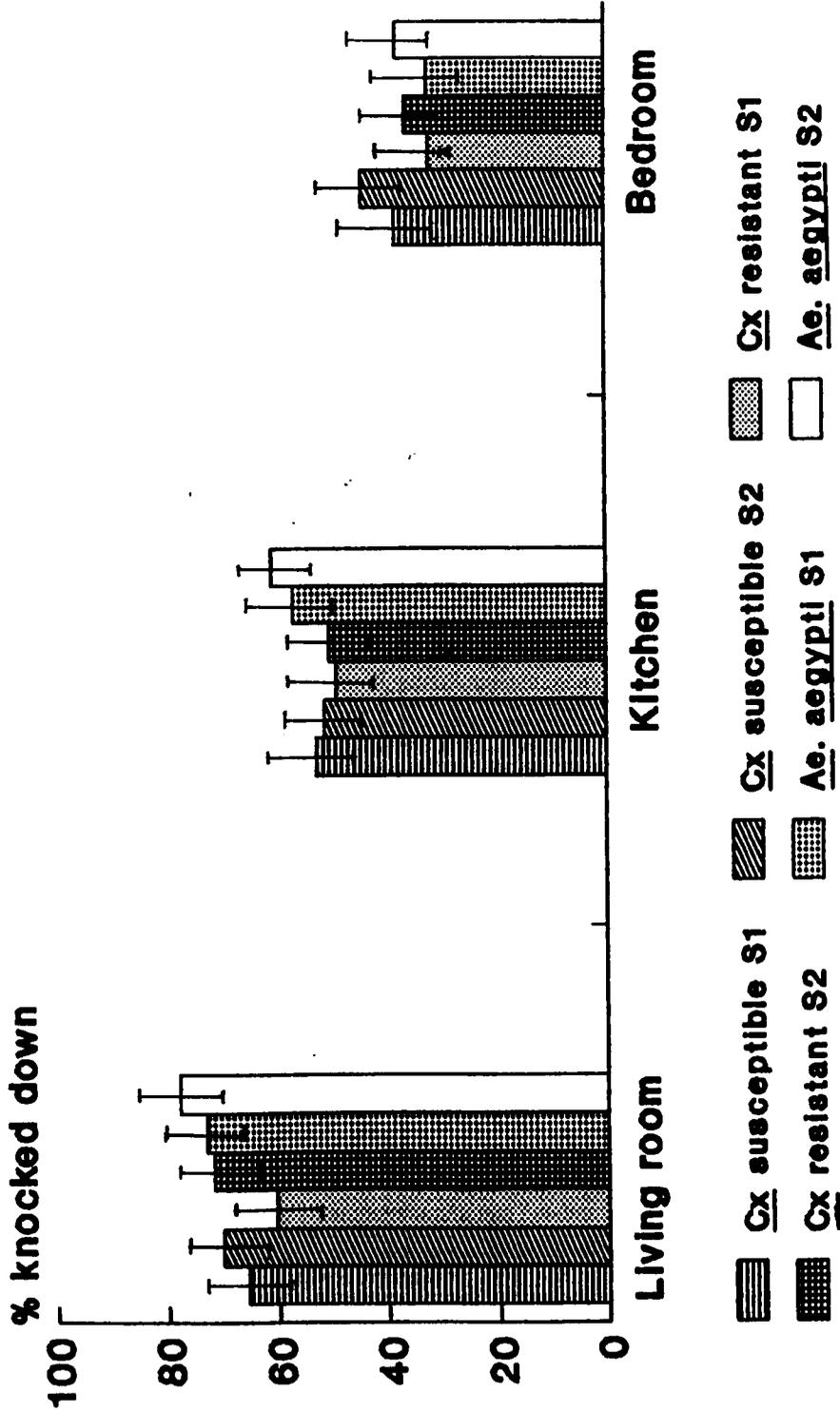
Species/ strain	Living room		Kitchen		Bedroom		Control	
	1st fogging	2nd fogging	1st fogging	2nd fogging	1st fogging	2nd fogging	1st fogging	2nd fogging
<u>Culex</u> susceptible	Dead	106	120	84	83	60	71	0
	Total	161	172	159	162	157	161	26
	% Mortality	65.8	70.0	52.8	51.2	38.2	44.1	0
	95 % confidence limits	57.7-72.9	61.9-76.2	45.1-61.0	42.6-58.5	31.8-47.4	36.0-51.8	-
<u>Culex</u> resistant	Dead	90	108	73	80	51	57	0
	Total	149	151	149	159	160	158	25
	% Mortality	60.4	71.5	49.0	50.3	31.9	36.1	0
	95 % confidence limits	52.4-68.4	63.4-78.4	41.2-57.6	42.6-58.5	28.3-42.1	30.2-45.5	-
<u>Ae.</u> <u>aegypti</u> susceptible	Dead	108	115	85	98	49	59	0
	Total	148	148	149	161	152	156	26
	% Mortality	73.0	77.7	57.0	60.9	32.2	37.8	0
	95 % confidence limits	65.5-80.2	70.5-84.3	49.0-65.3	52.6-68.2	26.9-42.5	31.8-47.4	-

Figure: 4.1
Mortality rates in malathion thermal fogging



N.B. S1 - 1st fogging S2 - 2nd fogging
95% confidence limits from tables based on the binomial distribution

Fig : 4.2
% knocked down by malathion thermal fogging



N.B. S1 - 1st fogging, S2 - 2nd fogging, Cx - Gulex
 95% confidence limits from tables based on the binomial distribution

Table : 4.3

Percentage knocked down in Resigen thermal fogging using the swing fog machine, on Ae. aegypti (susceptible) and Cx quinquefasciatus (susceptible and resistant strain)

Species/ strain	Living room		Kitchen		Bedroom		Control	
	1st- fogging	2nd- fogging	1st- fogging	2nd- fogging	1st- fogging	2nd- fogging	1st- fogging	2nd- fogging
<u>Culex</u> susceptible	Dead	153	156	138	129	140	0	0
	Total	153	156	153	161	159	25	25
	% Mortality	100	100	90.2	80.0	87.9	0	0
95 % confidence limits	97.6-100	97.8-100	92.0-98.6	84.0-94.3	72.9-85.9	82.1-92.7	-	-
<u>Culex</u> resistant	Dead	155	156	142	151	126	123	0
	Total	155	156	155	159	155	153	28
	% Mortality	100	100	91.6	95.0	81.3	80.4	0
95 % confidence limits	97.6-100	97.8-100	90.9-97.8	86.5-95.6	75.0-87.5	72.7-86.1	-	-
<u>Ae.</u> <u>aegypti</u> susceptible	Dead	152	150	144	151	138	126	0
	Total	152	150	160	156	161	158	26
	% Mortality	100	100	90.0	97.0	85.7	79.7	0
95 % confidence limits	97.6-100	97.6-100	84.2-94.2	92.8-99.0	79.2-90.7	72.9-85.9	-	-

Table : 4.4

Mortalities of Ae. aegypti (susceptible) and Cx quinquefasciatus (malathion resistant and susceptible strains) in different parts of houses subjected to Resigent thermal fogging.

Species/ strain	Living room		Kitchen		Bedroom		Control	
	1st fogging	2nd fogging	1st fogging	2nd fogging	1st fogging	2nd fogging	1st fogging	2nd fogging
<u>Culex</u> susceptible	Dead	153	156	145	144	145	0	0
	Total	153	156	153	161	159	26	25
	% Mortality	100	100	98.7	94.8	89.4	91.2	0
95 % confidence limits	97.6-100	97.8-100	95.6-99.9	89.7-97.7	83.5-93.7	85.7-95.1	-	-
<u>Culex</u> resistant	Dead	155	156	145	136	124	0	0
	Total	155	156	155	155	153	29	27
	% Mortality	100	100	98.1	87.7	81.0	0	0
95 % confidence limits	97.6-100	97.8-100	94.6-99.6	88.8-97.0	82.1-92.7	73.4-86.6	-	-
<u>Ae.</u> <u>aegypti</u> susceptible	Dead	152	150	147	140	142	0	0
	Total	152	150	156	161	158	28	29
	% Mortality	100	100	99.4	87.0	89.9	0	0
95 % confidence limits	97.6-100	97.6-100	96.6-99.9	86.5-95.6	80.6-91.7	84.2-94.1	-	-

malathion resistance gene(s) did not protect the mosquitoes under conditions of malathion thermal fogging. Rawlings et al. (1981) also showed that the highest dosage of HCH sprayed onto the walls and thatch roof of mud huts, killed all the three genotypes for dieldrin/HCH resistance in An. culicifacies in the first four weeks after spraying.

Yap et al. (1988) reported almost 100% kill with malathion thermal fogging, whereas in the present study only 48-99% kill was obtained. The difference in kill was probably due to the different methods of fogging adopted. In the trial of Yap et al., fogging was done inside the houses, beginning in the back kitchen area and progressing towards the front of the house to include living room, bedroom etc. In the present trials, as is routinely done, the sprayman stood at the front door and relied on the fog penetrating to other parts of the houses. This explains why a high kill was obtained in the living room where the sprayman was standing as compared to the kitchen and bedroom. The fog penetration into the bedroom could be affected by the presence of curtains in the bedroom doorways. Other barriers in the house structure preventing easy diffusion of the fog, could also partly explain the difference in kill between the rooms.

Malathion has poor larvicidal activity on larvae placed in bowls on the floor during fogging operations (Yap et al. 1988). Although malathion ULV is still effective in

controlling Ae. aegypti, it was noted that the mortality was highest in cages placed outdoors, followed by living rooms and kitchens respectively (Vythilingam and Panart 1991). In similar ULV trials conducted by Perich et al. (1990), 100% mortality was obtained in caged Ae. aegypti placed outdoors, while only 59% and 36% mortality were obtained with cages placed in bedrooms and beneath beds respectively. Thus it seems that during a dengue epidemic, ground ULV application alone would be insufficient because adult mosquitoes hiding in corners and under the beds would not be killed. ULV application from vehicles in the streets should thus be accompanied by thermal fogging in each room of each house, larviciding and other methods of control such as source reduction and environmental sanitation.

In India ULV application of malathion from vehicle mounted machines is frequently carried out to attempt to control the density of malaria vectors. However, in studies with caged mosquitoes it was found that this method of control was ineffective against An. stephensi and other mosquito species (Sharma et al. 1986). The failure was probably due to the fact that fogging was not carried out as recommended. Fogging was carried out at 4 to 6 week or at even longer intervals, instead of on a weekly basis as recommended. Another factor which could add to the failure was that people had the tendency to close doors, windows etc. instead of leaving them open when the fogging vehicles approached.

Resigen is a relatively new pyrethroid space spray formulation which has been registered with the Malaysian Pesticide Board since 1986 (Pesticide Registration Listing, Pesticide Board, Dept. of Agriculture, Kuala Lumpur). During the past few years a number of field trials have been carried out using this formulation. 100% kill was obtained in every trial carried out previously, e.g. Vythilingam, (1988). In the present trials using Resigen on caged adult Ae. aegypti and Cx quinquesfasciatus susceptible and resistant strains, 100% mortality was also obtained in the mosquitoes held in the rooms in each house nearest to the door at which the sprayman stood. Vythilingam (1988) also showed that Resigen was a good larvicide although it was not as good as fenitrothion. However, Resigen appears to be a safer insecticide with a higher LD50 for rats as compared with fenitrothion (Kenaga and Morgan, 1978). On the other hand Resigen was not as effective as a larvicide compared with malathion (Vythilingam and Panart 1990) in reducing the larval density in water pots which received the droplets of insecticide during fogging. Perhaps the concentration so far used has been inadequate.

Resigen was also noted to give a faster knockdown effect of all the strains within one hour after spraying when compared with malathion. This supports the finding of Yap et al. (1988). The presence of S-bioallethrin, a component of the Resigen formulation which is a knockdown agent (Roussel Uclaf 1974), could be a contributing factor

to the fast knockdown of the adult mosquitoes in the field. From this study, Resigen was found to be more effective for the control of Ae. aegypti and two strains of Cx quinquefasciatus when compared with malathion. This was shown by 100% and 84-100% kill obtained with Resigen and malathion respectively in the more exposed rooms in each house. Thus it seems that Resigen has a very promising future in the control of DF/DHF.

CHAPTER 5

MORTALITY OF TEMEPHOS RESISTANT AND SUSCEPTIBLE Culex quinquefasciatus AND Aedes aegypti LARVAE AT INTERVALS AFTER APPLICATION OF TEMEPHOS

5.1 INTRODUCTION

The organophosphate temephos (trade name Abate) is a selective insecticide and is very effective for the control of larvae of medically important insects especially mosquitoes. Temephos may be applied as sand granules impregnated with 1% of the insecticide to water containers to bring about control through slow release (WHO 1984a). It has been shown to have a low toxicity for mammals and it is safe for use in drinking water (Laws et al. 1968; WHO 1984a). In many countries, including Malaysia, temephos has been used to control Ae. aegypti which breeds in man-made habitats such as water storage jars, flower vases, discarded tins, tyres etc. In fact, Ae. aegypti is found breeding in virtually all available containers associated with man as long as they contain reasonably clean and clear water. In Malaysia, temephos has been widely used by the public since 1973 after an outbreak of dengue fever (DF) and dengue haemorrhagic fever (DHF). It is cheap and easily available at a cost of M\$1.00 - M\$1.50 (US\$0.3-0.4) per packet which will last for several months.

Even though piped water had been made available in almost all areas, including urban slums, the great majority of people still continue with the old tradition of storing water. This habit is made necessary by the unreliability of piped supplies. The water is used for drinking, cooking, bathing, washing etc., the containers usually being topped up after use. The stored water provides permanent mosquito-breeding places. The presence of wells in many houses, used or unused, provides another favourite breeding site for Ae. aegypti. Many of these containers are repeatedly treated with temephos 1% S.G. Those houseowners who do not use temephos in their containers are advised to empty and wash them at least once a week. However, this advice is seldom followed. As a result of the use of temephos and other methods of control, the Ae. aegypti Breteau index (the number of containers with mosquito breeding as a percentage of the number of houses) has been brought down from 72% in 1973 (Wallace et al. 1980) to 14% in 1980 (Cheong unpublished data).

Despite the long term use of temephos against Ae. aegypti larvae in many countries, they still show susceptibility to the compound and to organophosphates in general, but are reported to be becoming more tolerant (Chen and Sudderrudin 1978; Lee et al. 1984; Georghiou et al. 1987). As mentioned in chapter 2 in certain parts of Malaysia Ae. aegypti larvae were reported to have developed resistance to DDT, fenthion and malathion (Thomas 1970). It

was an aim of the present study to find out what effect the use of temephos at the recommended dosage has on the survival of resistant and susceptible Ae. aegypti larvae, taking into consideration that some of the water is frequently removed and replaced with clean water thus diluting the insecticide. The amount of water used and replaced varies from person to person and house to house.

As indicated in chapter 2, I was in fact unable to find a strongly resistant Ae. aegypti larvae and observed that they did not respond very much to selection. Therefore resistant Cx quinquefasciatus larvae produced as described in chapter 3 were used instead.

5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

5.2.1.1 Mosquitoes

5.2.1.1.1 Aedes aegypti

The AT strain larvae described in chapter 2 were used in this experiment.

5.2.1.1.2 Culex quinquefasciatus

JUT-Strain : This is a temephos selected strain of Cx quinquefasciatus as described in chapter 3.

JUS-Strain : the unselected susceptible field stock from which JUT had been derived.

5.2.1.2 Insecticide

Temephos or Abate (0,0,0,0-tetramethyl-0,0-thiodi-p-phenylene phosphorothioate) is usually applied at a dosage of 1 ppm of active ingredient (10 gm of sand granules containing 1% temephos added to 100 litres of water).

5.2.2 METHODS.

5.2.2.1 Application of temephos

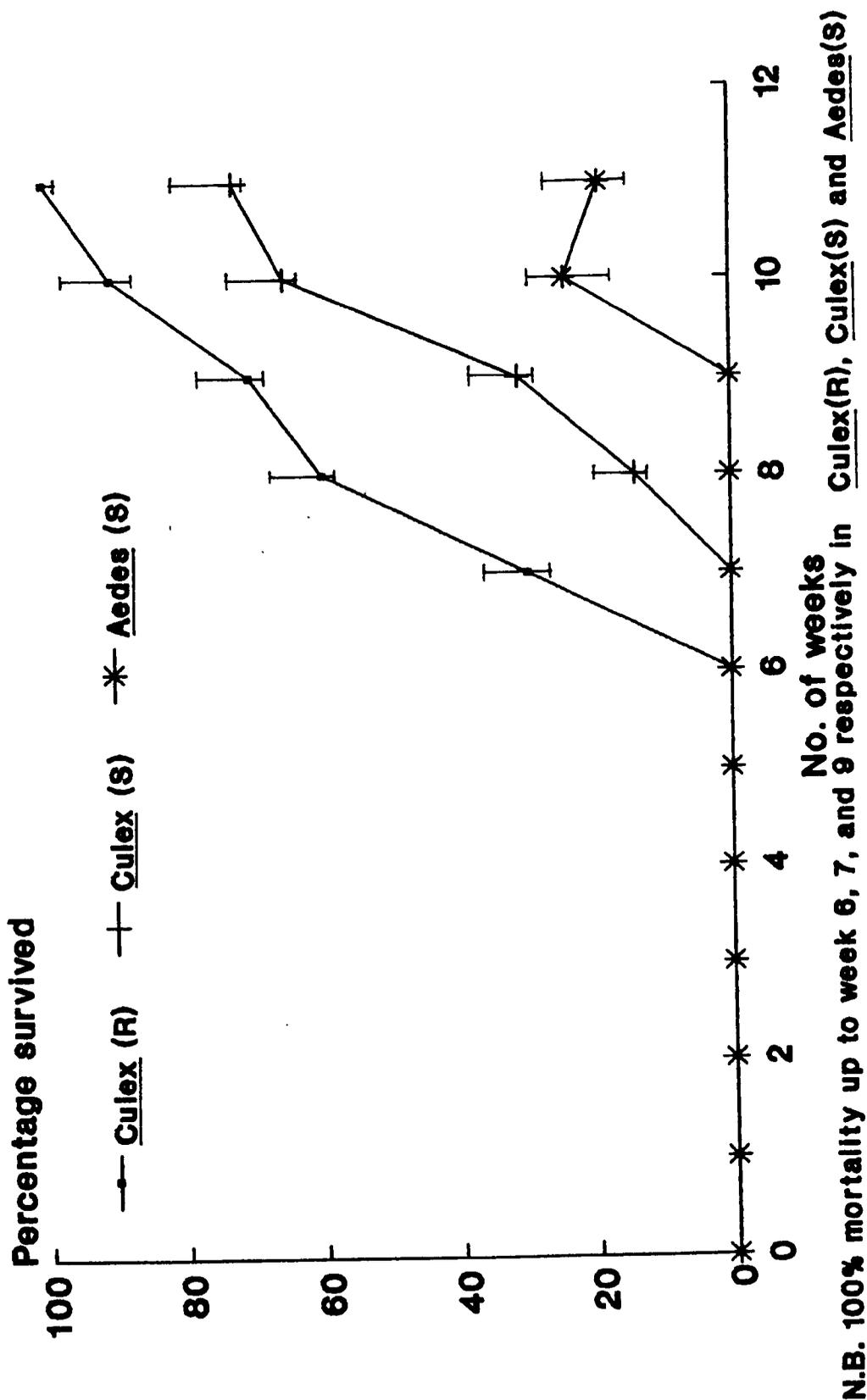
Eight approximately equal sized containers (average 30-40 litres) were placed in the open air in Kuala Lumpur, Malaysia. Four of these containers were plastic and four were earthenware jars. The volume of each container was measured and they were numbered 1 to 8. They were then topped up with tap water to the rim. An appropriate amount of Abate 1% S.G. was added to each container so as to give a concentration of about 1 ppm a.i. Larvae of Ae. aegypti and the JUT and JUS strains of Cx quinquefasciatus were reared in the insectarium and transported to the location of the containers in their rearing bowls covered with plastic covers. 25-30 fourth instar larvae were placed in plastic cups containing 250ml of water taken from the containers

and the mortalities were counted after 24 hours. Controls were set up with tap water. This procedure was repeated weekly for eleven weeks. After every test the containers were topped up to the rim with about five litres of tap water thus diluting the insecticide in the container. The removal and topping up of the containers was to simulate the dilution process in a lightly used domestic container. The containers were covered to minimise evaporation which would have increased the insecticide concentration prior to the regular topping up with tap water.

5.3 RESULTS

On exposure to water samples from the plastic containers, 100% mortality was observed in all three strains in the first six weeks of this experiment (Fig 5.1). Resistant Cx quinquefasciatus started to survive in the seventh week after the treatment and the proportion of survivors increased rapidly to 100% by week eleven. The susceptible strain of Cx quinquefasciatus began to survive the bioassays in the eighth week and the survival increased steadily to 75% in week eleven. Ae. aegypti was the most susceptible strain with 100% mortality up to week nine declining to 78% by week 11. The non-overlap of the 95% confidence limits (based on the binomial distribution) indicates that there were very significant differences in

Fig : 5.1
Bioassays from temephos in water in plastic containers

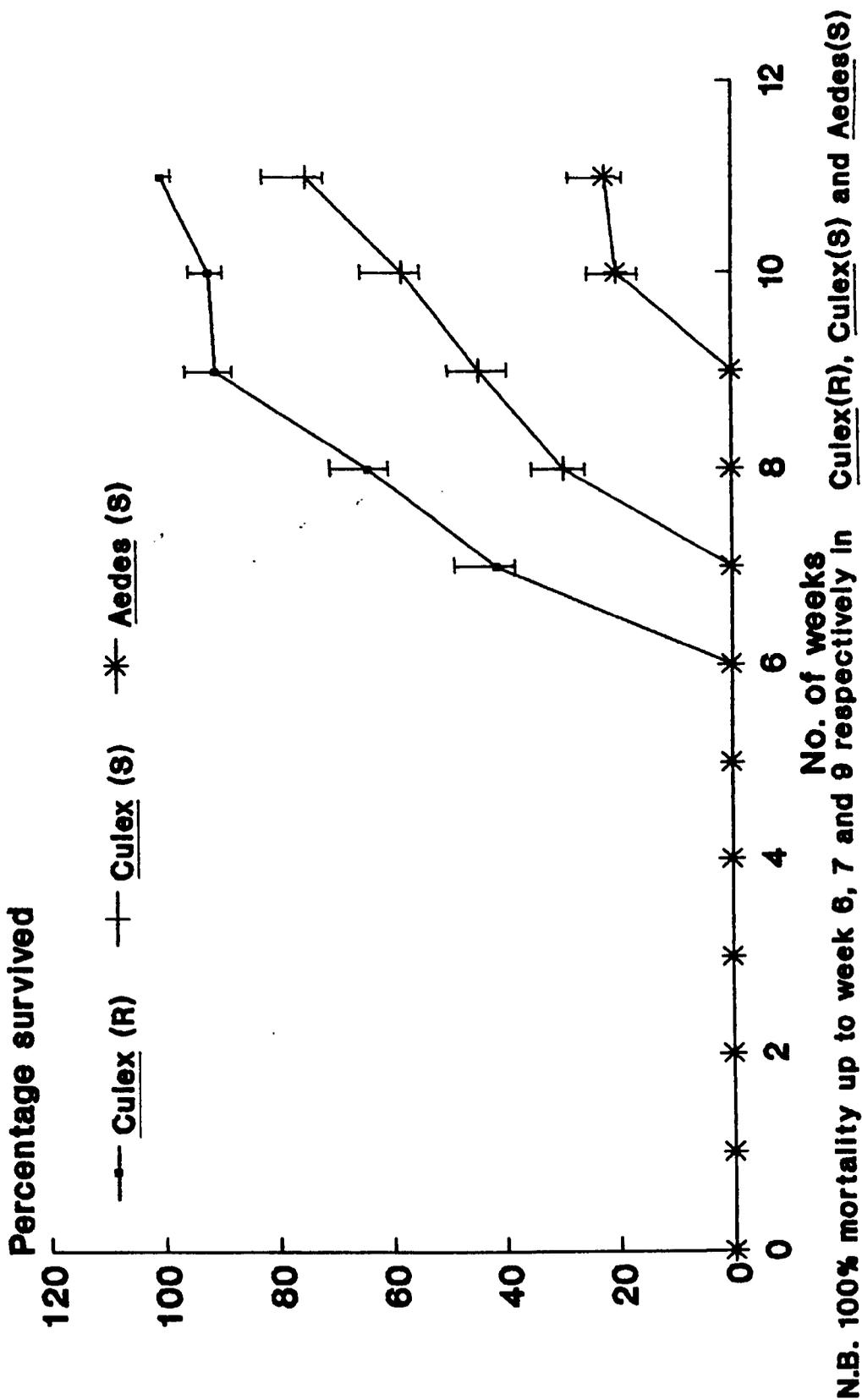


the mortality rates of the three strains. The same trends were observed in the earthenware jars (Fig. 5.2).

5.4 DISCUSSION

It appears that, despite a level of resistance comparable to that in the JUT-Strain, routine larviciding with temephos sand granules would be effective for at least six weeks after treatment. However, water from these containers was completely non-toxic to partially resistant JUT larvae of Culex at week eleven. Culex has been shown to possess a high potential for developing resistance to temephos (Georghiou et al. 1975, 1985) and the local Malaysian population showed the genetic potential for tolerance to this compound. The Aedes strain was the most susceptible of the three strains tested, and a single temephos application at the recommended dosage, with repeated removal and replacement of a proportion of the water, resulted in a very low survival in the bioassays up to three months. This justifies the recommendation that temephos sand granules should be re-applied every three months. Bang et al. (cited in Bang and Pant 1972) reported that a single mass treatment with Abate 1% S.G. at a level of 1 ppm gave good control of Ae. aegypti for periods of up to 6 - 24 weeks. The present study indicated persistence towards the lower end of this range.

Fig : 5.2
Bioassays from temephos in water in earthenware jars



Based on the measured water volumes of the containers (30-40 litres) and the measured volumes of water removed and replaced from each container each week (about 0.4 litres), dilution factors of each container have been calculated (Table 5.1), assuming that the insecticide was homogeneously distributed in the water. These calculations take no account of insecticide bonded to the sand granules and slowly becoming detached nor to the decay of the insecticide. Nevertheless, these dilution factors could explain the 9 weeks duration of control in this study and the 6 - 24 weeks of Bang et al. One should bear in mind that the effective duration could vary from a few days to several months depending on the extent of water-exchange in a particular container.

The duration of control in this experiment was rather similar to the findings of Curtis et al. (1984) from the very different environment of pit latrines. They showed that in most cases there was sufficient residue of chlorpyrifos to kill all susceptible Cx quinquefasciatus larvae for at least 10 weeks, but resistant larvae survived from about two weeks after spraying of chlorpyrifos at a target dosage 1 ppm. It has been reported that different types of container behave very differently in retaining insecticide and releasing it gradually over a prolonged period (Bang and Pant 1972). However, in this study no difference in mortalities was observed in water samples from plastic or earthenware jars. Brooks et al.(1967) and Laws

Table : 5.1 Estimate of the weekly dilution factor in the containers to which temephos was added.

Assuming homogeneous distribution and no decay of the insecticide,

Let V = Volume of the container in litres

Let t = gm. of temephos a.i. added

Therefore initial concentration of temephos = t/V

Let volume removed for the bioassays = R

R litres of tap water are replaced

After one week's water exchange, concentration of temephos

$$= \left[\frac{V - R}{V} \right] \times \frac{t}{V}$$

After n weeks, concentration will be,

$$= \left[\frac{V - R}{V} \right]^n \times \frac{t}{V}$$

For the plastic containers average $V = 50.8$ litres

For the earthenware jars average $V = 49.1$ litres

Volume, R , removed for the bioassays = 5.5 litres

Weeks	Estimated concentration	
	Plastic containers	Earthen jars
1	0.8917 ppm	0.8880 ppm
3	0.7090 ppm	0.7002 ppm
5	0.5637 ppm	0.5521 ppm
7	0.4483 ppm	0.4354 ppm
9	0.3565 ppm	0.3433 ppm
11	0.2835 ppm	0.2707 ppm

et al. (1968) showed no cumulative increase of Abate following continued re-treatment with the sand granule formulation. Larviciding provides longer lasting control than does the use of fogs or aerosols against adults but is difficult to manage because of the large number and variety of larval habitats. Bang et al (cited in Bang and Pant 1972) suggested that three monthly interval between mass treatment gave adequate control.

It is concluded from the present study that the normal application of temephos 1% S.G. at a dosage of 1 ppm a.i. to water containers with constant removal and replacement of a small proportion of the water would be sufficient to kill even the resistant larvae of Culex for about six weeks. The duration could be longer for a more susceptible species such as Ae. aegypti. It seems that resistance gene(s) of the type found in current Malaysian populations of Cx quinquefasciatus would have little impact on the effectiveness of the present larviciding programme.

CHAPTER 6

PROTECTION CONFERRED BY, AND EFFECTIVE DOMINANCE OF, DDT
RESISTANCE IN Anopheles gambiae

6.1 INTRODUCTION

Insecticide resistance mechanisms do not usually confer absolute protection against the insecticide concerned. Decisions about whether a particular example of resistance will interfere with control operations and force a change of insecticide depend on how much protection the resistance gene gives under realistic conditions. When a resistance gene is rare the Hardy-Weinberg ratio indicates that it will occur almost entirely as heterozygotes. It is at this time that resistance management strategies have the best chance of working, so it is important to assess the effective dominance of examples of resistance (Curtis et al. 1978), i.e. to what extent the heterozygote is protected under field conditions.

Most of the studies to date has been done under laboratory conditions (Taylor and Georghiou 1979; Wood and Mani 1981; Roush and Plapp 1982). In the laboratory studies, using the standard WHO test kits the conditions are very artificial, the dose applied is fixed according to those recommended by WHO to detect resistance

and the exposure time is also fixed and the excito-repellent effect of such chemicals as DDT is not allowed for. However in the real situation in the field, the mosquitoes can "choose" either to stay on the sprayed surfaces for a long period of time or to leave seconds after contact with the insecticide. Furthermore the uniform paper surfaces onto which the insecticide is deposited differ from conditions in the field where the insecticide is sprayed onto different kinds of material such as mud walls and thatch roofs of houses and the insecticide is left there as a residue for months during which it may decay or diffuse into the substrate. These differences will influence the results obtained from field and laboratory studies. Emphasis is therefore needed on field trials or at least simulations of the real situation in the field. It was thus the aim of this experiment to study the question of the protection of homozygotes and heterozygotes for resistance. The example chosen was DDT resistance in An. gambiae s.s.

Mosquitoes of the An. gambiae complex are Africa's principal malaria vector as well as being a vector of bancroftian filariasis and certain arboviruses (White 1974). Among the reasons that Africa has by far the worst malaria problem in world is the high vectorial capacity of An. gambiae s.l. because of its long survival and the strong tendency to bite man in most members of the An. gambiae complex. Davidson (1956a,b, 1962) showed that An. gambiae was not just a single species but a species complex. This

complex comprised of six species, namely the fresh water breeding, partially or completely anthropophilic An. gambiae s.s. and An. arabiensis, the zoophilic An. quadriannulatus and An. bwambae (which is apparently restricted to the forest areas of the Rift Valley between Zaire and Uganda). Another two species are mainly salt water breeders, i.e. An. melas of West Africa and An. merus of East Africa (Service 1985). An. gambiae s.s. feeds mainly on humans and is more anthropophilic than An. arabiensis. An. gambiae s.s. usually rests indoors after a blood meal and only exits on the 2nd or 3rd night to seek a suitable oviposition site. Thus there is ample opportunity for contact with a residual insecticide sprayed in houses, provided that this insecticide is not so irritant that it greatly reduces the normal indoor resting period.

As in many other Anopheles species An. gambiae s.l. has been reported to develop resistance to the organochlorine compounds used against it, such as DDT, dieldrin and HCH (Davidson 1956a,b; Davidson and Hamon 1962, WHO 1980b). An. arabiensis in Sudan has also developed malathion resistance (Hemingway 1983).

DDT house spraying has been carried out since the Second World War. It was adopted as a method of malaria control in many parts of the world because it is one of the safest and most effective insecticides with a long residual life and it is one of the cheapest methods available for use

on a large scale. It was first carried out in the West Pacific area during 1944 (Bang et al. 1947). This method of control was shown to be successful in many parts of the world e.g. in Bangkok (Metselaar 1961) and in Papua New Guinea (Sweeney 1983). In Africa, there has been much less vector control than in other malarious continents but malaria control operations using DDT spraying of indoor surfaces has been tested in most of the types of malarious area (Kouznetsov 1977). DDT house-spraying in these regions has been carried out since 1948 with the assistance of UNICEF and WHO. This was generally of limited scope, and in response to epidemics. These early programmes were not very successful due to a number of factors such as high intensity of transmission, the behaviour of the vector An. gambiae, the effect of sorption on mud surfaces of the insecticide, the mobility of the population and the limited size of the trial zones (Kouznetsov 1977). With better management of the methods of application it is possible to ensure that the density of An. gambiae decreases very drastically and that of An. funestus, the other important malaria vector, almost or completely disappears (Kouznetsov 1977).

A large proportion of the African population of rural malarious zones lives in houses made of mud with a thatch roof. It is therefore of great importance to determine the effectiveness of DDT sprayed on the walls and roofs of mud huts. This strategy is based on the assumption that malaria vectors, being anthropophilic, would enter human habitations

at night to seek a blood meal (i.e. show endophagic behaviour). Thompson (1951) and Gillies (1954) demonstrated that An. gambiae s.l. remained indoors after successful engorgement until fully gravid (i.e. showed endophilic behaviour). During this period of indoor resting, one might expect that mosquitoes would pick up a lethal dose of DDT in a sprayed hut and would be killed and thus break the extrinsic malaria cycle in the invertebrate host. The problem is that not all An. gambiae remain inside sprayed huts long enough to pick up a lethal dose. This is due to the excito-repellent effect of DDT (Kennedy 1947). Experiments have shown that the numbers of mosquitoes entering sprayed huts may be 25% less than those entering unsprayed huts and, among those that managed to enter, the feeding rate was less than normal due to irritability from the DDT deposit (Kuhlow 1962; Sweeney 1983; Mpofo et al. 1988). For the endophilic mosquito, An. punctulatus, the reduction in density is very dramatic in sprayed huts (Laird and James 1983).

The residual effect of DDT deposits on various materials has been studied by several workers (Bordas et al. 1953; Mpofo et al. 1988). In most programmes a dosage of 2 gm/sq m has been targeted and it has been shown that this dosage on most surfaces will maintain a residual toxicity for several months or even up to a year or more (Taylor et al. 1981; Mpofo et al. 1988). However the duration of DDT activity varies greatly depending on the nature of the soil

used to make mud walls (Bordas et al. 1953) and contradictory reports have been published on the effectiveness of residual DDT deposits in the control of Anopheles. The higher the humidity the faster the adsorption process of DDT by the soil (Down and Bordas 1951). Fontaine (1983) also pointed out that the residual activity of malathion may exceed three months on wood, but on some mud surfaces its activity can be reduced to a very low level after only three weeks.

In this experiment an attempt was made to simulate the conditions in the field by spraying a miniature mud hut with DDT wettable powder in order to study the survival of resistant and susceptible homozygotes and the effective dominance of the heterozygotes for DDT resistance in An. gambiae s.s. released to fly freely in a room. For comparison the three genotypes were also tested in WHO bioassay cones, with a fixed exposure period of one hour.

6.2 MATERIALS AND METHODS

6.2.1 MATERIALS

6.2.1.1 Mosquitoes used

6.2.1.1.1 An. (Cellia) gambiae s.s. (ZANDS strain)

This stock was mass selected from the ZANU Strain for DDT resistance. It also shows some dieldrin resistance.

This strain has been colonised in this laboratory since 1982 when it was collected from Zanzibar, Tanzania, where DDT house spraying has been used by the anti-malaria programme intermittently for more than 20 years. DDT resistance in this strain is mainly due to an increase in the enzyme DDT - dehydrochlorinase (Hemingway et al. 1986), a type of glutathione S-transferase (Clark and Shamaan 1984) which detoxifies DDT to DDE. A further unknown mechanism may also be operating in this strain (J. Hemingway, pers. comm.).

6.2.1.1.2 An. (Cellia) gambiae (KWA Strain)

This strain was obtained from Kwale, 35 km north of Tanga, Tanzania. It has been colonised from pooled eggs sent from the field by F. Bushrod in 1975. It is susceptible to DDT, dieldrin, organophosphates and carbamates.

6.2.1.2 Insecticides used

75% DDT wettable powder from Tanzania being used in the malaria control programme, brought to London by J.D. Lines. 4% DDT papers were obtained from WHO Headquarters, Geneva.

6.2.2 METHODS

6.2.2.1 Establishment of DDT resistant and susceptible colonies

6.2.2.1.1 Selection of DDT resistant colony

The selection of a DDT resistant colony from the ZANU strain of Anopheles gambiae from Zanzibar was carried out as follows :-

6.2.2.1.2 Mass selection

Mass selection was carried out in order to raise the level of resistance in what was already a markedly resistant strain. The WHO standard adult test methods was used. One-day-old male and female mosquitoes were separated and exposed to 4% DDT papers. A series of exposure times from one to eight hours were used. At successive generations the exposure periods were increased slowly until about 30% survival was obtained after 8 hours exposure to 4% DDT (Table 6.1). The survivors from the exposure were collected and transferred into clean cages and allowed to mate among themselves. The mosquitoes were then blood fed twice a week in order to obtain eggs for the next generation. When the average mortality of the population became stable at about 70% after an 8 hours exposure period, the selection method was changed.

6.2.2.1.3 Single family selection

In order to try to obtain a pure homozygous colony, single family selection was carried out after 13 generations

of mass selection. This method was chosen because mass selection cannot be relied upon to discriminate between homozygous and heterozygous resistant individuals. The survivors of the last round of mass selection with 8 hours exposure were collected and allowed to mate among themselves. Three days later a blood meal was offered in order to obtain eggs. The gravid females were put individually into glass vials (7.5 x 2.5 cms). Each tube was lined with filter paper pieces about 2 cm high. The vials were covered with netting, labelled and a small amount of water added. Only one egg batch was taken from each female and the eggs were allowed 48 hours to hatch. The larvae of each single female were reared separately in bowls of 30 cms diameter. The newly emerged adults were exposed to 4% DDT for 8 hours. The families that had the lowest mortalities after the treatment were kept as parents of the next generation. These mosquitoes were allowed to mate among themselves and after a blood meal, gravid females were again tubed individually for egg laying. This inbreeding process was repeated for four generations and a line that showed the lowest mortality after the treatment in every generation was selected. This line was considered to be probably homozygous resistant and was checked by test crossing to susceptibles to make sure that no susceptible homozygotes were produced. These are recognisable because the WHO recommended discriminating dosage of 4% DDT for 1 hour exposure has been chosen to reliably kill more than 99% of susceptible homozygotes.

6.2.2.1.4 Selection of a DDT susceptible colony

Because the original susceptible strain from Kwale, Tanzania, did not give 100% mortality after an exposure to 4% DDT for one hour, re-selection for DDT susceptibility was carried out by the single family selection method as described above, but the selection was done in the reverse direction. Only a sample of the emerged adults from each family were exposed to 4% DDT for one hour. The full sibs of those samples which showed 100% mortality after the treatment were kept as parents of the next generation. This procedure was repeated for 3 generations. One of the families that showed 100% mortality in every generation was selected to produce the designated susceptible colony.

6.2.2.2 The Experimental Huts

Two mud and thatch huts were constructed to test susceptibility to DDT under realistic conditions. Each mud hut consisted of 3 mud walls, a curtained opening on its fourth side and a palm thatch roof of approximately 1 square metre (see photograph). There was a total of approximately 4 sq. metre of sprayable walls and ceiling. The walls were made of mud bricks (not mortared together) that were specially made for this experiment from London clay by The Building Research Centre, Garston, near Watford. The size of each brick was 25 x 10 x 7 cm and 110 of these were required to build one hut. The thatch roof was made up of

Photograph: 6.1 The experimental miniature hut



palm leaves (local name makuti) which were brought from Tanzania.

The curtain over the hut entrance was lowered when releases of mosquitoes were made. This curtain was free from insecticide. Each mud hut was situated in a temperature controlled room measuring about 1.6 x 2.8 x 1.6 metres. The only exits to these rooms were by a door approximately 1.5 metre high by 0.8 metre wide. These doors fitted tightly and curtains made from netting material were fitted with Velcro just inside each so that mosquitoes could not escape from the room.

The floor of these rooms was covered with white paper and any hole between the paper and the wall or floor was carefully sealed up so that mosquitoes could not creep into these holes. The experiment was carried out after one of the mud huts was sprayed with DDT. The other hut was kept as an unsprayed control throughout.

Before the spraying operation was carried out the outside surfaces of each brick were labelled with a marker pen so that spraying could be done on the opposite side. The hut was then dismantled and all the bricks and the thatch roof were taken outside where the spraying was done. The bricks were lined up against a wall which was previously covered with a plastic sheet. The bricks and the roof were sprayed with an aqueous suspension of DDT made from 75%

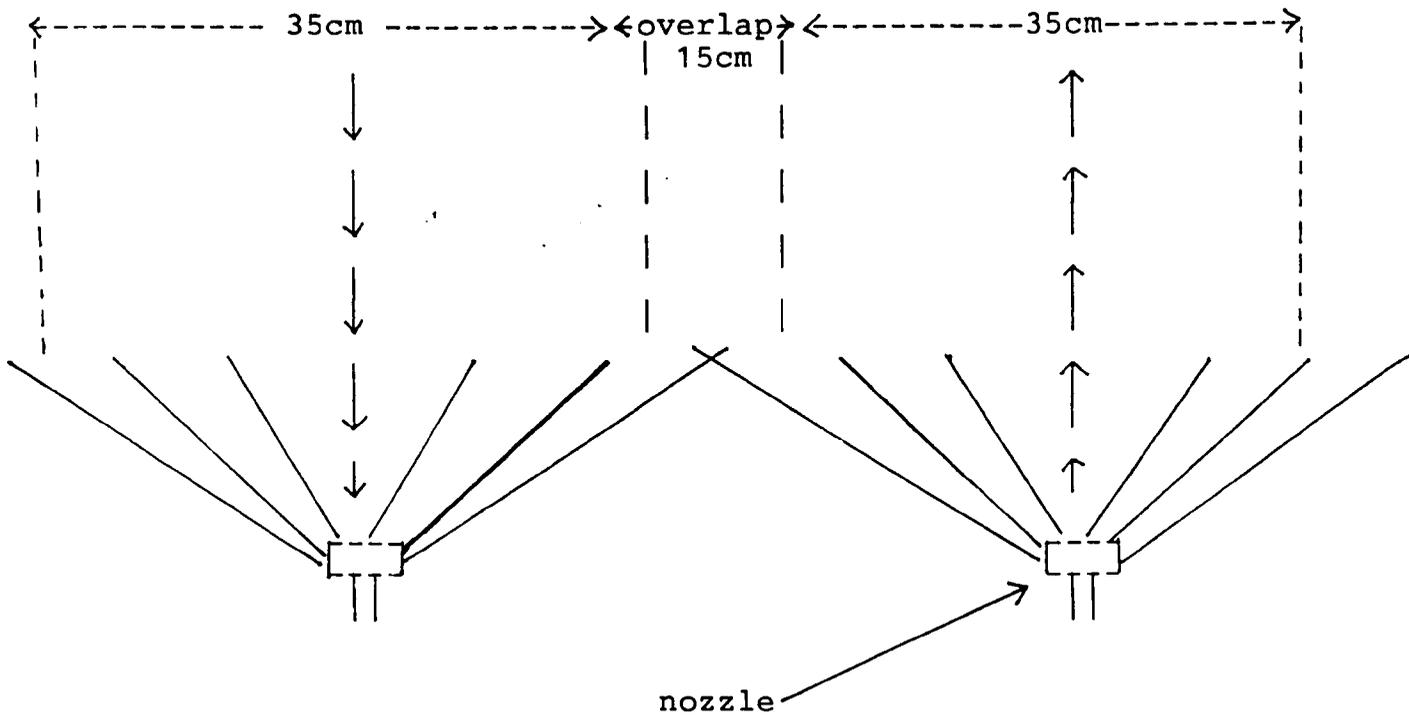
wettable powder. The spraying machine was a Hudson X-part of the type used in malaria control operations, operating at 2.8 kg/sq cm in conjunction with a fan-type spray nozzle with a spray angle of about 80° which was held about 45 cm from the bricks (Fig. 6.1). Calculations were made of the weight of powder required per litre of water to obtain 2 gm of DDT per square metre on the assumption that the rate of application would be 750 ml per minute (Davidson 1981). The volume left in the machine after spraying was measured and the dosage estimates corrected accordingly.

The bricks were allowed to dry for about 2 hours before they were taken to the room where the hut was to be situated. The bricks were re-assembled making sure that the sprayed surfaces formed the inside wall of the hut. The curtain cloth was fixed in position and the hut was ready for the experiment. During the course of this experiment it was observed that released mosquitoes often hid under the lowest layer of bricks, i.e. the spaces between the mud bricks and the floor. To ensure that the mosquitoes did not creep into these spaces, they were sealed up with cotton wool both on the inside and outside of the mud hut.

At monthly intervals after spraying the hut, the selected susceptible strain and a colony which had been selected for DDT resistance as described above were used for releases into the hut. Hybrid mosquitoes were the F1 generation produced by crossing the two selected stocks.

Fig : 6.1

Diagram to show the tracks followed during the spraying process.



These three types of mosquitoes are referred to as SS, RR and RS, though as indicated above, the resistance is probably not only due to a single gene. All these mosquitoes were reared from the early larval stages onward in the room where the unsprayed hut was situated so that their circadian rhythms were adjusted to the lighting conditions of the rooms. The lights in the control and sprayed hut rooms were automatically switched off from 11.00 hr till 23.00 hr and were on from 23.00 hr to 11.00 hr. Thus for most of normal working hours night conditions were simulated. The adult mosquitoes from one batch of eggs were collected in a cage and held until all of them were at least 2 days old before a release experiment.

6.2.2.3 Preparation of mosquitoes for release into the huts.

Preparation of the mosquitoes for release was done outside the room. Mosquitoes were aspirated out of their cages and counted. They were then transferred into paper cups which were covered with netting. The three genotypes of mosquitoes were dusted lightly with different, readily distinguishable, fluorescent powders by the method described by Curtis and Rawlings (1980). They were then released into the sprayed and control huts where anaesthetised guinea-pigs were placed. The releases were made at 11.00 hr just after the lights went off. The curtain over the hut doorway was

drawn and the mosquitoes were released from the paper cups inside the huts by removing the netting material.

Gentle tapping of the sides of the cups usually induced most mosquitoes to fly out. Some injury inevitably occurred during handling and dusting, and the mosquitoes that were unable to fly out of the cups were kept, counted and subtracted from the totals released. The remaining mosquitoes were free to fly about and feed on the guinea-pigs if they "chose" to do so. Movement in and out of the huts was limited to the crevices between the bricks and spaces (varying in width between 6 - 12 cm) between the roof and the top row of mud bricks.

The mosquitoes were collected from the huts and rooms at 17.00 hr in the dark with the help of an ultraviolet lamp. The different genotypes could be distinguished easily by the different fluorescent marking on them. These mosquitoes were collected using a battery operated aspirator and were classified as knocked down or active, fed or unfed and whether they were found inside or outside the hut. They were kept overnight in the paper cups with access to damp cotton wool which had been soaked in glucose solution. The mortality of each genotype was counted the next morning. The same procedure was repeated for the control, unsprayed, hut.

Because of the poor blood feeding rates after these releases, the whole procedure was repeated with female

mosquitoes of the three genotypes of An. gambiae s.s. which had been fully fed on guinea-pigs prior to release.

6.2.2.4 Cone bioassays

Tests with WHO standard bioassay cones (WHO 1984b) were carried out with the susceptible and resistant strains of An. gambiae s.s. and their hybrids. Fully fed female mosquitoes were exposed for 1 hour inside the cones on the sprayed surfaces of the walls and roof of the mud huts. 3 - 4 sites on the walls and the roof of the hut were chosen for this experiment. These exposures were started during week 3. The same procedure was repeated for the control hut.

6.3 RESULTS

6.3.1 Selection of DDT resistant and susceptible colonies

The results of mass selection are shown in table 6.1 which gives data on the mortality at each exposure time. The parental stock showed 59% mortality following exposure to 4% DDT for 1 hour. At generations F1 and F2 the mortality increased with increasing exposure time of one to three hours. From generation F8 onwards, 8 hours exposure was used to select the stock and this gave decreasing mortality until at F12 about 71% mortality was observed.

Table: 6.1. Mass selection for a DDT resistant colony (ZANDS) of An. gambiae. The figures indicate the percentage mortality following the indicated exposure period (Number tested in parentheses). At each generation, survivors of the longest exposure period shown were used as parents of the next generation.

Generations	Exposure period (hrs)							
	1	2	3	4	5	6	7	8
P	59.2(191)							
F1	76.5(169)	82.3(198)	88.3(445)					
F2	60.5(260)	66.4(422)	71.1(698)					
F3		61.9(163)	82.9(263)	86.9(170)	83.1(254)			
F4				86.8(281)				
F5					91.2(441)			
F6						82.0(337)		
F7						79.7(212)		
F8							82.4(198)	90.4(126)
F9							70.4(196)	82.5(125)
F10								70.6(150)
F11								69.7(105)
F12				28.6(200)	35.3(156)	40.2(178)	50.6(176)	71.3(121)

After four generations of single family selection, the strain still showed about 38% mortality on 8 hours exposure (Fig. 6.2). The selection was then stopped and the family with the lowest mortality was taken as being as resistant as could be achieved in the time available. It is referred to as the RR-strain. Fig 6.3 shows the single family selection of the KWA strain. Only a sample of the emerged adults from each family were used for the test. Where the sample showed 100% mortality after treatment, the remainder of the family were kept for continuing selection. Only a family which showed 100% mortality in three consecutive generations was chosen to form the fully susceptible colony. At F3 all the families showed 100% mortality, but the one with the largest sample (21 mosquitoes) was chosen and designated as the SS strain.

Table 6.2 shows the results of test crossing the RR strain to the SS strain. Zero mortality on exposure to 4% DDT for one hour indicates that all test cross progeny were RS, in contrast with the SS strain which showed 100% mortality. Thus the RR strain was taken as homozygous for DDT resistance.

6.3.2 Mosquito survival after release into DDT sprayed hut followed by 24 hours holding period.

Figs 6.4 and 6.5 show the percentage survival of the three genotypes released either pre-fed or unfed into the DDT

Fig : 6.2

Single family selection for homozygous DDT resistant colony of the ZANDS strain of *An. gambiae*. The figures indicate mortality on exposure to 4% DDT for eight hours. The black dots indicate the families which were selected because of their lowest mortality.

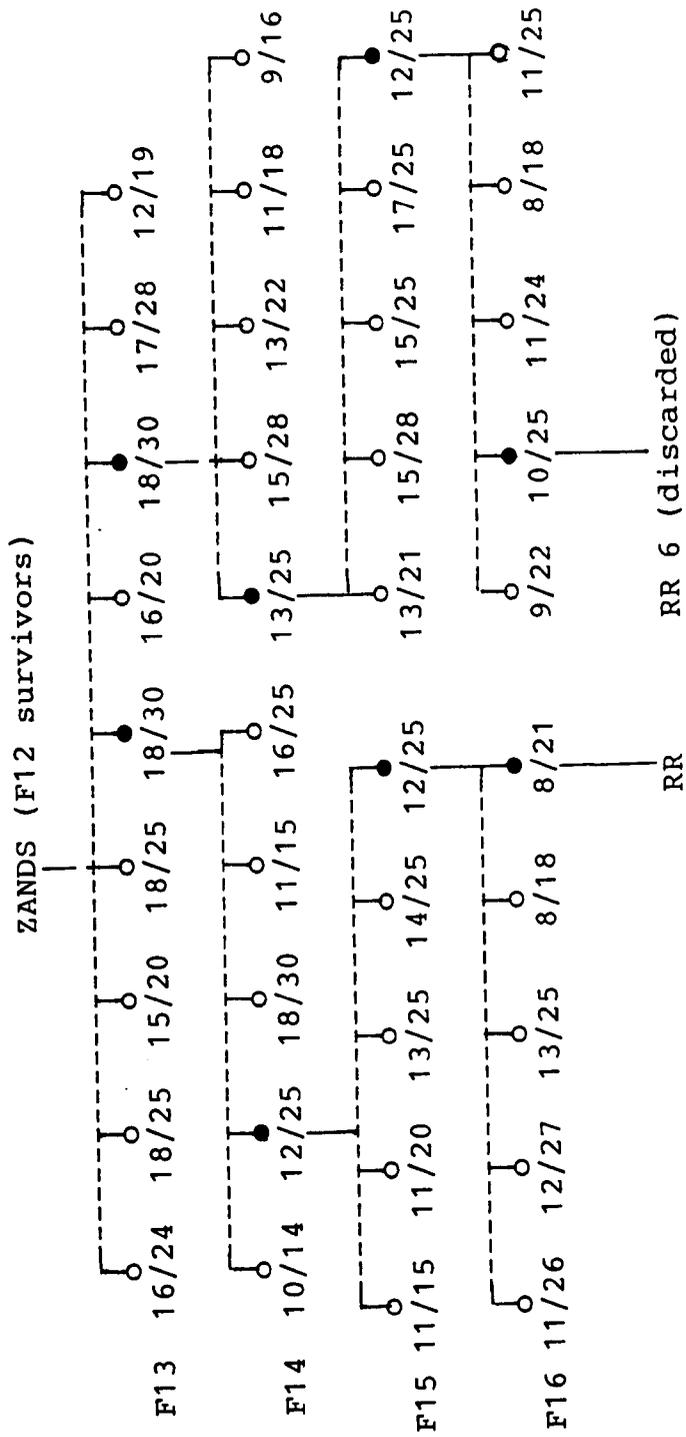


Fig : 6.3

Single family selection for DDT susceptible homozygote colony of the KWA strain of *An. gambiae* s.s. The figures show mortality on exposure to 4% DDT for one our of a sample of each family.

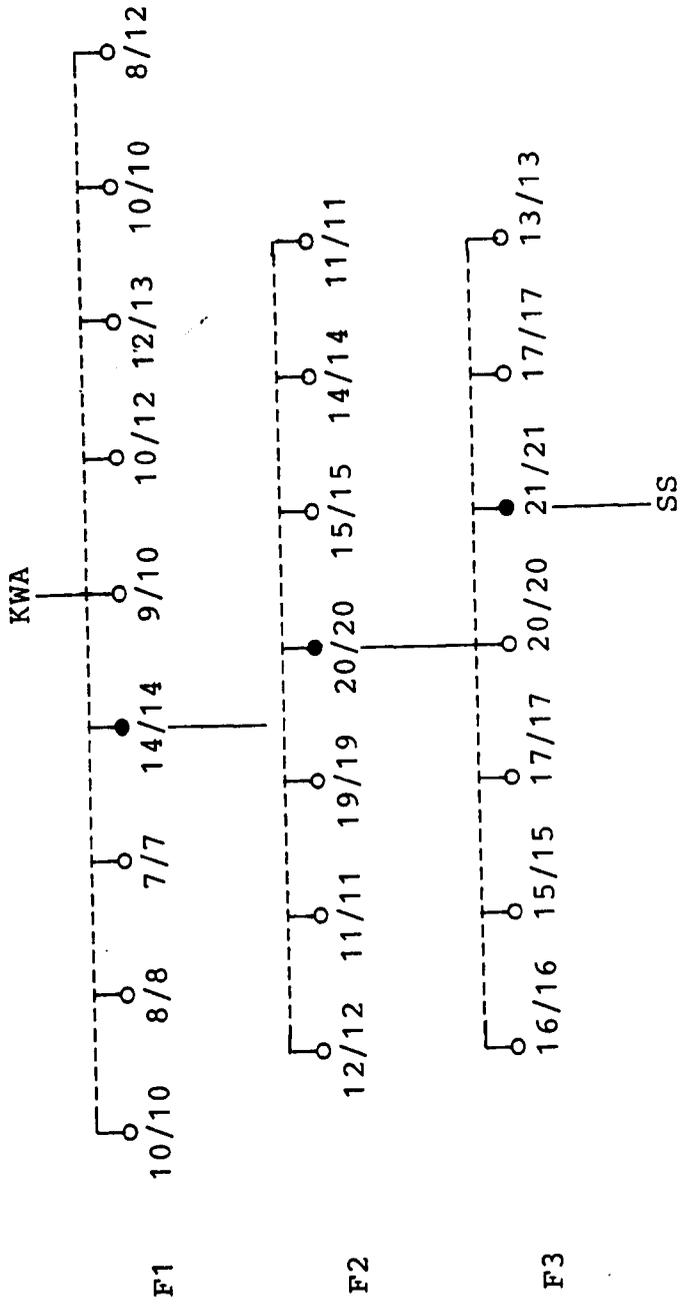
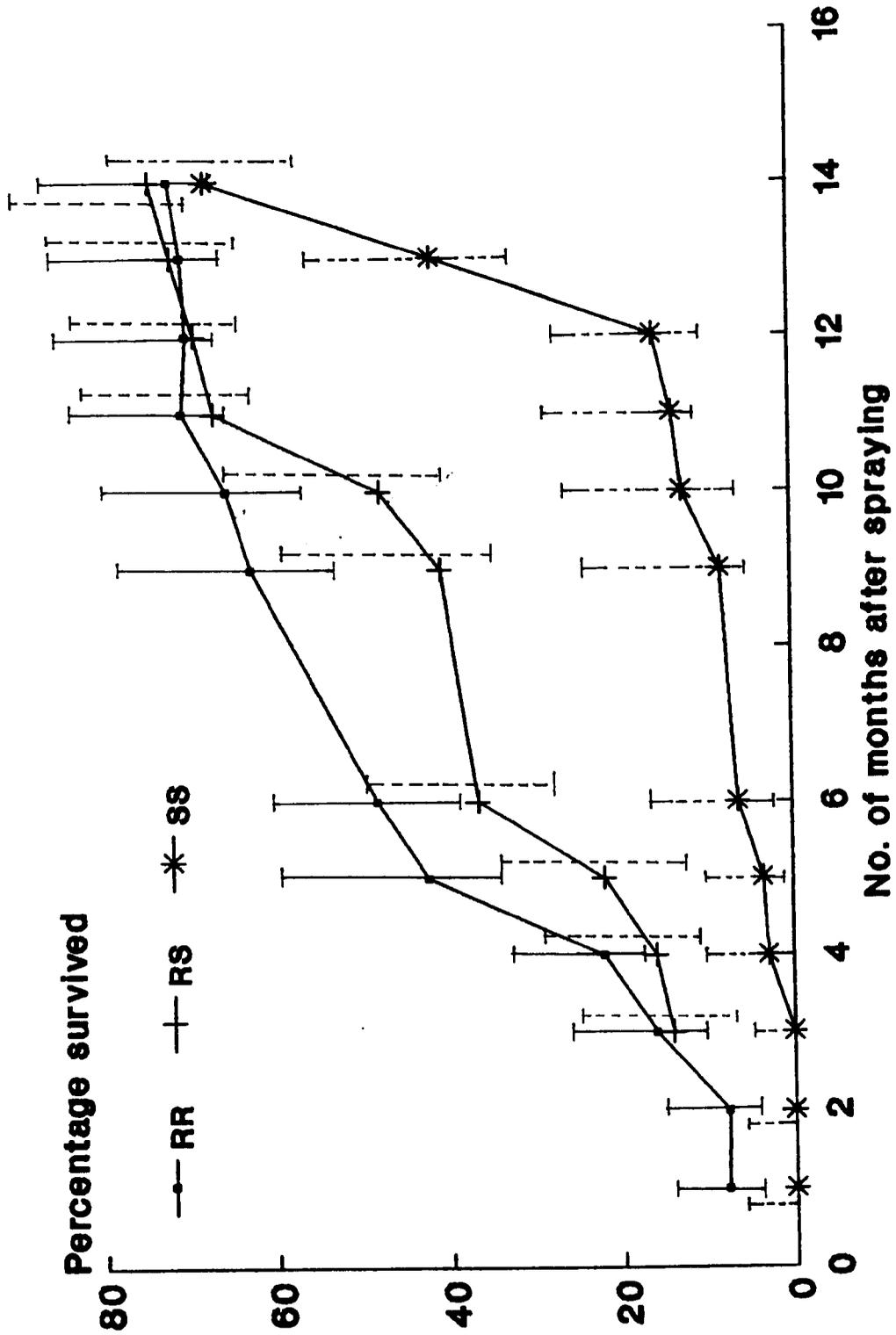


Table: 6.2

Test-cross results of RR-strain to the homozygous susceptible stock (SS) on exposure to 4% DDT for one hour.

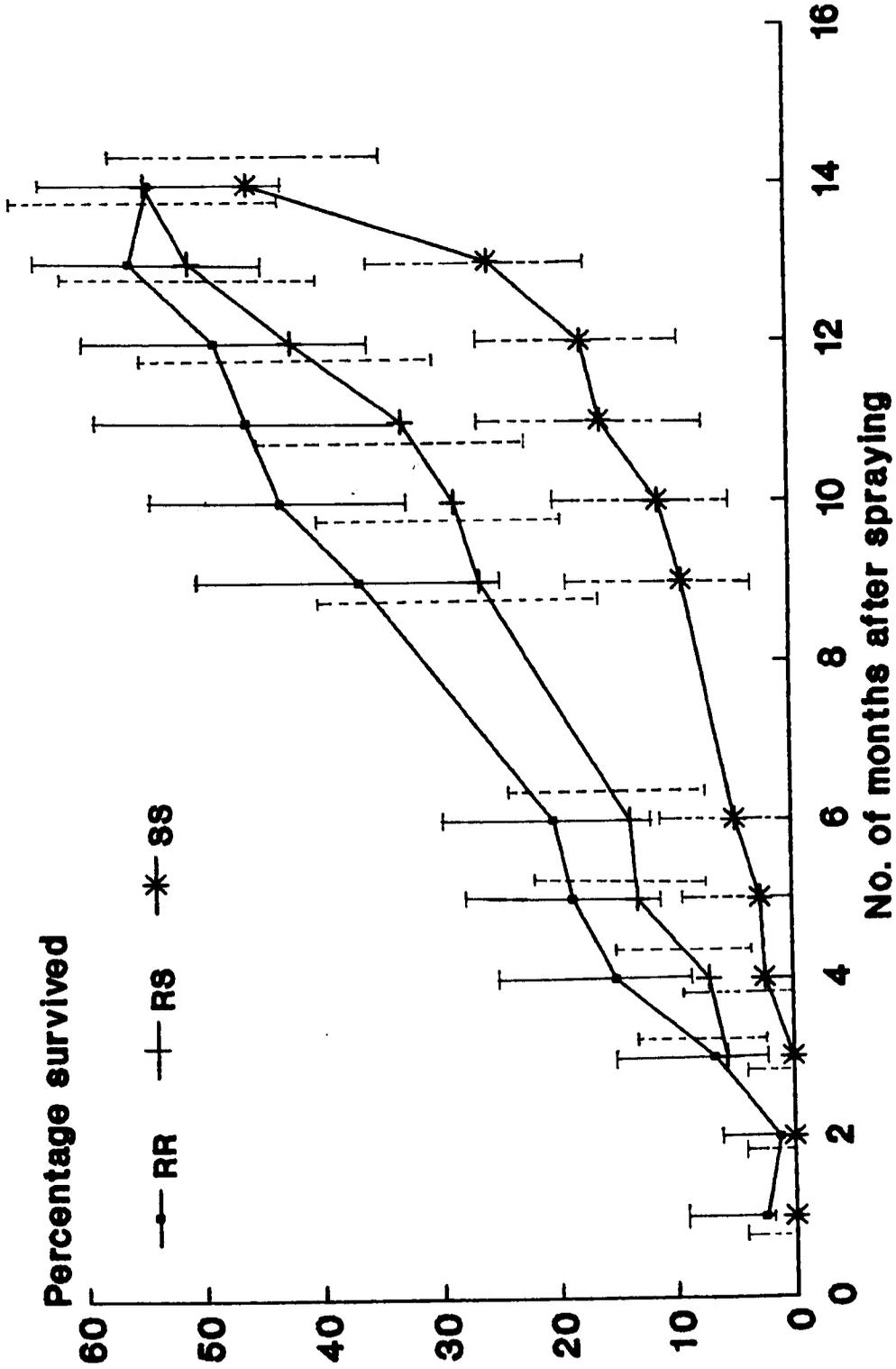
Origin of mosquitoes	No. tested	Dead	Mortality
F1 (RRXSS)	324	0	0.0
S stock	250	250	100.0

Fig : 6.4
% survival of unfed mosquitoes released
into DDT sprayed hut



N.B. 100% mortalities were obtained for SS up to month 3

Fig : 6.5
% survival of pre-fed mosquitoes
released into DDT sprayed hut



N.B. 100% mortalities were obtained for SS up to month 3

sprayed hut. Details of the sample sizes are given in Tables 6.3 and 6.4. When the DDT was fresh all the SS mosquitoes and almost all the RR were killed. As the deposit decayed RR gave increasing survival and eventually SS started to show some survival. At almost every test, survival of the genotypes was in the order $RR > RS > SS$ so that it can be concluded that resistance was neither completely dominant nor recessive. However, more detailed examination of the data showed that for females released unfed there was often an overlap in the 95% confidence limits of the survival between RR and RS genotypes but no such overlap between RS and SS genotypes up to 13 months after spraying (Fig 6.4). Furthermore the survival of RS almost always exceeded the mean of that of RR and SS (i.e. the mid-parental value, Table 6.3) and was on average 34% greater, i.e. resistance was incompletely dominant.

Among the pre-fed females, there was generally overlap of the confidence limits between RR and RS and between RS and SS genotypes but there was no overlap between RR and SS genotypes from month 4 to month 13 after spraying (Fig 6.5). At a given time after spraying and for a given genotype, higher survival of females released unfed than fed was observed. Mortalities of both unfed and pre-fed females in the control hut were very low (0 - 13% for unfed and 0 - 5% for pre-fed mosquitoes, Table 6.5 and 6.6).

Table : 6.3 Percentage of survival and feeding of females of the different genotypes released unfed into the DDT sprayed hut.

Month	Genotype	No. survived	No. fed	No. recaptured	% survived	Mid-parental value	95% confidence limits
1	RR	7	5	90	7.8	-	3.2-15.4
	RS	-	-	-	-	-	-
	SS	0	0	80	0	-	0.0-4.5
2	RR	6	5	78	7.7	-	2.9-16.0
	RS	-	-	-	-	-	-
	SS	0	3	72	0	-	0.0-5.0
3	RR	11	2	69	15.9	-	8.2-26.7
	RS	8	0	57	14.0	8.0	6.3-25.8
	SS	0	1	81	0	-	0.0-4.5
4	RR	18	7	82	22.0	-	15.6-35.1
	RS	13	7	81	15.9	13.7	10.8-28.7
	SS	2	4	69	2.9	-	0.4-10.1
5	RR	25	5	59	42.4	-	32.7-59.3
	RS	16	3	73	21.9	24.7	13.1-33.1
	SS	3	5	85	3.5	-	0.7-10.0
6	RR	40	5	83	48.2	-	38.2-60.6
	RS	26	3	71	36.6	27.9	25.5-48.9
	SS	4	2	63	6.3	-	1.8-15.5
9	RR	35	3	56	62.5	-	52.2-78.2
	RS	24	6	59	40.7	39.6	32.8-59.3
	SS	5	7	61	8.2	-	5.8-24.2
10	RR	47	7	72	65.3	-	56.0-78.6
	RS	31	7	65	47.7	41.2	39.5-64.9
	SS	7	6	56	12.5	-	6.4-26.2
11	RR	57	10	81	70.4	-	63.1-83.2
	RS	56	9	84	66.7	46.3	61.8-81.8
	SS	11	9	81	13.5	-	10.8-28.7
12	RR	58	14	83	69.9	-	65.3-84.6
	RS	58	13	84	69.0	47.0	63.1-82.8
	SS	13	9	83	15.7	-	10.5-28.1
13	RR	57	18	81	70.4	-	65.8-85.3
	RS	53	16	74	71.6	60.6	62.8-83.8
	SS	27	14	65	41.5	-	32.3-57.5
14	RS	51	13	71	71.8	-	66.0-86.5
	RS	54	17	73	74.0	72.6	71.5-90.2
	SS	50	10	74	67.6	-	55.7-78.0

Table : 6.4 Percentage survival of prefed females of the different genotypes released into the DDT sprayed hut.

Month	Genotype	No. survived	No. recaptured	% survived	Mid-parental value	95% confidence limits
1	RR	2	81	2.5	-	0.3-8.6
	RS	-	-	-	-	-
	SS	0	84	0	-	0.0-4.3
2	RR	1	82	1.2	-	0.0-6.6
	RS	-	-	-	-	-
	SS	0	81	0	-	0.0-4.5
3	RR	5	75	6.7	-	2.2-14.9
	RS	5	87	5.7	3.4	1.9-12.9
	SS	0	83	0	-	0.0-4.4
4	RR	12	80	15.0	-	8.0-24.7
	RS	6	84	7.2	8.7	2.7-14.9
	SS	2	82	2.4	-	0.3-8.5
5	RR	16	86	18.6	-	11.0-28.5
	RS	11	84	13.1	10.7	6.7-22.2
	SS	2	74	2.7	-	0.3-9.4
6	RR	17	84	20.2	-	12.3-30.4
	RS	10	73	13.7	12.5	6.8-23.8
	SS	4	84	4.8	-	1.3-11.8
9	RR	20	55	36.4	-	23.8-50.4
	RS	15	57	26.3	22.8	15.5-39.7
	SS	6	66	9.1	-	3.4-18.7
10	RR	37	86	43.0	-	32.4-54.2
	RS	23	81	28.4	27.0	18.9-39.5
	SS	9	82	11.0	-	5.1-19.8
11	RR	27	59	45.8	-	32.7-59.3
	RS	22	67	32.8	30.9	21.9-45.4
	SS	11	69	15.9	-	8.2-26.7
12	RR	33	68	48.5	-	36.2-61.0
	RS	29	69	42.0	33.0	30.2-54.5
	SS	10	57	17.5	-	8.8-29.9
13	RR	45	81	55.6	-	44.1-66.6
	RS	42	83	50.6	40.6	39.4-61.8
	SS	20	79	25.3	-	16.2-36.4
14	RS	41	76	53.9	-	42.1-65.5
	RS	44	81	54.3	49.9	42.9-65.4
	SS	36	79	45.6	-	34.3-57.2

Table : 6.5 Percentage of survival and feeding among females of the different genotypes released unfed into the unsprayed control hut.

Month	Genotype	No. survived	No. fed	No. recaptured	% survived	95% confidence limits
1	RR	99	27	99	100	96.3-100.0
	RS	-	-	-	-	-
	SS	92	31	92	100	96.1-100.0
2	RR	76	35	81	93.8	86.2-98.0
	RS	-	-	-	-	-
	SS	86	30	89	96.6	90.5-99.3
3	RR	87	28	90	96.7	90.6-99.3
	RS	78	23	79	98.7	93.2-100.0
	SS	87	33	91	87.0	89.1-98.8
4	RR	93	28	93	100.0	96.1-100.0
	RS	96	26	96	100.0	96.2-100.0
	SS	88	31	89	98.9	93.9-100.0
5	RR	77	23	82	93.9	86.3-98.0
	RS	79	19	84	90.8	86.7-98.0
	SS	82	29	87	94.3	87.1-98.1
6	RR	97	32	97	100.0	96.3-100.0
	RS	92	31	93	98.9	94.2-100.0
	SS	91	28	92	98.9	94.1-100.0
9	RR	86	27	88	97.7	92.0-99.7
	RS	87	26	87	100.0	95.9-100.0
	SS	80	25	81	98.8	93.3-100.0
10	RR	90	30	91	98.9	94.0-100.0
	RS	95	25	97	97.9	92.8-100.0
	SS	88	20	90	97.8	92.2-99.7
11	RR	86	22	86	100.0	95.8-100.0
	RS	84	20	85	98.8	93.6-100.0
	SS	82	17	82	100.00	95.6-100.0
12	RR	82	25	90	92.2	84.6-86.8
	RS	91	21	93	97.8	92.5-99.7
	SS	93	23	98	94.9	88.5-98.3
13	RR	88	26	92	95.7	89.2-98.8
	RS	87	23	90	96.7	90.6-99.3
	SS	89	21	91	97.8	92.3-99.7
14	RS	87	18	88	98.8	93.8-100.0
	RS	87	15	89	97.8	92.1-99.7
	SS	79	19	80	98.8	93.2-100.0

Table : 6.6 Percentage recaptured of prefed female mosquitoes released into the control hut (found inside or outside)

Month	Genotype	Number released	Inside		Outside		Percent recaptured	% Mortality
			Dead	Alive	Dead	Alive		
1	RR	99	0	78	1	20	92.0	1.0
	RS	-	-	-	-	-	-	-
	SS	100	1	73	1	18	93.0	2.2
3	RR	100	2	76	2	12	92.0	4.3
	RS	95	0	58	3	28	93.7	3.4
	SS	97	0	70	0	27	100.0	0.0
5	RR	99	0	61	3	32	97.0	3.1
	RS	89	1	63	0	25	100.0	1.1
	SS	100	0	89	0	11	100.0	0.0
9	RR	95	4	69	0	21	98.9	4.3
	RS	95	0	49	3	40	96.8	3.3
	SS	95	0	56	1	38	100.0	1.1
11	RR	97	0	59	0	35	96.9	0.0
	RS	89	2	62	0	21	95.5	2.4
	SS	95	0	66	0	27	97.9	0.0
13	RR	99	0	72	1	19	92.9	1.1
	RS	101	2	57	3	39	100.0	5.0
	SS	99	1	72	2	21	97.0	3.1

6.3.3 Mosquito recoveries (dead or alive)

In table 6.7 it is shown that 80 - 100% of the mosquitoes released unfed were recovered (whether inside or outside the sprayed hut). 100% recoveries were obtained for the first 2 months, almost all the mosquitoes being found dead on the floor. Later the recovery rate declined somewhat, probably because the mosquitoes did not die so quickly and so had more time to hide between the bricks and in the thatch. A slightly higher recovery rate (86-100%) was obtained in the control hut (Table 6.7).

6.3.4 Location and feeding of the recovered mosquitoes

During the first 3 months most of the dead unfed mosquitoes were found inside the sprayed hut but, as the deposits decayed, slightly more deads were found outside the hut until month 9. From month 9 onwards about equal numbers of the dead and of the live mosquitoes were found inside and outside the hut (Fig 6.6). Among the mosquitoes released unfed (Fig 6.7) almost the same trend was observed. In the control hut, most of the fed and alive mosquitoes were found resting inside the hut either on the wall or on the thatch roof (Fig. 6.8).

Table : 6.7 Percentage recoveries of the total number released of the different genotypes released unfed into DDT sprayed and control huts.

Month	Genotype	DDT sprayed hut		Unsprayed hut	
		No. released	Percent recaptured	No. released	Percent recaptured
1	RR	90	100	99	100
	RS	-	-	-	-
	SS	82	100	92	100
2	RR	78	100	81	100
	RS	-	-	-	-
	SS	72	100	89	100
3	RR	80	86.3	95	94.7
	RS	71	80.3	79	100
	SS	90	90.0	96	94.8
4	RR	90	91.1	95	97.9
	RS	85	95.3	94	98.9
	SS	75	92.0	89	100
5	RR	68	86.8	82	100
	RS	80	91.3	90	93.3
	SS	90	94.4	90	96.7
6	RR	85	97.6	97	100
	RS	80	88.8	96	96.9
	SS	75	84.0	94	96.8
9	RR	65	86.2	100	88.0
	RS	65	90.8	100	87.0
	SS	70	87.1	90	90.0
10	RR	80	90.0	91	100
	RS	75	86.7	97	100
	SS	69	81.2	95	94.7
11	RR	85	95.3	90	95.6
	RS	86	97.7	99	85.9
	SS	90	90.0	94	87.2
12	RR	91	91.2	90	100
	RS	88	95.5	93	100
	SS	89	93.3	99	99.0
13	RR	90	90.0	96	95.8
	RS	85	87.1	90	100
	SS	76	85.5	91	100
14	RR	80	88.8	92	95.7
	RS	82	89.0	89	100
	SS	79	93.7	90	88.9

Fig: 6.6
% survival and mortality of prefed
mosquitoes released into DDT sprayed hut

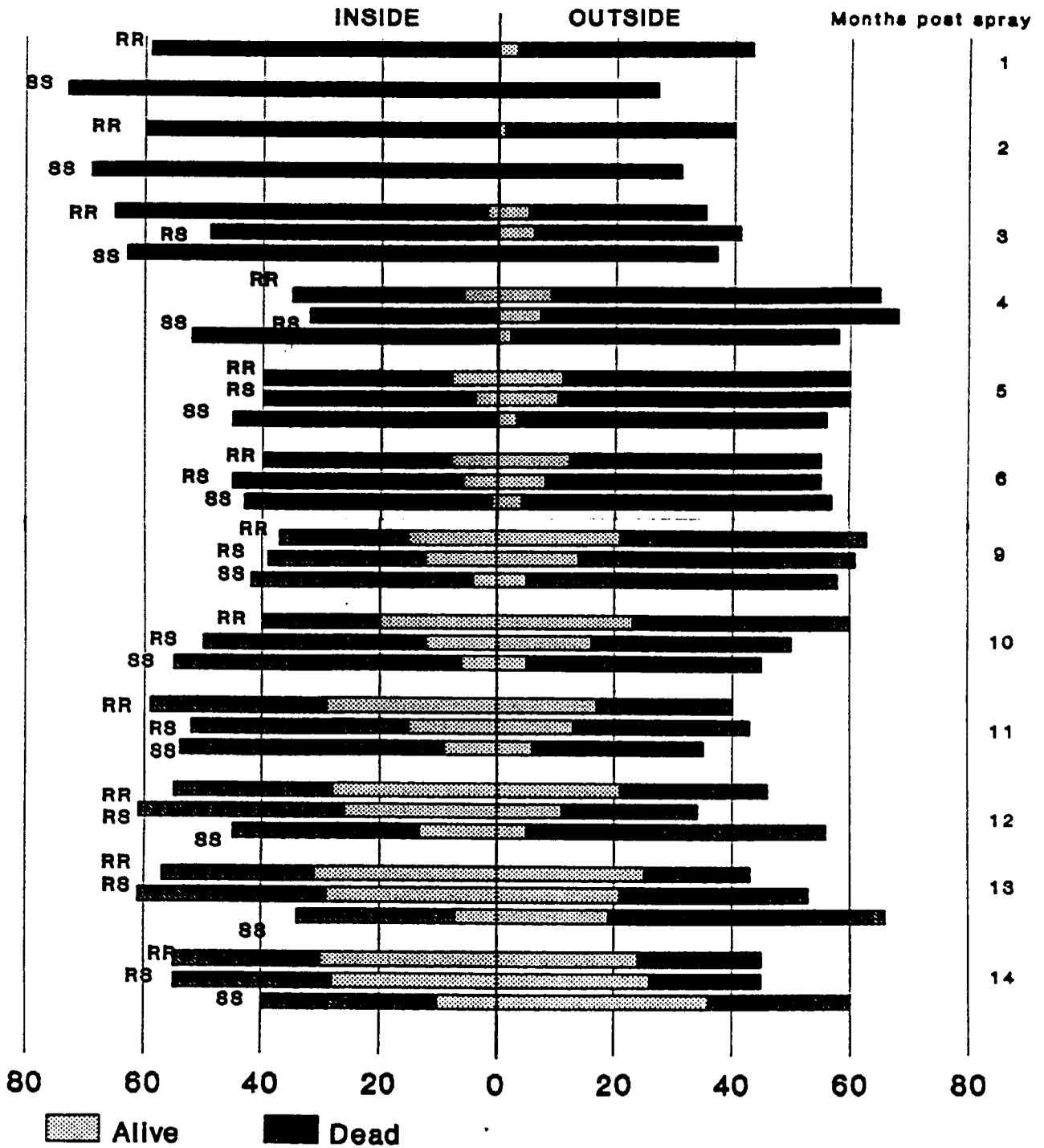


Fig: 6-7

Percentage survival and mortality of fed and unfed females released unfed into DDT sprayed hut.

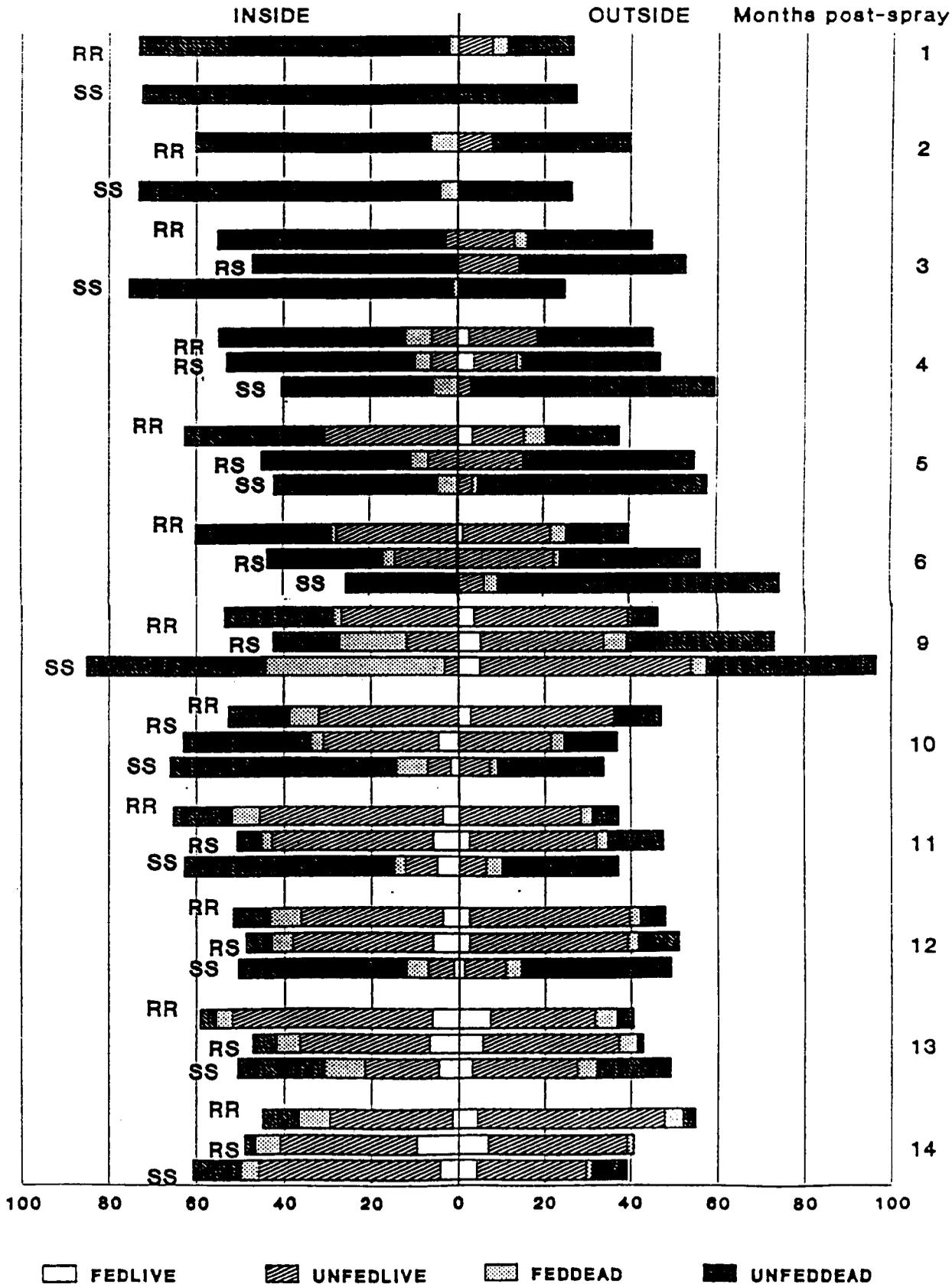
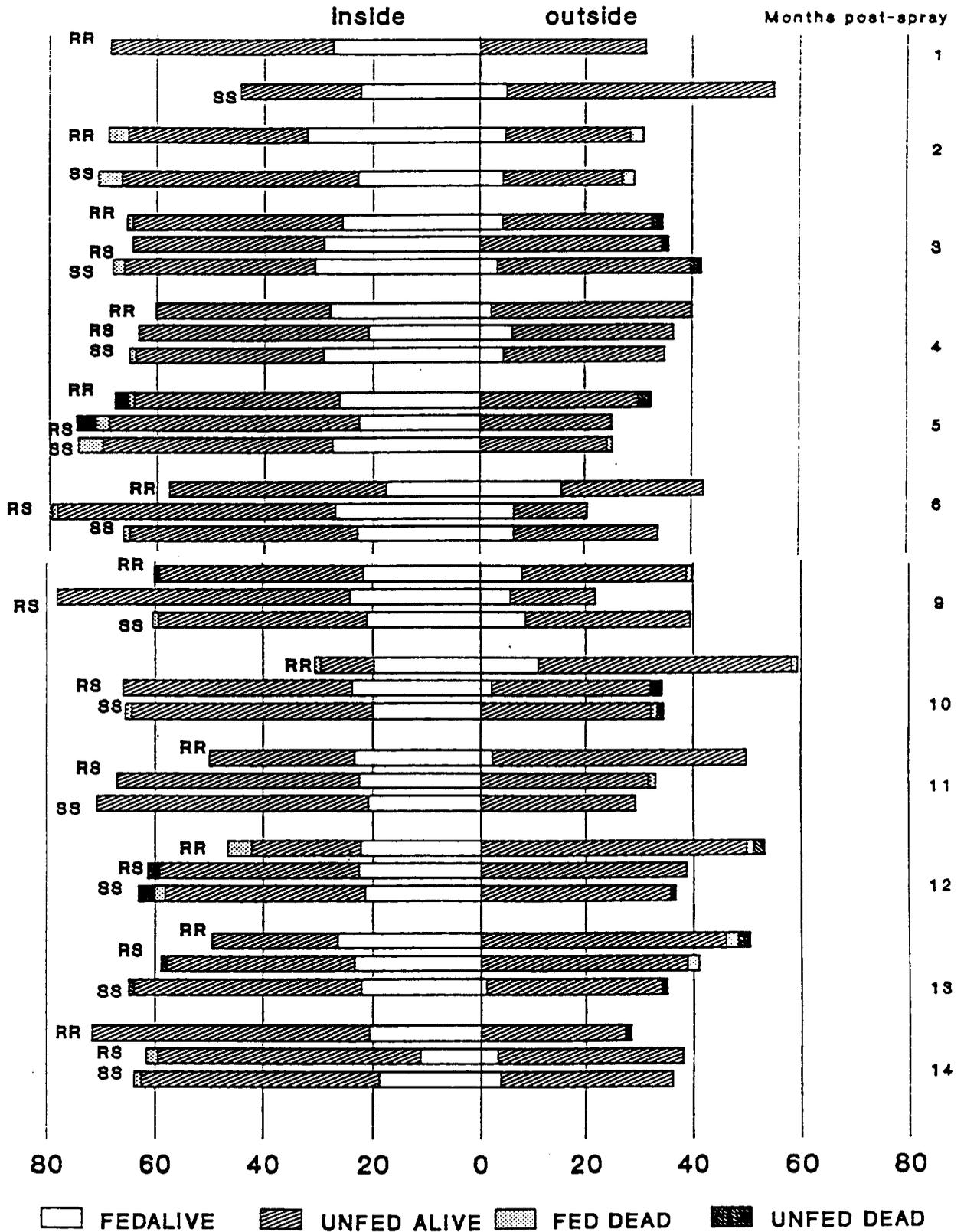


Fig: 6.8
 % survival and mortality of fed/unfed females released unfed
 into unsprayed control hut.



6.3.5 Percentage feeding of females released unfed into DDT sprayed hut.

Fig. 6.7 shows the percentage of feeding among mosquitoes released unfed and found outside or inside the sprayed hut. The percentage of fed mosquitoes ranged from 0 to 23%. With increasing age of the deposits, a slightly higher rate of feeding was obtained. It was interesting to notice that there was no significant difference in the feeding rate between the three genotypes as shown by the trends in fig 6.9a,b and by the overlapping of the 95% confidence limits. In the first 3 months, none of the fed mosquitoes of any of the genotypes were found alive (Fig.6.7). During this period only unfed RR mosquitoes were found alive outside the hut. From month 4 onwards increasing numbers of fed mosquitoes, especially of RR genotype, were found alive inside the hut followed by RS and SS genotypes.

In comparison, the feeding rate in the control hut was slightly higher for all the three genotypes: it ranged from 17 to 43%, most of the fed mosquitoes being found resting inside the hut (Fig. 6.8).

6.3.6 Bioassays on the treated mud walls

Fig 6.10 and Table 6.8 show the survival of pre-fed females of the different genotypes obtained from cone

Fig : 6.9a
Feeding rates of mosquitoes released
unfed into unsprayed control hut

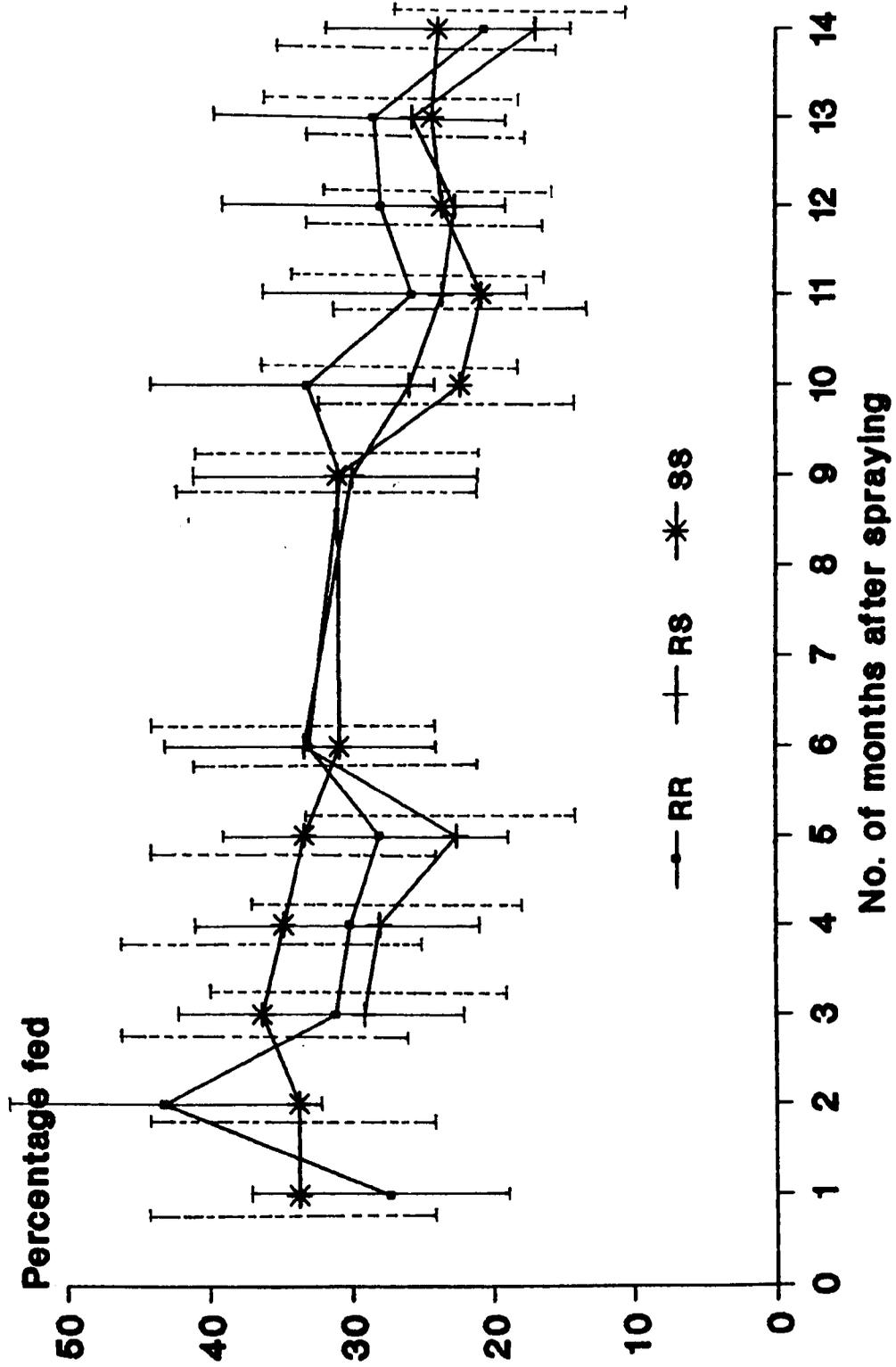
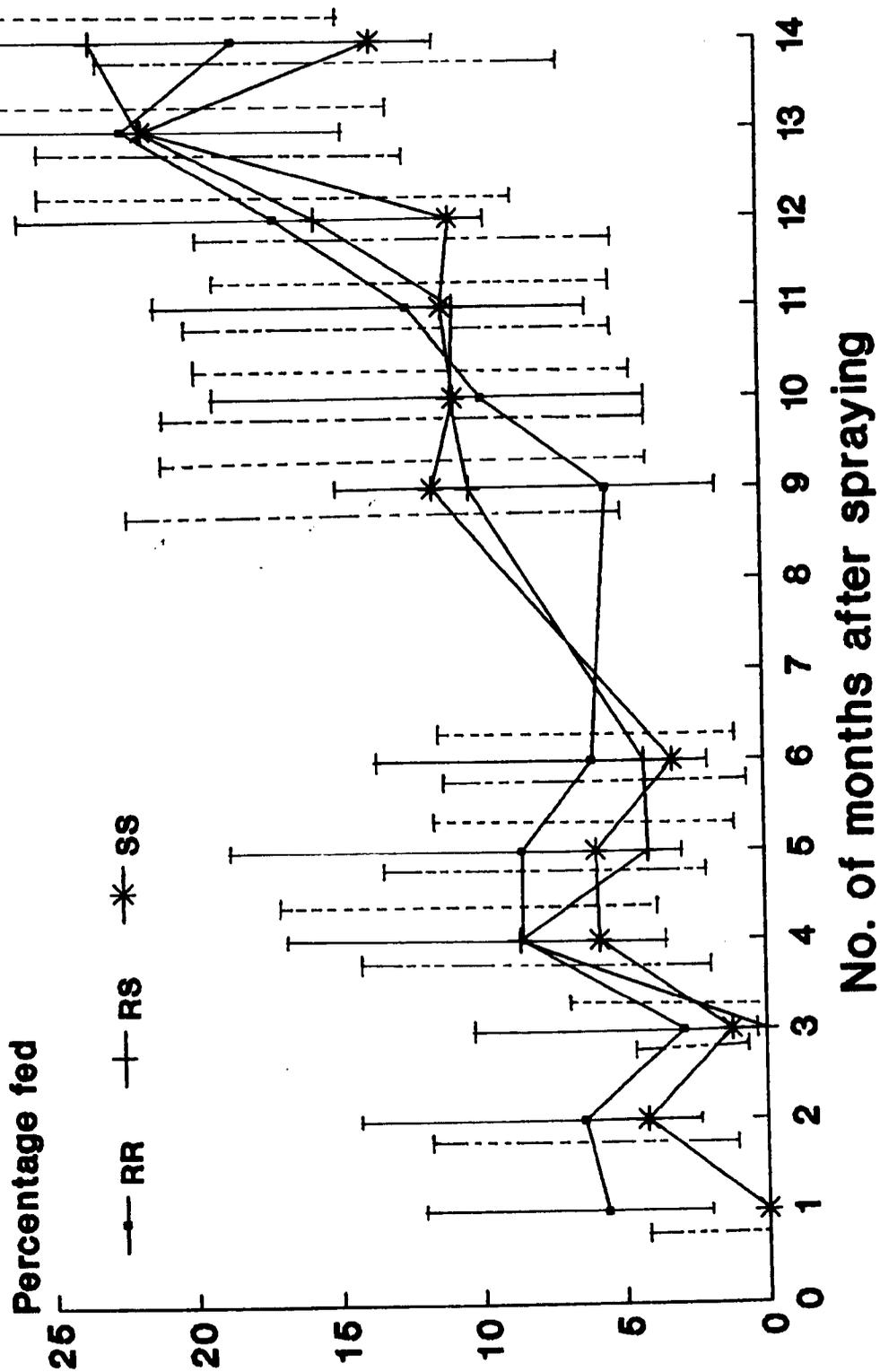


Fig : 6.9b
Feeding rates of mosquitoes released
into DDT sprayed hut



bioassays on the DDT sprayed walls. Very similar results in bioassays of unfed mosquitoes with one hour exposure were obtained, but the percent survival was very much lower. None of the SS genotypes could survive until 6 months after spraying and the percent survival was very low, with about 13% survival at month 14. Generally there was an overlap of the confidence limits between the RS and RR genotypes but not between RS and SS (Fig 6.10). Generally the survival of RS was greater than the mid-parental value by about 18%.

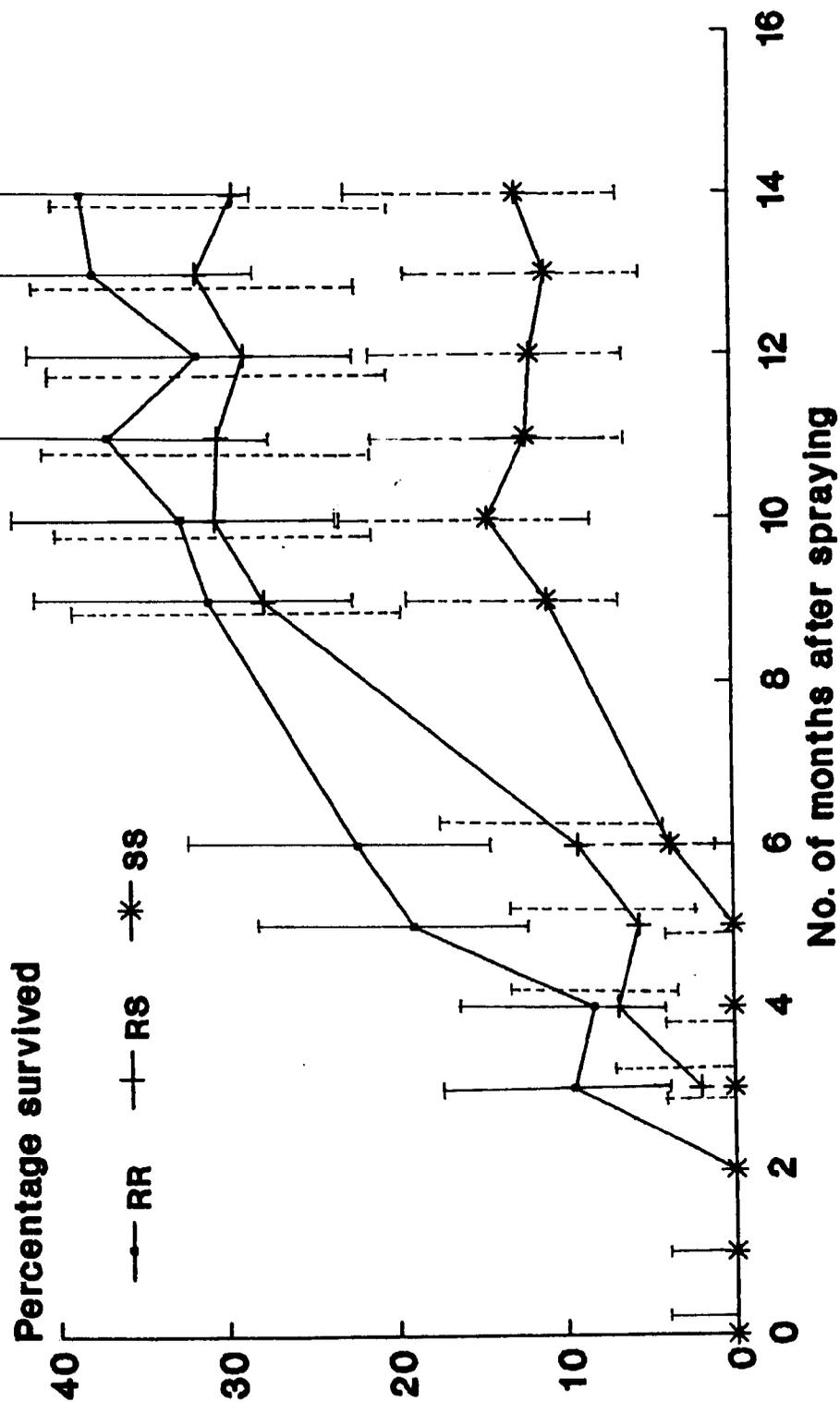
6.3.7 Bioassays on the treated thatch roof.

There was no definite trend in the thatch bioassays (Fig 6.11 and Table 6.9). This test was more difficult to perform than those on the walls. In most cases mosquitoes were irritated and disorientated by the insecticide and penetrated the thatch and were trapped in it. A very high mortality was obtained throughout the experiment, with only about 20% survival of the RR genotype at month 6 and 10.

6.4 DISCUSSION

In malaria control programmes by house spraying, it is assumed that many malaria vectors enter human habitations at night to seek a blood meal and remain indoors to digest the blood. During this period of indoor resting the mosquitoes

Fig: 6.10
% survival of pre-fed females observed
in cone bioassays on DDT sprayed wall

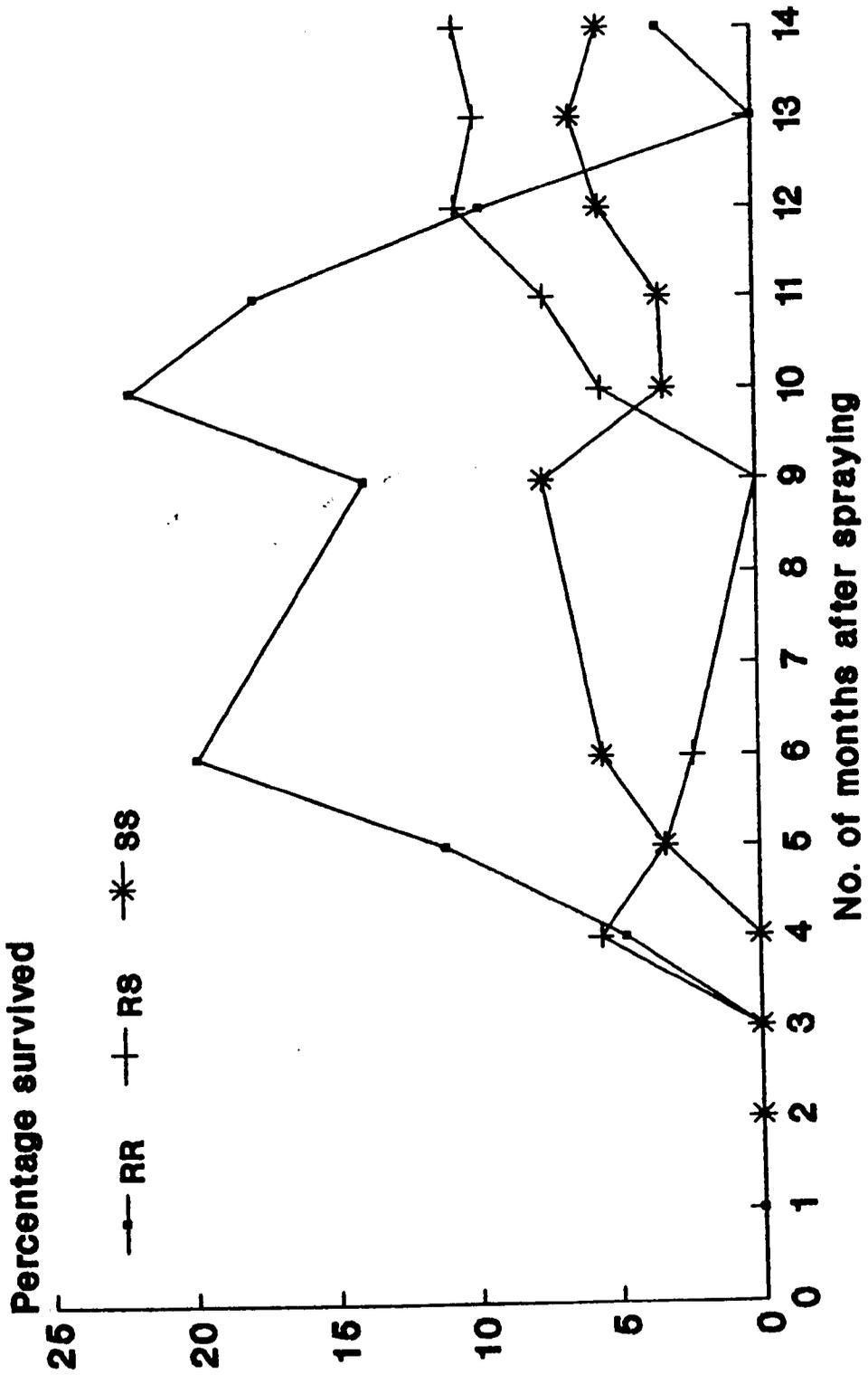


N.B. 100% mortalities were obtained for RR and SS up to month 2 and 6 respectively

Table : 6.8 Percentage survival of prefed females of the different genotypes observed in the cone bioassays on the DDT sprayed wall.

Month	Genotype	No. survived	No. recaptured	% survived	Mid-parental value	95% confidence limits
1	RR	0	99	0	-	0.0-3.7
	RS	-	-	-	-	-
	SS	0	100	0	-	0.0-3.6
2	RR	0	103	0	-	0.0-3.5
	RS	-	-	-	-	-
	SS	0	100	0	-	0.0-3.6
3	RR	10	105	9.5	-	4.7-16.9
	RS	2	980	2.0	4.8	0.3-7.2
	SS	0	98	0	-	0.0-3.7
4	RR	8	96	8.3	-	3.7-15.8
	RS	7	102	6.9	4.2	2.6-13.0
	SS	0	101	0	-	0.0-3.5
5	RR	18	95	18.9	-	11.6-28.3
	RS	5	89	5.6	9.5	1.9-12.6
	SS	0	98	0	-	0.0-3.7
6	RR	22	99	22.2	-	14.5-31.7
	RS	9	98	9.2	13.0	4.3-16.7
	SS	4	109	3.7	-	1.0-9.1
9	RR	30	97	30.9	-	21.9-41.1
	RS	27	98	27.6	20.9	19.9-39.0
	SS	11	101	10.9	-	5.7-18.9
10	RR	29	89	32.6	-	23.0-43.3
	RS	29	95	30.5	23.5	21.5-40.8
	SS	14	97	14.4	-	8.1-23.0
11	RR	32	87	36.8	-	26.7-47.8
	RS	27	89	30.3	24.5	21.0-41.0
	SS	11	91	12.1	-	6.2-20.6
12	RR	28	89	31.5	-	22.0-42.2
	RS	25	87	28.7	21.7	19.5-39.4
	SS	10	85	11.8	-	5.8-20.6
13	RR	35	93	37.6	-	27.8-48.3
	RS	28	89	31.5	24.3	22.0-42.2
	SS	10	92	10.9	-	5.3-19.1
14	RR	36	94	38.3	-	28.5-48.9
	RS	27	92	29.3	25.5	20.3-39.8
	SS	11	87	2.6	-	6.5-21.5

Fig: 6.11
% survival of pre-fed females observed
in cone bioassays on DDT sprayed roof



N.B. 100% mortalities were obtained for RR, RS and SS up to month 3

Table : 6.9 Percentage survival of prefed females of the different genotypes observed in the cone bioassays on the sprayed roof.

Month	Genotype	No. survived	No. recaptured	% survived	95% confidence limits
1	RR	0	91	0	0.0-4.0
	RS	-	-	-	-
	SS	0	92	0	0.0-3.9
2	RR	0	92	0	0.0-3.9
	RS	-	-	-	-
	SS	0	89	0	0.0-4.1
3	RR	0	93	0	0.0-3.9
	RS	0	91	0	0.0-4.0
	SS	0	100	0	0.0-3.6
4	RR	4	85	4.7	1.3-11.6
	RS	5	89	5.6	1.9-12.6
	SS	0	85	0	0.0-4.3
5	RR	10	90	11.1	5.5-19.5
	RS	3	91	3.3	0.7-9.3
	SS	3	90	3.3	0.7-9.4
6	RR	17	86	19.8	12.0-29.8
	RS	2	87	2.3	0.3-8.1
	SS	5	91	5.5	1.8-12.4
9	RR	12	87	13.8	7.3-22.9
	RS	0	91	0	0.0-4.0
	SS	7	93	7.5	3.1-14.9
10	RR	20	91	22.0	14.0-31.9
	RS	5	93	5.5	1.8-12.1
	SS	3	94	3.2	0.7-9.0
11	RR	15	85	17.6	10.2-27.4
	RS	7	95	7.43	3.0-14.6
	SS	3	92	3.3	0.7-9.2
12	RR	8	83	9.6	4.3-18.1
	RS	10	95	10.5	5.2-18.5
	SS	5	92	5.4	1.8-12.2
13	RR	0	85	0	0.0-4.3
	RS	9	92	9.8	4.6-17.8
	SS	6	94	6.4	2.4-13.4
14	RR	3	90	3.3	0.7-9.4
	RS	9	86	10.5	4.9-19.0
	SS	5	92	5.4	1.8-12.2

would presumably pick up a lethal dose of the insecticide in a sprayed hut and would be killed. In this study it was found that when DDT was fresh all the SS and almost all the RR genotypes, were killed (Figs 6.4 and 6.5). This result is comparable to that obtained by Curtis et al. (1984) on Cx quinquefasciatus in a breeding site sprayed with chlorpyrifos, where resistant mosquitoes were also killed by freshly applied insecticide (Table 6.10). With HCH resistance in An. culicifacies (Rawlings et al. 1981) some resistant homozygotes survived even on a freshly sprayed deposit. With malathion and An. arabiensis, all of the partially resistant wild population was killed during the first 2 weeks after spraying (Lines and Curtis unpublished data quoted in Curtis 1987, Table 6.10).

In the present trial, it was unfortunately not possible to release the RS hybrids in the first two months after spraying. Thus comparisons of the hybrids with the selected resistant or susceptible stock could not be made during this period. However, it was noticed that as the deposit decayed or diffused into the mud bricks the RR showed increasing survival and later the SS started to show some survival. At the end of the experiment, i.e. 14 months later, there was almost no difference in the mortalities. Up to that time the survival of the RR genotype was always more than that of RS and this was more than SS (i.e. $RR > RS > SS$). This indicates that the DDT resistance gene in An. gambiae s.s. does have a protective effect and the effect is greater when

Table : 6.10 Survival of resistant homozygotes (RR), heterozygotes (RS) and susceptible homozygotes (SS) when exposed to insecticide deposit in field conditions.

Species	Insecticide	Genotype	Survival at following no. of weeks since spraying :-							
			1	2	3	4	5	6	7	8
1. <u>An. culicifacies</u>	HCH	RR	9.3%	23.1%	34.0%	38.4%				
		RS	0	0	0	0.8%				
		SS	0	0	0	0				
2. <u>Cx quinquefasciatus</u>	Chlorpyrifos	RR	0	100%	100%	100%				
		RS	0	0	0	0				
3. <u>An. arabiensis</u>	Malathion	mixed res.	0	14%	22%	-				
		SS	0	0.5%	16%	-				
4. <u>An. gambiae s.s.</u>	DDT	RR	0	2.5%	0	1.2%				
		RS	-	-	-	-				
		SS	0	0	0	0				

1 = Rawlings et al. 1981

2 = Curtis et al. 1984

3 = Lines and Curtis, unpublished data, quoted in Curtis 1987.

4 = First two months of present study (For subsequent data see Figs 4.4 & 4.5)

homozygous than heterozygous. Comparison of RS survival with the mid-parental value indicates that resistance was incompletely dominant.

The fact that the hybrid genotype, RS, always survived better than the susceptible SS indicates that apparently it will not be possible to make the resistance recessive. As emphasised by Georghiou and Taylor (1977b) and Curtis et al. (1978), in the field even a very small deviation from complete recessiveness of resistance would be very disadvantageous, as this would allow the heterozygotes to gain a selective advantage leading to accumulation of the rare resistance allele and rapid evolution of insecticide resistance would begin.

Rawlings et al. (1981) managed to obtain a dose which reliably killed all the heterozygotes and susceptible homozygotes in the first six weeks of their experiment on An. culicifacies with HCH.

In the present study, cone bioassays of fed mosquitoes with one hour exposure on the walls gave a similar picture to the experiment with free flying mosquitoes (Fig 6.10). However, the value of bioassays in testing insecticide residual activity is doubtful. High mortalities are observed in mosquitoes that have been forced into contact with sprayed surfaces. They have no freedom to "choose" their resting sites, unlike free flying mosquitoes.

Histograms of where the mosquitoes were found show that excito-repellency of DDT tends to drive them out of the hut (Fig 6.6 and 6.7). With SS, when the DDT was fresh it seems that many died before they could leave the hut. DDT irritability has been demonstrated by several workers (Kennedy 1947; Cullen and de Zulueta 1962; Kuhlow 1962; Sweeney 1983; Mpofo et al. 1988). In contrast, in the control hut more of the pre-fed female mosquitoes (60-90%) remained inside (Table 6.6), resting on the walls and roof. During the experiment in the unsprayed hut, it was observed that more mosquitoes were resting on the roof than the wall. Those that were on the wall were found on the upper layers of bricks near to the roof. In hot tropical climates the thatch is relatively cool by day (Schofield and White 1984) and this provides a cool resting place for the mosquitoes.

When the mosquitoes were released unfed into the sprayed hut very few of them fed on the guinea-pigs (range from 0 to 23%). Some of the fed mosquitoes were found resting outside the hut (Fig 6.7) presumably because of the excito-repellent effect of DDT which could also be the reason for the low feeding rate in the sprayed hut. Sloof (1964, cited from Sweeney 1983) pointed out that the feeding rate of An. punctulatus in a DDT sprayed hut was 15 - 30% less than that in a control hut. In the control hut a higher percentage of feeding was observed and most of the fed mosquitoes were found resting inside the hut mainly on the roof (Fig 6.8). It was noticed that there was no

difference in the number that fed between the three genotypes in the sprayed hut (Fig 6.9) and the feeding rates were uniformly higher in all three genotypes in the control hut (Fig 6.9). This suggests that the resistance would not protect against feeding inhibition but only against killing, i.e. spraying of houses should still protect a person from being bitten when the mosquitoes become resistant, but the community would not benefit so much from mosquito mortality where there is resistance.

The percentage recaptured in this experiment was always high (Table 6.6 and 6.7) compared to the result obtained by Mpofo et al. (1988). This could be explained by the fact that the huts used in this study were situated in well sealed rooms. There was no way the mosquitoes could escape from the room except by following the experimenter out of the room immediately after the release. Another factor ensuring a higher recovery rate compared with field studies could be the lack of scavenging arthropods such as ants and spiders in these rooms. These arthropods are known to prey on mosquitoes as proven by Service (1973) through precipitin test. By thorough searching some of the dead mosquitoes were found hidden in the cracks or spaces between the mud blocks or entangled in the thatch roofs.

The results of this study demonstrate that DDT spray deposits are effective in killing An. gambiae for up to 12 months post-spray for susceptible mosquitoes, with about 82%

mortalities for mosquitoes released unfed or fed. Other workers have given various estimates of the durability of the DDT residual activity in killing vector mosquitoes (Taylor et al. 1981; Mpofu et al. 1988). Rawlings et al. (1983) obtained 100% mortality of An. culicifacies in palm-leaf huts, two months after malathion spraying. The resistant mosquitoes however survived better at 12 months post-spray with about 25% and 52% mortalities for mosquitoes released unfed or pre-fed, respectively. It seems that the unfed females survived better than the pre-fed ones (Figs. 6.5 and 6.4). This result was in contrast to An. punctulatus where mortality was much higher in unfed than engorged mosquitoes in sprayed huts (Sweeney 1983). Better survival of the unfed females could be due to the fact that they are more active and more easily stimulated to leave DDT sprayed surfaces.

CHAPTER 7

CHEMICAL ANALYSIS OF DDT ON MUD AND THATCH ROOF

7.1 INTRODUCTION

This study was undertaken in association with the study described in chapter 6 on the mortality of resistance and susceptibility genotypes in simulated mud huts. The intention was to relate the declines in mortality of the three genotypes with time to changes in the actual amount of DDT present. It was also the aim of this study to determine the chemical persistence of DDT on mud bricks and thatch and to determine how far DDT had migrated into the bricks at intervals after spraying.

In most malaria control programmes the target dosage applied is 2gm/sq m: this has been shown to maintain a residual toxicity for several months or up to a year or more (Bordas et al. 1953). There have been, however, several contradictory reports on the effectiveness of residual DDT in the control of Anopheles which have led to some confusion. There are many factors which could influence the effectiveness or the life span of DDT and these include factors which relate to the behaviour of the species, factors which involve the insecticides themselves such as formulations, technique of application, repellent effect

etc. and the types of materials or the chemical nature of the sprayed surfaces. For example, small crystal formulations of DDT give higher kills than larger crystals at least shortly after treatment (Hadaway and Barlow 1951). DDT crystals from a wettable powder formulation produce very different results from the DDT in solution in Risella oil in the WHO test papers and it is likely that similar differences will be observed with plaster, brick, stone, mud, thatch and other building materials.

The inactivation of DDT deposits on dry mud surfaces is primarily due to adsorption of the DDT deposits present on the mud surface, and according to Bordas et al. (1953), is also due to catalytic breakdown of the insecticide. They believed that adsorption represents the initial step of the catalytic breakdown of DDT. The adsorption of DDT by soil is influenced by environmental relative humidity: the higher the humidity the faster the absorption. Downs and Bordas (1951) observed a long duration of insecticidal effectiveness of DDT deposits on mud (from two to three years) which could be due to the reactivation of the DDT under high atmospheric humidity.

Studies on other insecticides such as malathion also showed that there are great variations in the effectiveness of the insecticides on mud and other surfaces. A recent study by Hemingway et al. (1989) showed that malathion was rapidly absorbed into mud walls within 3 months after

treatment. Similar results were obtained by Smith and Hocking (1962) working with An. gambiae and malathion in Tanzania. They also showed that organophosphate and carbamate insecticides persist longer on cajan (palm) or grass thatch. Fontaine (1983) pointed out that malathion persists longer on wood, residual activity sometimes exceeding three months, but on some mud surfaces its activity can be reduced to a very low level after only three weeks.

7.2 MATERIALS AND METHODS

7.2.1 Samples taken from the inner surface of the mud hut walls

An area of 2 sq cm was marked on one of the mud bricks and the area was dug out with a scalpel to a depth of 1 cm. All the dust was collected and placed in labelled tubes. Five samples from different bricks were taken every month and the dust samples from all five replicates were pooled and stored at -70° C until High Pressure Liquid Chromatography (HPLC) analysis was done. Each stored sample was weighed and then isopropanol was added (3x the weight of the sample) to extract the DDT. The mixture was shaken well to dissolve all the DDT and was then centrifuged at 10,000g for 10 minutes. The supernatant was removed and centrifuged again at 60,000g for another 10 minutes. The supernatant

was again removed and filtered through a 0.22μ filter and dried with a stream of air. It was then resuspended in 0.3 ml isopropanol and $20\mu\text{l}$ of this was injected onto an Ultrasphere ODS column run with a mobile phase of 45:45:10 Acetone: Acetonitrile: Water. Two replicate injections were carried out with each extract sample. The column was calibrated with a known quantity of pure DDT under the same conditions. Peaks were detected at 290nm and integrated on a Beckman SP 1442 integrator.

The same procedure was repeated for the following samples:

7.2.2 Samples taken from the thatch roof.

Three samples of a 4 cm long section of the leaf blade from the thatch roof were taken every month and labelled. These were stored at -70°C until extraction with isopropanol and HPLC analysis was done as described above.

7.2.3 Samples taken at the same time from multiple sites.

Samples were taken from 5 different sites on the mud hut walls and 5 sites on the roof, eleven months after spraying.

7.2.4 Samples taken at different depths into the mud hut bricks, eleven months after spraying.

The samples were taken on the same day and at different depths into the mud bricks. The bricks were about 13 cm thick and the samples examined included ones from the opposite surface of the brick from that to which the spray had been applied. Unlike the previous studies the top 1 cm was split into 0.5 cm sections.

7.2.5 Samples taken from freshly sprayed bricks for 12 successive weeks.

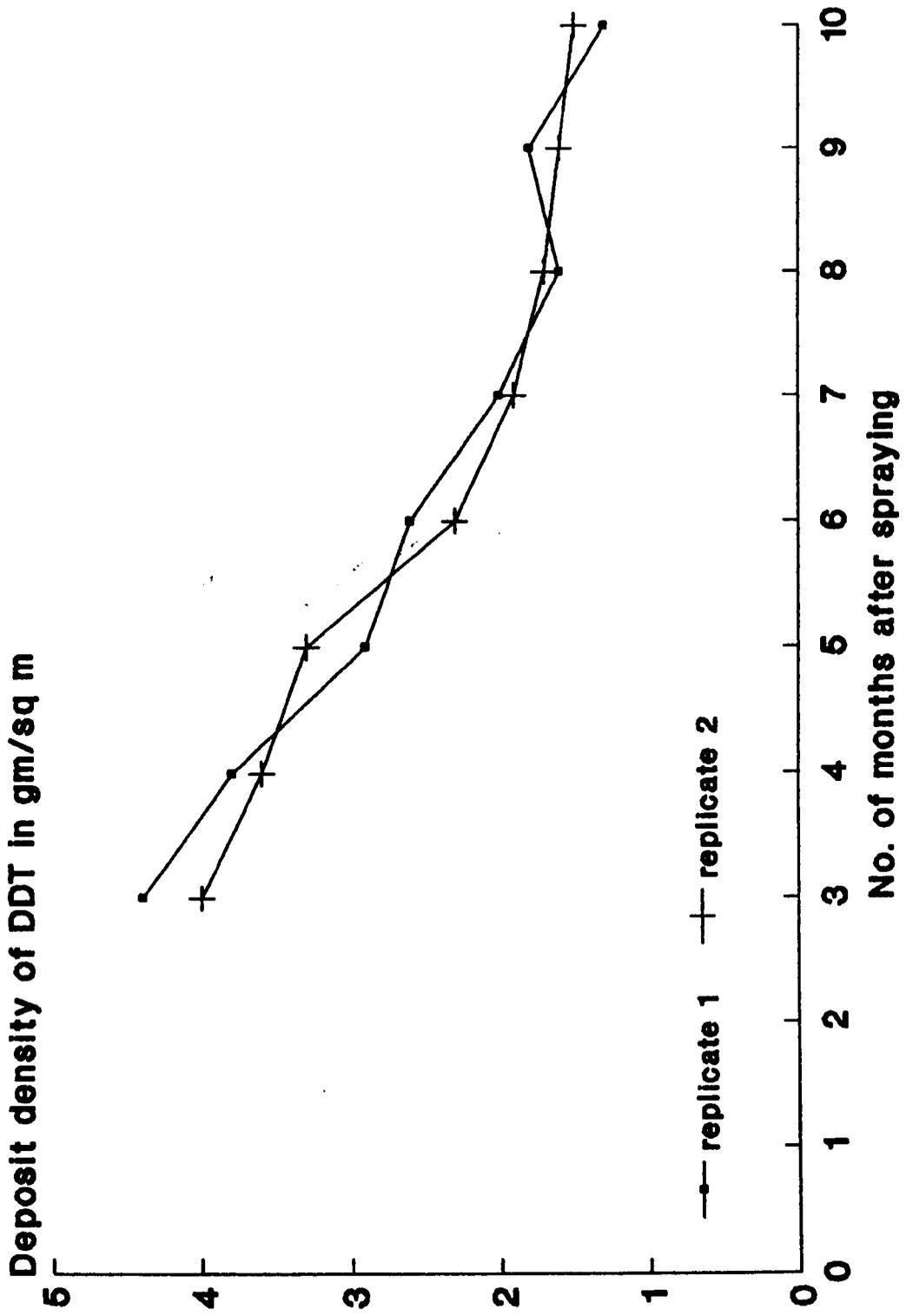
The bricks were sprayed specially for this study on a different occasion from the main spraying of the hut.

7.3 RESULTS

7.3.1 Samples taken from the inner surface of the mud hut walls.

The results in Fig 7.1 indicate that the deposit density was about 2x higher than the targeted dosage of 2 gm/sq m even at 3 months after spraying. The observed deposit density was 4.2 gm/sq m. The deposit density gradually decreased to about 1.6 gm/sq m 8 months after

Fig : 7.1
Amount of DDT in mud wall samples



spraying and remained more or less the same for another 2 months.

7.3.2 Samples taken from the thatch roof.

The initial deposit density in the thatch was also found to be about twice as high as the targeted dosage of 2 gm/sq m (Fig. 7.2). The amount obtained was 3.6 gm/sq m, 3 months after spraying. The deposit density dropped quite rapidly to about 1.7 gm/sq m by month 5 and remained more or less the same up to 10 months after spraying.

7.3.3 Samples taken at the same time from 5 different sites on the mud hut walls and roof eleven months after spraying.

Table 7.1 shows that the deposit density in the mud varied from 1.1 to 2.8 gm/sq m. The deposit density observed at month 10 in fig. 7.1 lies within this range.

Table 7.1 also shows that the deposit density in the thatch varied from 1.0 to 1.7 gm/sq m which is a somewhat narrower range compared to that found on the mud bricks. The mean of 1.36 gm/sq m is however slightly lower than what was recorded in month 10 in fig. 7.2 but the range is similar to that recorded in fig. 7.2 for months 5 - 10.

Fig : 7.2
Amount of DDT in roof samples

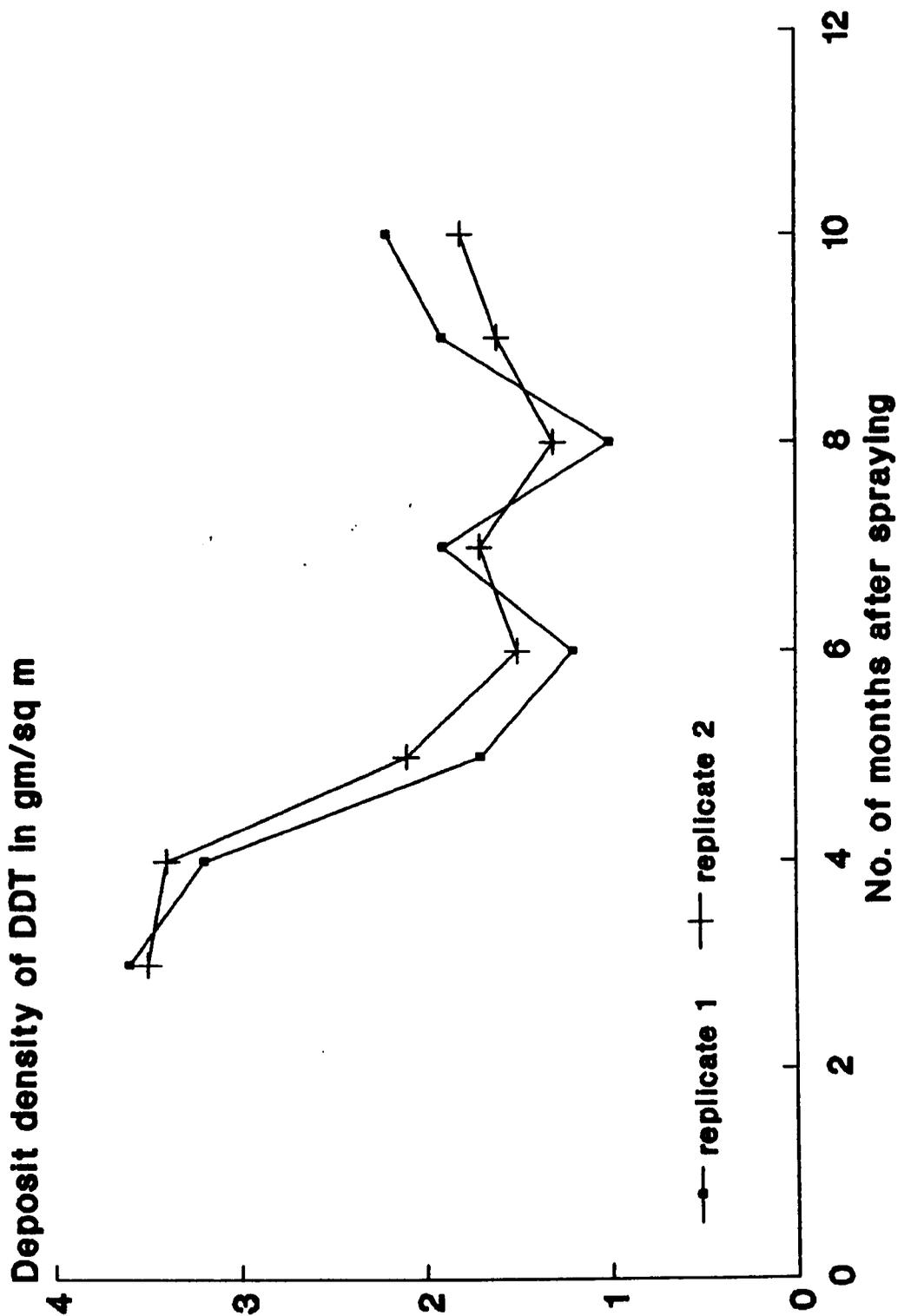


Table: 7.1 Amount of DDT present at different sites in the outer 1cm of the mud hut bricks and on the thatch roof taken eleven months after spraying.

Sites	Amount of DDT (gm/sq m)	
	Brick wall	Thatch roof
1	2.8	1.0
2	2.5	1.6
3	1.1	1.5
4	2.4	1.7
5	1.6	1.0

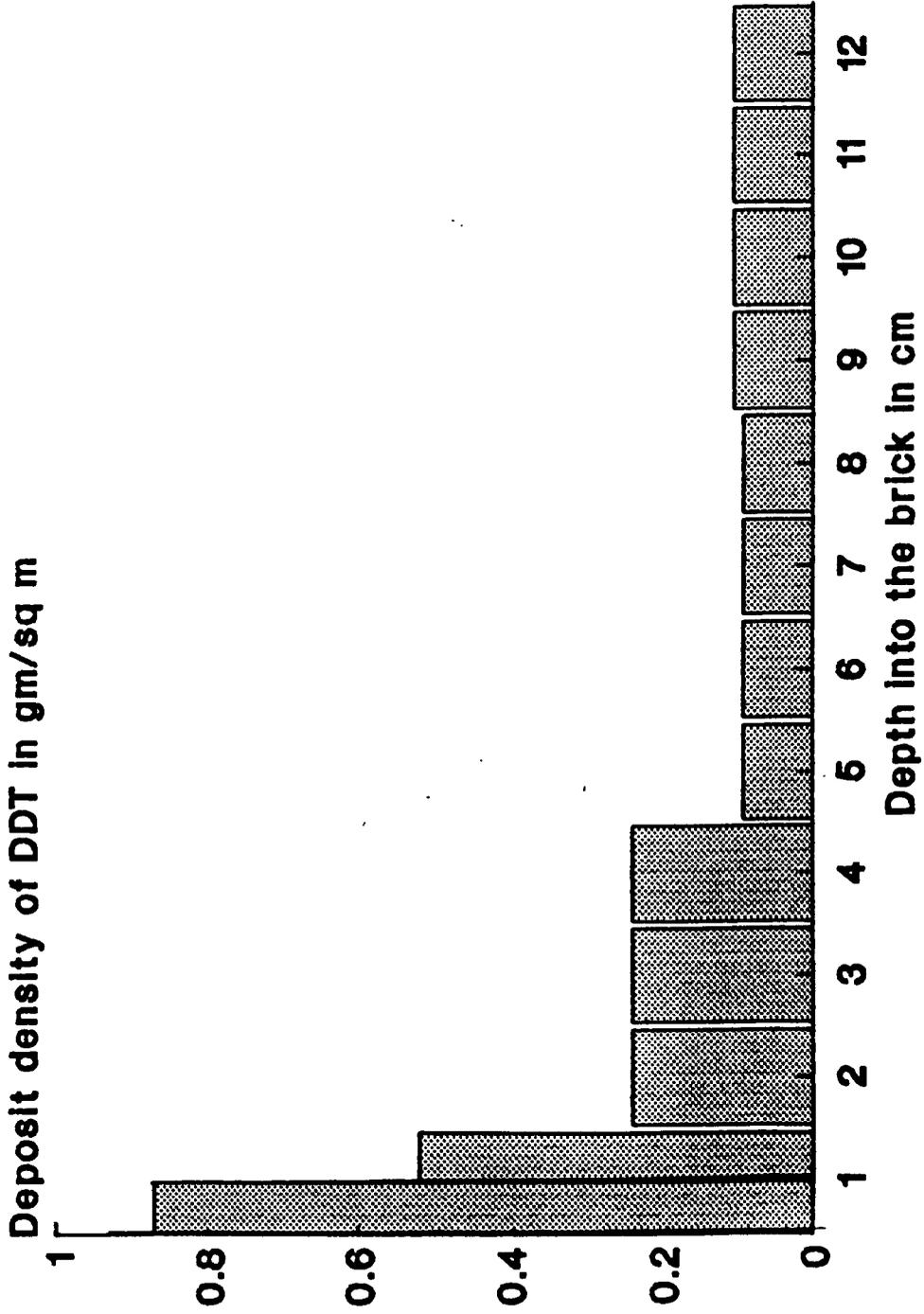
7.3.4 Samples taken at different depths into the mud hut bricks, eleven months after spraying.

Figs. 7.3 and 7.4 show that the deposit density of DDT still remained highest in the 1 cm of the bricks nearest to the sprayed surfaces. Adding that observed in the 1st and 2nd half cm yielded 1.39 gm/sq m in fig. 7.3 and 2.0 gm/sq m in fig. 7.4 respectively. However some DDT could be found at all depths into the bricks, right to the layer furthest from the sprayed surface. When the amounts present at each depth was added together, a total amount of 2.9 gm/sq m was obtained from fig 7.3 and 3.8 gm/sq m from fig 7.4. These amounts were slightly lower than the amount of DDT (4.2 gm/sq m) found in the top 1 cm of the bricks after 3 months (Fig 7.1). Samples in fig 7.3 were taken from 4 corners of a brick and those in fig 7.4 were taken from 2 holes in one brick.

7.3.5 Samples taken from freshly sprayed bricks for 12 successive weeks.

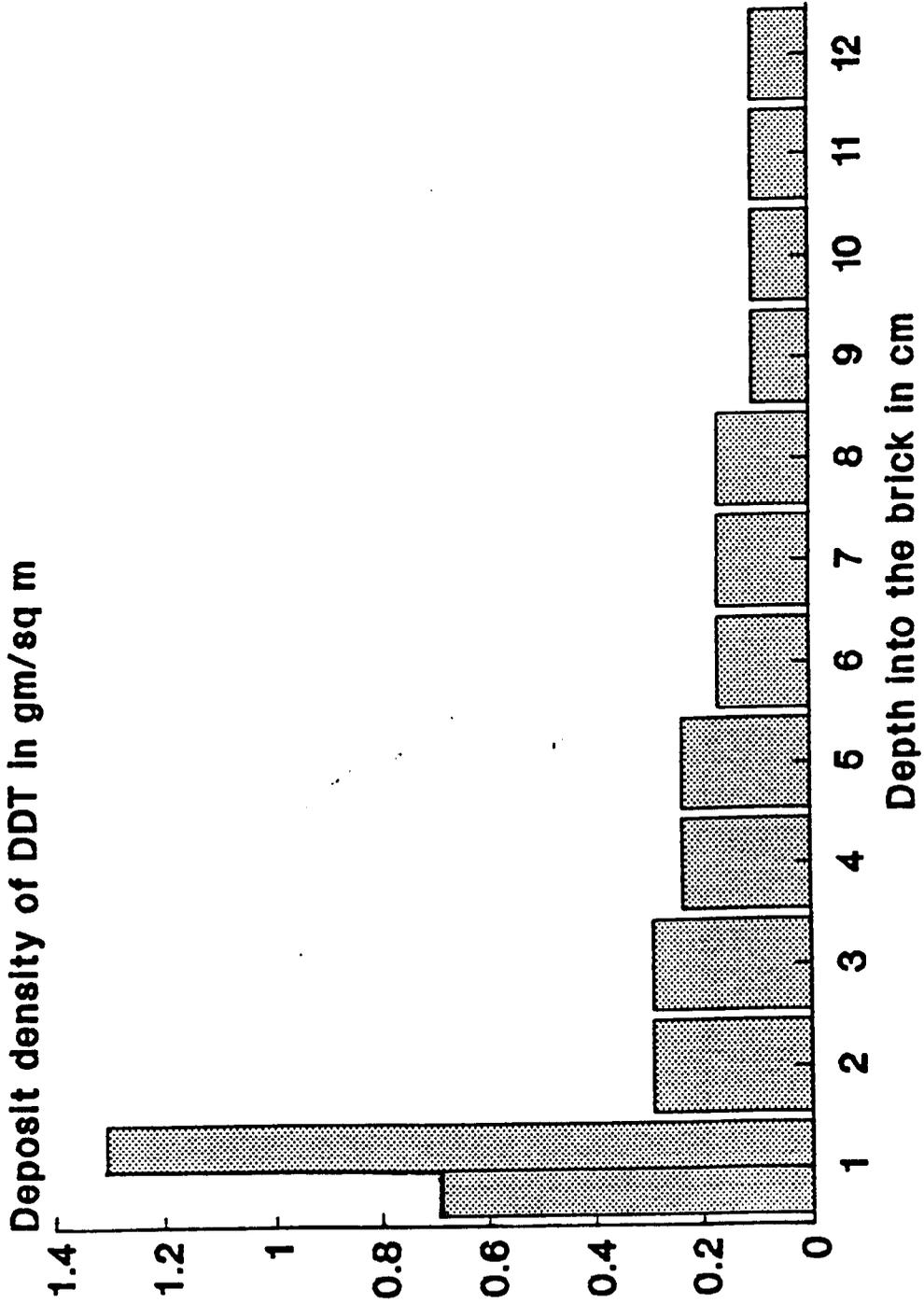
Because the study illustrated in fig 7.1 failed to cover the first 3 months after spraying, a second spraying was carried out and the results followed over a 3 month period. Fig 7.5 shows the amount of DDT present in the top 1cm. The deposit density observed initially was higher (3.0 gm/sq m) than the targeted dosage of 2.0 gm/sq m. The amount found varied from 2.0 gm/sq m to 3.5 gm/sq m at different

Fig : 7.3
Amount of DDT present at different depth
taken from 4 corners of one brick



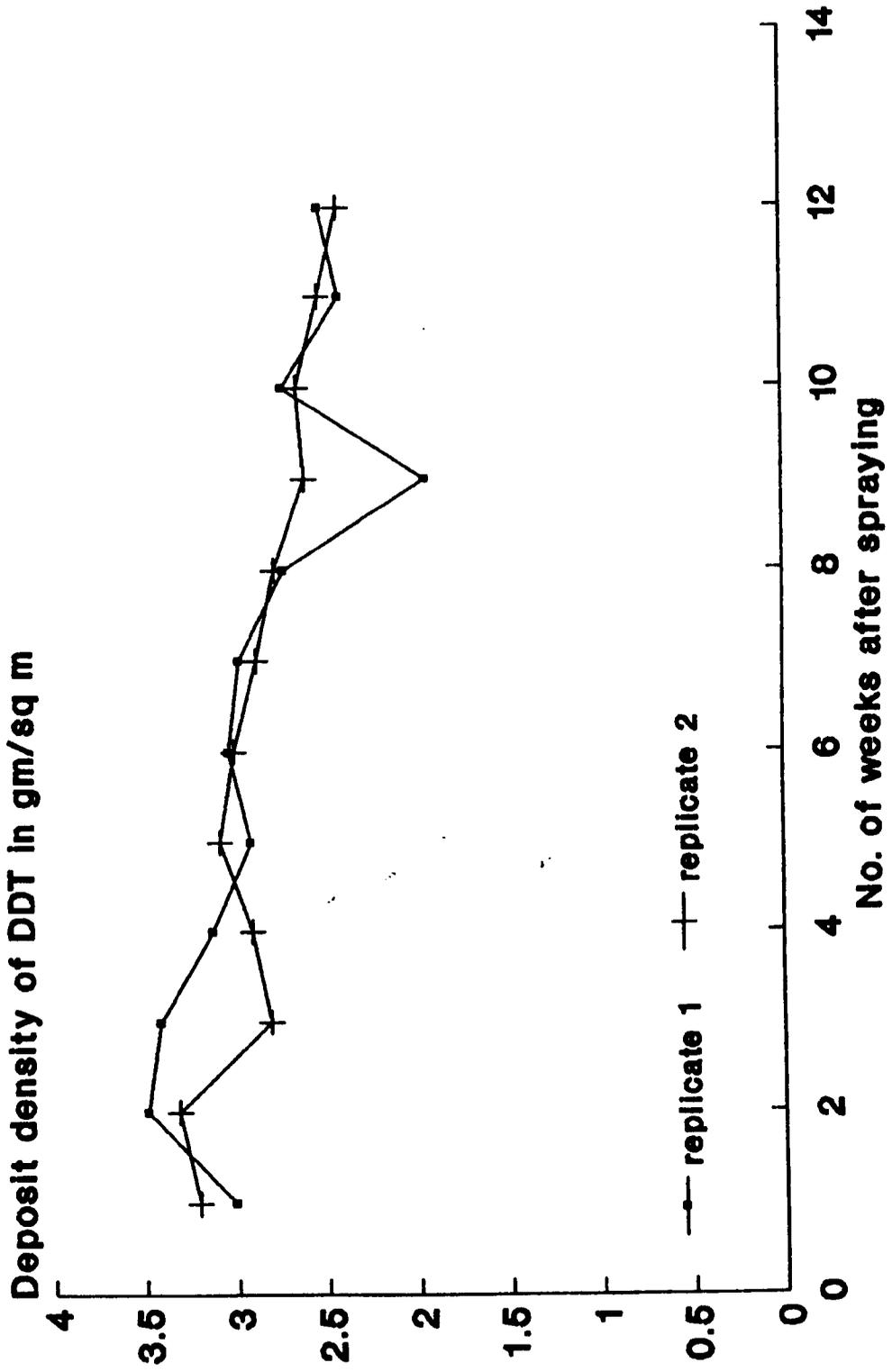
Notes:- the amount of DDT is an average from 2 readings
 - samples taken 11 months post-spray

Fig : 7.4
Amount of DDT present at different
depth from 2 holes in one brick



Notes: the amount of DDT is an average from 2 readings
 -samples taken 11 months post-spray

Fig : 7.5
Amount of DDT in the outer 1cm of the brick samples taken
between 1st and 12th week post-spray



times within the first 3 months after spraying but there was generally a downward trend suggesting that the amount of DDT lost within this period was 0.5 - 1.0 gm/sq m.

7.4 DISCUSSION

The results of the analysis showed that the amount of DDT in the samples taken were far higher than the expected dose of 2 gm/sq m a.i. (active ingredient) even 3 months after spraying (Fig 7.1 and 7.2). This seems to suggest that either the technique or the equipment was at fault but it could also be due to the fact that the hut had previously been sprayed by another experimenter 8 months before. Thus the levels obtained in this study could be the accumulation of two sprayings. The gradual decrease of the DDT deposits on the mud wall could reasonably be explained as due to diffusion of the deposits deeper into the mud. This was proved by the existence of DDT deposits at different depths into the mud bricks right to the bottom-most layer (Fig 7.3 and 7.4). The loss of the DDT deposits could also be partly due to chemical degradation of the insecticide as suggested by Bordas et al. (1953). He showed that mud surfaces containing iron oxides rapidly inactivate DDT spray deposits.

Chemical degradation could be the reason for the difference in the total amount of DDT present at all depths

at month 11 (Table 7.1) as compared to the amount in the top 1 cm at month 3 (Fig 7.1). Bordas et al (1953), however, claimed that the loss of insecticidal activity of DDT deposits on dry mud surfaces was primarily due to adsorption so that they are not picked up by insects. The decrease observed on the mud wall could also be partly due to attrition of the DDT deposits from the walls and thatch during the carrying out of the experiment. This is particularly likely explanation for the decline observed on the thatch roof (Fig 7.2) which was shaken during each mosquito collection to drive out mosquitoes hiding in it. It was apparent that the DDT deposits remained on the surface. Thus with shaking and rubbing of the leaves against each other some of the deposits would be expected to fall off. Mud on the other hand absorbs the spray deposits rapidly. Langford (as cited by Kuhlow 1962) reported that DDT deposits are lost faster from a thatch roof than from mud. This is in contrast to the findings of this study and those of Mpofo et al. (1988). The same trend as in this study was also observed by Taylor et al. (1981) but with different insecticides. Thatch is also said by Mpofo et al. (1988) to be less biologically active than mud surfaces.

Variation in the spray deposit and in the rate of diffusion and chemical degradation between the different mud blocks could be the reasons for the wide variation (1.1 - 2.8 gm/sq m) of DDT deposits obtained at eleven months after spraying (Table 7.1). The DDT deposit density recorded on the wall

at month 10 (1.3 gm/sq m) in fig 7.1, however falls into the lower range of that in table 7.1. This shows that there was an actual loss of DDT deposit from the wall surfaces eleven months after spraying and it was not just due to sampling or technical error.

Taylor et al. (1981) have also shown a wide variation in insecticide deposit following spraying. They considered the reason for this was mainly due to the technique of application. The variation of DDT deposits recorded on the roof was slightly less and ranged from 1.0 - 1.7 gm/sq m (Table 7.1). The range of DDT deposit density recorded from months 5 - 10 of 1.0 - 2.4 gm/sq m in fig 7.2 was similar to that recorded for the samples taken at different sites on the roof eleven months after spraying (Table 7.1). The DDT deposits remained predominantly (49%) in the top 1.0 cm of the brick even at eleven months after spraying (Fig 7.3 and 7.4). Similarly, Hadaway and Barlow (1951) found that 50% of the DDT formulation they used remained in the top one millimetre of the brick at five months after spraying.

The total amount (2.9 gm/sq m) recorded from four corners of one brick (Fig 7.3) was less than that obtained from the middle of the bricks (3.8 gm/sq m in Fig. 7.4). The difference could be due to faster run-off of the spray deposits from the corners.

From the second spraying illustrated in fig. 7.5 it

appears that the amount of DDT deposit started to decline gradually during the three months after spraying, with an average loss of 0.25 gm/sq m per month. Thus it could be concluded that the original amount sprayed onto the mud hut walls and roofs was higher than that observed at month 3 in fig. 7.3 and was probably 4.5 - 5 gm/sq m.

The mosquito mortality reported in chapter 6 can be related to the amount of DDT deposit present on and near the mud surfaces (Fig. 7.6a,b). At month 9, when the DDT deposit density in the top 1.0 cm of the mud and in the thatch was approximately the normal target of 2.0 gm/sq m there was approximately 87% mortality of the SS genotype, 51% of RS and 34% of RR (Fig 7.6b). After month 9, when the DDT deposit was less than 2 gm/sq m, the mortalities of the 3 genotypes (especially of SS) declined very rapidly.

Fig : 7.6a
Mortalities of prefed mosquitoes and
amount of DDT in mud wall and roof

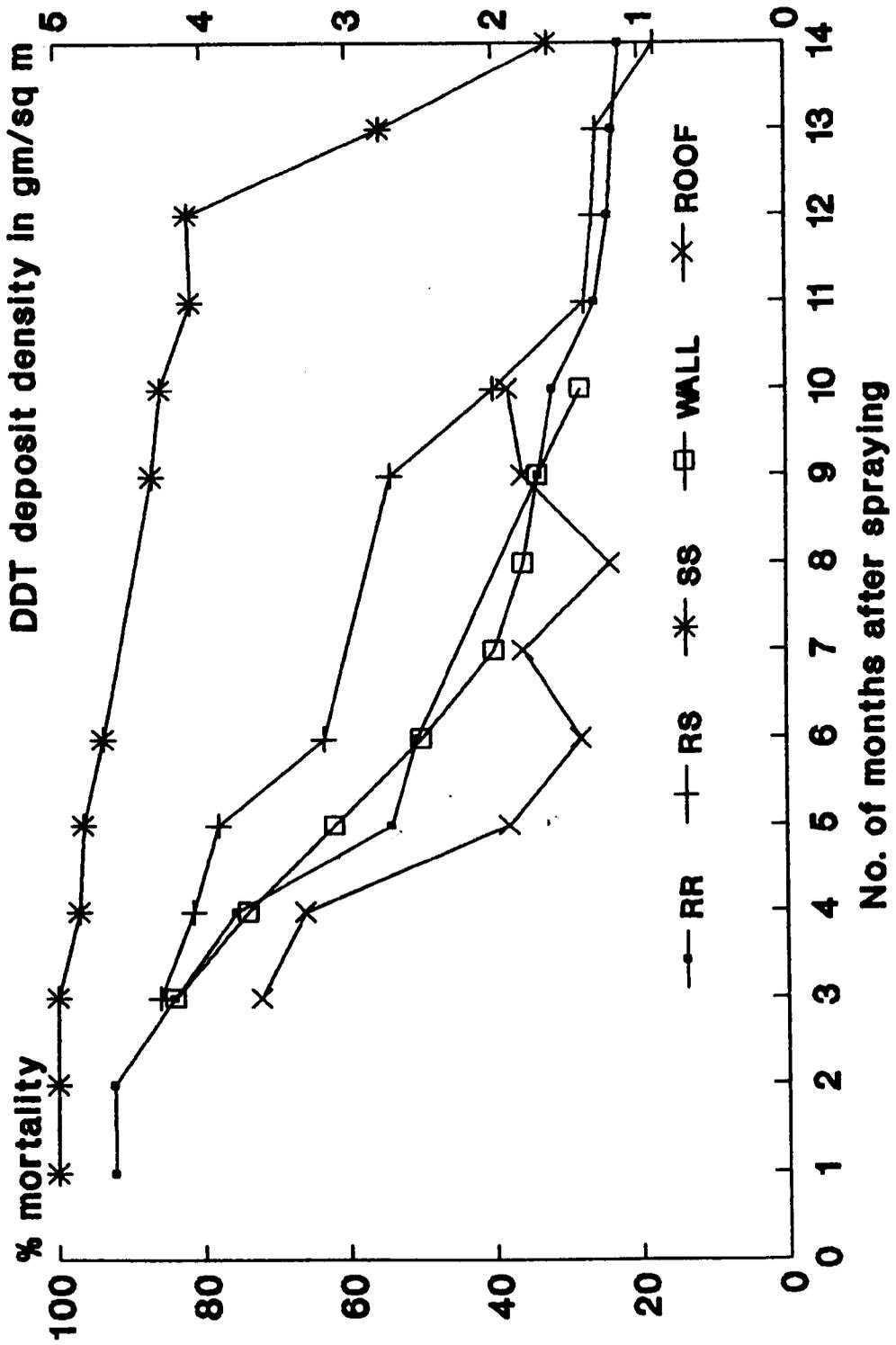
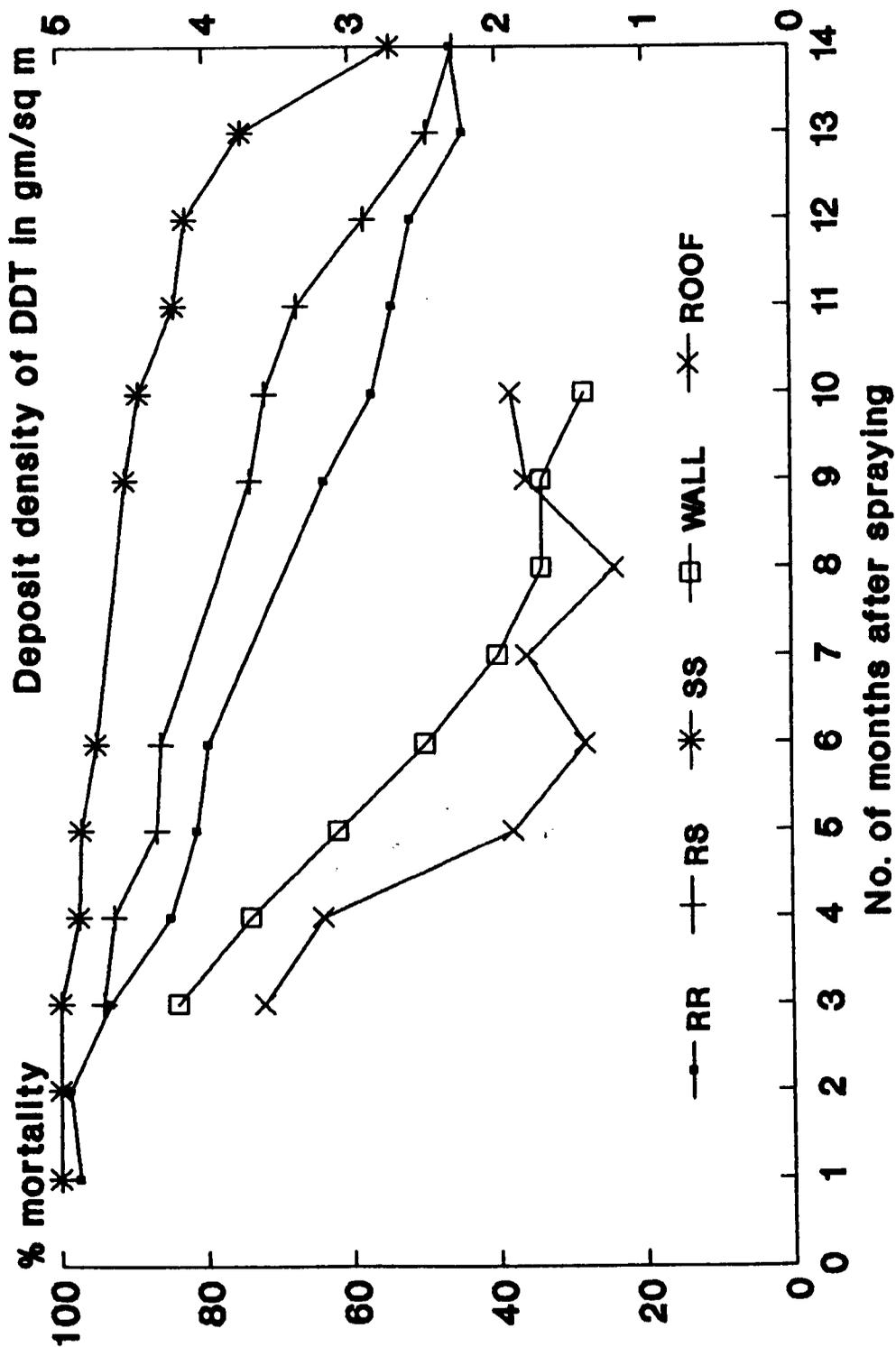


Fig : 7.6b
Mortalities of unfed mosquitoes and
amount of DDT in mud wall and roof



CHAPTER 8

LINKAGE OF PROPOXUR AND DIELDRIN RESISTANCE GENES IN
Anopheles albimanus FROM EL SALVADOR

8.1 INTRODUCTION

Anopheles albimanus Wiedemann is mainly a tropical lowland species found from the lower Rio Grande Valley through Mexico and Central America and along the coast of Colombia to the Paria Peninsula in Venezuela and on some of the Caribbean islands (Hobbs et al. 1986). In Central American and Caribbean countries, this species is an important, and sometimes the only, vector of human malaria. In El Salvador, for example, it is responsible for the annual transmission of malaria to thousands of people.

An. albimanus has two sharp biting-peaks, one at sundown and one at dawn. It appears to feed indiscriminately on man and the larger domestic animals, such as donkeys, mules, cows etc. In general the biting activity of An. albimanus is outdoors but, during the rainy season when the population density is high, they tend to feed indoors (Breeland 1972). Breeland (1972) summarised the behaviour of this species as more zoophilic than anthropophilic, more exophagic than endophagic and more

exophilic than endophilic. Thus it seems probable that residual house spraying for malaria control would not be very effective against this species.

To date An. albimanus has become resistant to several compounds such as DDT, dieldrin, malathion, propoxur etc (Breeland et al. 1970; Ariaratnam and Georghiou 1971; Georghiou 1972b; Ariaratnam and Georghiou 1975; Ayad and Georghiou 1975; Herath and Davidson 1981; Hemingway and Georghiou 1983; Hemingway et al. 1986). Tests for resistance in An. albimanus started in 1960, with populations from Panama and Haiti, but no resistance was reported. The first appearance of malathion resistance, accompanied by cross-resistance to carbamates in a field population of An. albimanus from El Salvador, was reported by Breeland et al. (1970). Resistance to carbamates was also reported in this species by Georghiou (1972b) and is thought to have been originally selected by the application of carbaryl in agriculture. This is thought to have been intensified by the use of propoxur in the anti-malaria programme since 1968. This species is also known to have developed resistance or cross-resistance to pyrethroids in El Salvador and Guatemala (Brogdon and Barber 1990).

The multiple resistance to a range of organophosphates and carbamates is due to an alteration in the acetylcholinesterase (AChE) target site. In the case of propoxur resistance, it is inherited as a single autosomal

semi-dominant gene (Davidson and Sawyer 1975; Hemingway et al. 1986). Ayad and Georghiou (1975) showed that the propoxur resistance in this species is due to their AChE being insensitive to this insecticide. This mechanism is stronger against the carbamates than the organophosphate compounds.

Most cases of insecticide resistance in natural populations have been shown to be due mainly or entirely to single genes of major effect (Wood 1981). In anophelines and culicines and in other insects of medical importance where resistance has been found, dieldrin resistance has usually been found to be incompletely dominant (i.e. hybrids are intermediate in resistance) (Davidson 1957; Brown 1967). However, Rozeboom and Johnson (1961) reported an exceptional case of dieldrin resistance in An. albimanus which showed completely dominant inheritance due to a single autosomal factor. Dieldrin resistance in this species from El Salvador was reported soon after DDT-resistant strains first appeared in 1958 (Brown and Pal 1971). The dieldrin and DDT resistance genes were found to be linked and located on chromosome 3 (Davidson and Curtis 1979; WHO 1980a).

With the common semi-dominant form of dieldrin resistance, the three genotypes, namely the susceptible homozygote SS, the heterozygote RS, and the resistant homozygote RR, may be separated by the following diagnostic dosages of the insecticide: one-hour exposure of adults to

0.4% dieldrin impregnated papers can separate the SS homozygotes by killing them but not RS or RR, and exposure to 4% papers separates the RR homozygotes by leaving them as survivors but killing RS and SS (Davidson 1963).

The present study was intended to determine the extent of linkage, if any, between the dieldrin and propoxur resistance genes in the FEST population from El Salvador. Since few visible markers are available in Anopheles species, linkage between resistance genes must be tested directly, by measuring recombination between the genes themselves. The doubly resistant FEST strain was crossed and backcrossed to the susceptible PANAMA strain. The F1 were tested with both insecticides to ensure that they were doubly heterozygous. The backcross progeny were tested first with one insecticide and then with the other. Three linkage possibilities may be distinguished :- (1) if there is no linkage between the two genes, 50% mortality will be expected in the backcross progeny on exposure to the second insecticide (Fig. 8.1a), (2) if there is a tight linkage, we expect zero mortality in the backcross progeny on exposure to the second insecticide (Fig. 8.1b), and (3) if there is moderate linkage between zero and 50% mortality will be obtained in the F1 progeny after treatment with the second insecticide (Fig. 8.1c). Note that, on exposure to the first insecticide 50% mortality is expected regardless of the linkage relationships. This follows from the fact that each resistance is under the control of a single gene.

Fig:8.1 A series of diagrams to show the three possible linkage situations between dieldrin and propoxur resistant genes.

A -If no linkage between the genes

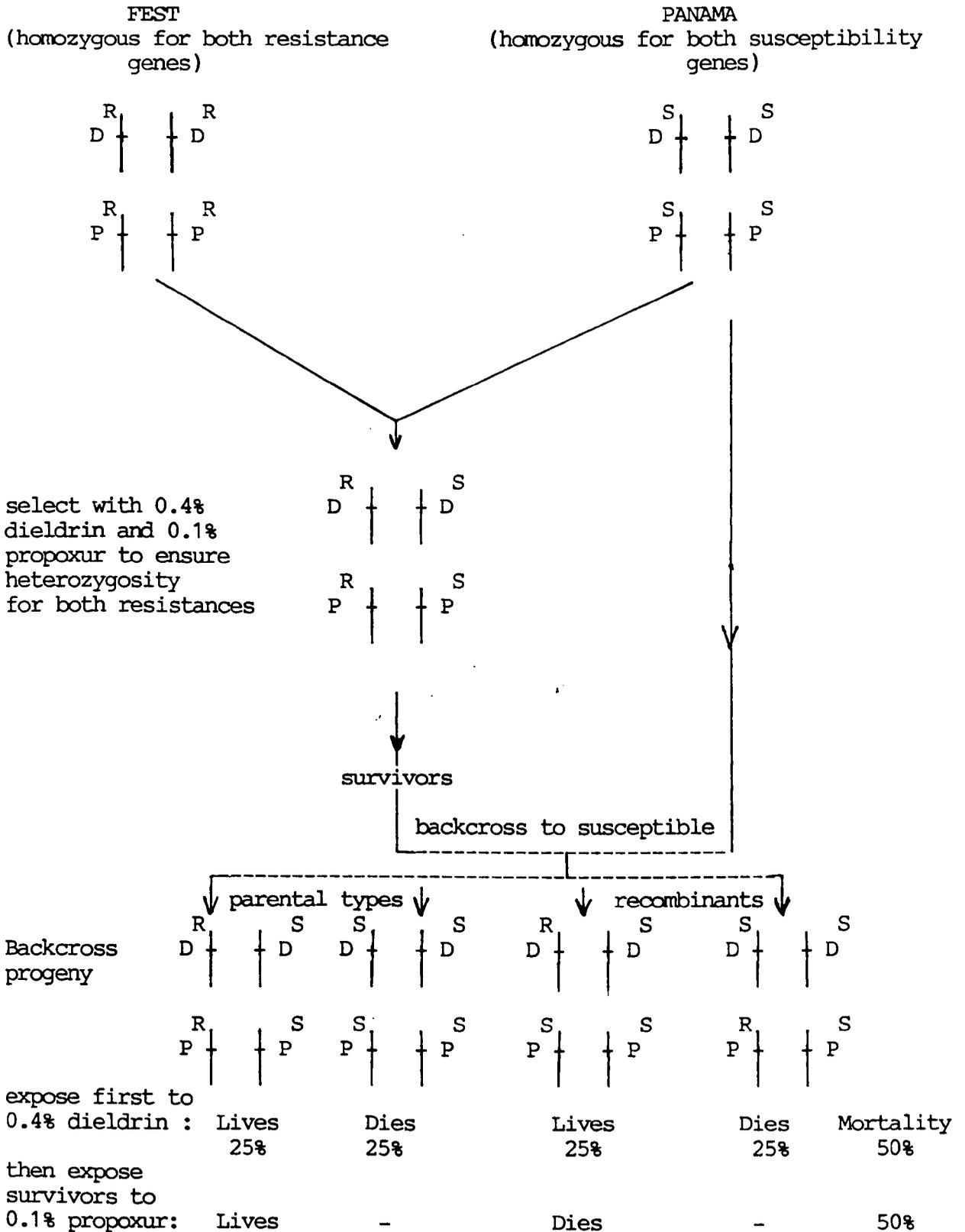


Fig : 8.1B If tight linkage between the genes

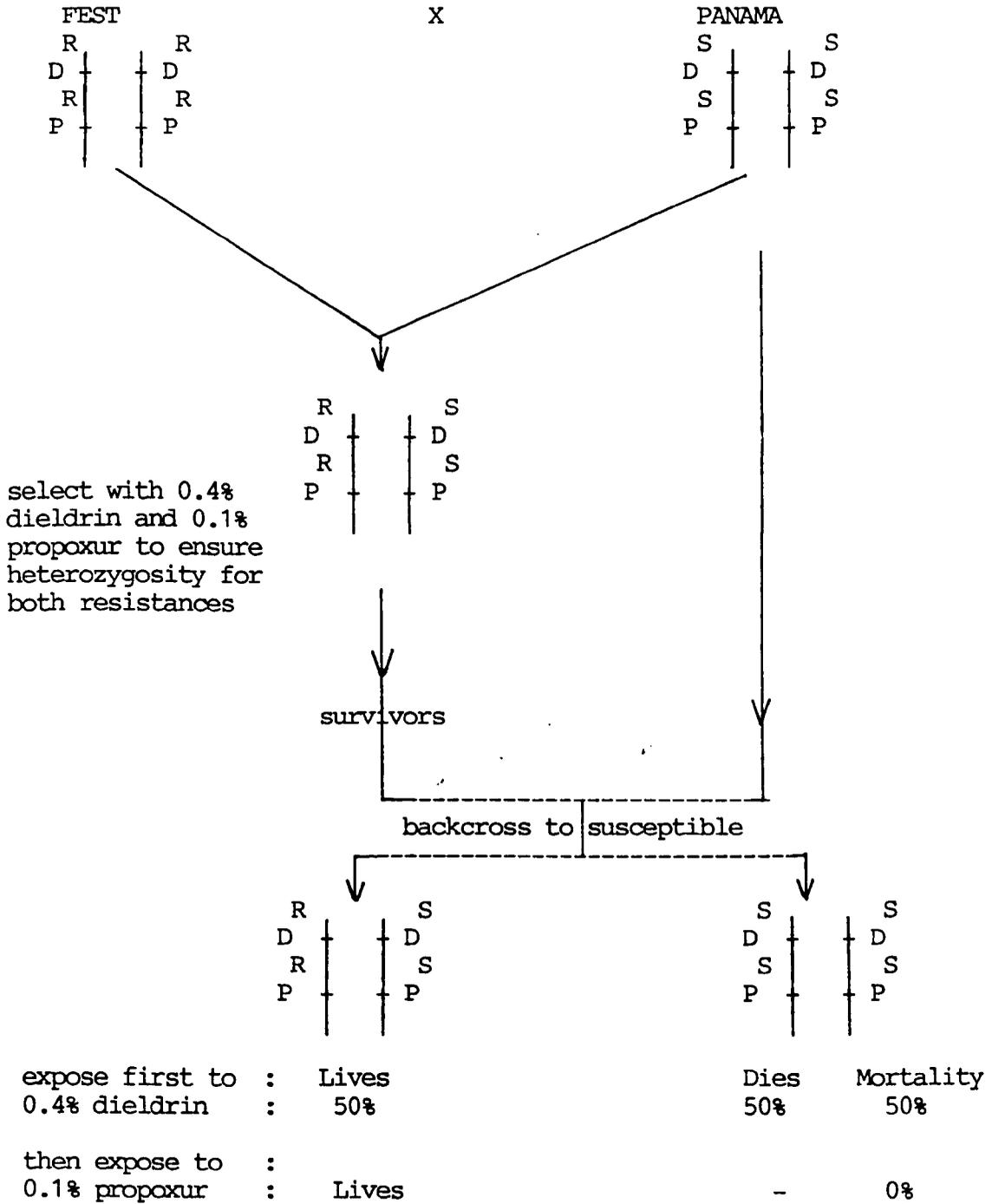
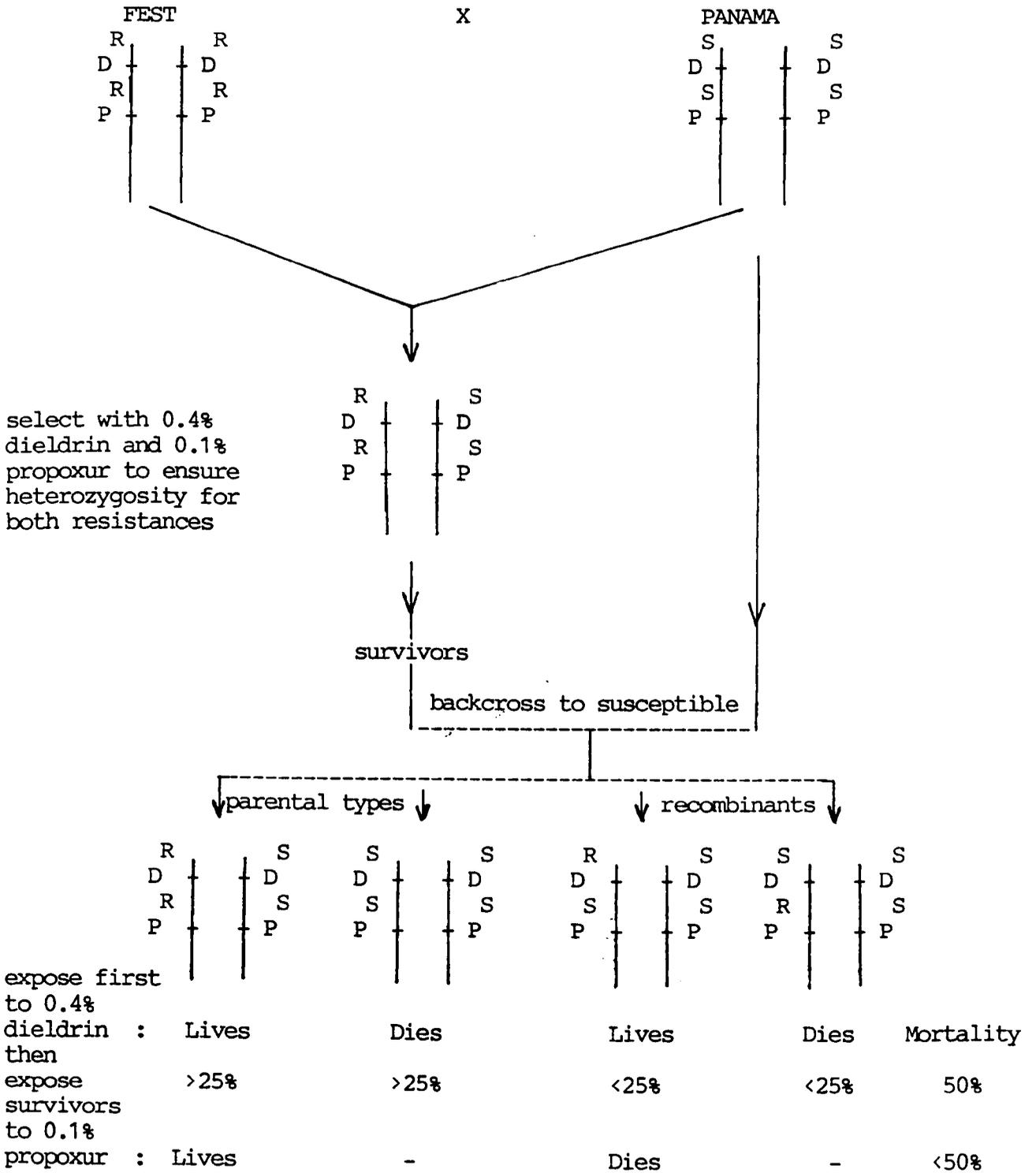


Fig : 8.1c Partial linkage (i.e. some crossing over takes place).



An alternative procedure was reported by Lines, French Constant and Kasim (1990) whereby a dieldrin bioassay is used and then the dead and live mosquitoes for this test are subjected to a biochemical assay for the forms of AChE which are, or are not, readily inhibited by insecticide. These types correspond to the propoxur resistant and susceptible alleles. The practical work for these tests was mainly carried out by the present author and the jointly published results are summarised in this chapter.

8.2 MATERIALS AND METHODS

8.2.1 MATERIALS

8.2.1.1 Mosquitoes

8.2.1.1.1 FEST strain

An. albimanus {An. (Nyssorchynchus) albimanus}.

This strain was obtained from Fernando, El Salvador, Central America. It was collected from the field in 1975 and was colonised in London in 1981. The original colony was lost. However, a sub-colony had been set up by J.A. Seawright, United States Department of Agriculture, Gainesville, Florida, and he kindly sent back eggs with which to re-start the London School of Hygiene and Tropical Medicine (LSHTM)

colony. This strain is known to be resistant to propoxur, malathion, dieldrin and HCH (the same gene giving cross resistance to dieldrin and HCH).

8.2.1.1.2 PANAMA Strain

An. (Nyssorchynchus) albimanus

This is a susceptible strain which came from Panama, in Central America. It was obtained from the Center for Disease Control, Atlanta, Georgia, U.S.A, where it has been in their insectary for about 20 years. It has been colonised in London since 1986 to replace the closely related PALB strain which was lost.

8.2.1.2 Insecticides

Dieldrin : 0.4% and 4% dieldrin impregnated papers, supplied by WHO.

Propoxur : 0.1% propoxur papers. These were impregnated by the author in London as WHO impregnated papers have only a short shelf life. The procedure was as follows: a stock solution of 0.1% propoxur in olive oil was prepared from 96% pure propoxur solution. 0.7ml of this stock solution was taken and added to 1.3ml acetone to make a total volume of 2.0ml. All of this was then slowly and evenly spread onto pieces of Whatman's No. 1 filter paper measuring 12 x 15 cms. The paper had previously been labelled with pencil.

8.2.2 METHODS

Selection of a fully dieldrin and propoxur resistant colony from the FEST strain was carried out as follows:-

8.2.2.1 Mass selection

The mass selection methods was used in order to produce a highly resistant population. The WHO adult test methods (WHO 1970) were used. These methods have been revised in 1975 (mimeographed documents WHO/VBC/75.581 and 582) and again in 1981 (documents WHO/VBC/81.805 and 806).

8.2.2.2 Single family selection

One-day-old adults were exposed first to 4% dieldrin for 1 hour and the survivors of this exposure were then exposed to 0.1% propoxur for 1 hour. The survivors from these exposures were then collected and transferred to a clean cage. They were allowed to mate among themselves and, two days after the treatment, they were given a blood meal. The gravid females were individually tubed in glass vials for single family selection. This selection was done mainly to obtain a homozygous propoxur resistant colony, as the treatment with 0.1% propoxur would allow both the heterozygous and homozygous resistant individuals to survive. (There is no discriminating dose between these genotypes for propoxur, unlike dieldrin). Eggs from each

female were collected separately and allowed to hatch within 48 hours. Larvae from each female were reared in different bowls and allowed to grow into adults. One-day-old adults from each female were again exposed to 0.1% propoxur for 1 hour and the survivors were kept for the next generation. This selection process was continued for eight generations until a line with no mortality on either insecticide was obtained. This line was considered to be probably homozygous resistant to both dieldrin and propoxur .

8.2.2.3 Selection of a dieldrin and propoxur susceptible colony of An. albimanus.

Because the original susceptible PANAMA strain did not give 100% mortality after an exposure to either 0.4% dieldrin or 0.1% propoxur for 1 hour, it was decided to re-select for a dieldrin and propoxur susceptible colony by the single family selection method as described above, but applying the selection process in the reverse direction.

Samples of the emerged adults from each family were exposed to 0.4% dieldrin and to 0.1% propoxur for 1 hour. The sibs of those which showed 100% mortality in both the samples tested were kept to be parents of the next generation. This procedure was repeated for several generations until a family that showed 100% mortality in every generation was obtained. This family was considered

to be a homozygous doubly susceptible colony of the PANAMA strain.

8.2.2.4 Cross of FEST X PANAMA

About 100 virgin female FEST were put into an empty cage and 100 PANAMA males were introduced into the cage and were allowed to mate freely. They were then fed on an anaesthetised guinea pig and, after two days, F1 eggs were collected. The eggs were hatched and the larvae were reared to the adult stage.

8.2.2.5 Backcross test

8.2.2.5.1 First experiment

Virgin F1 female offspring were backcrossed to PANAMA males. The resulting progeny is designated as FPXP. The backcross progeny were then tested in two groups, one receiving propoxur treatment followed by dieldrin, the other, dieldrin followed by propoxur.

8.2.2.5.2 Second experiment

F1 male survivors were backcrossed to virgin PANAMA females. The resulting progeny is designated as PXFP. The progeny were tested on each insecticide in turn, in both reciprocal orders, as before.

8.2.2.6 Combining bioassays and biochemical methods of detecting resistance

Another batch of the backcross progenies (i.e. FPxP and PxFP) were first exposed to the discriminating dosage of 0.4% dieldrin for one hour in standard WHO test kits. After a 24 hour holding period the dead and live mosquitoes were counted separately and put into labelled tubes marked SS for dead, and RS for live, mosquitoes. These were then frozen at -20°C overnight.

Individual mosquitoes from the SS group were then put into the wells of 96 well (NUNC- Immuno-plate II) microtitre plates using alternate rows of wells. Mosquitoes were thoroughly homogenized in $20\mu\text{l}$ of Triton phosphate buffer using a multiple homogenizer. A further $180\mu\text{l}$ of buffer was added to each well. The homogenates were again thoroughly mixed with the homogenizer. This second mixing washed away any residue remaining on the rods. $185\mu\text{l}$ of homogenate from each well was then transferred into the adjacent empty well of the microtitre plate. This was to avoid interference in the assay from fragments of cuticle.

Another plate corresponding to the first one was prepared and $135\mu\text{l}$ Triton buffer was added to each well. $10\mu\text{l}$ of DTNB and $30\mu\text{l}$ of a homogenized insect extract were added to each well. AChE activity was measured in the wells using ASCHI as the substrate. $25\mu\text{l}$ of substrate alone or

substrate plus insecticide was added to start the reaction. Where insecticide was to be added a stock solution of 0.1M propoxur was used to give a final concentration of 1.25mM propoxur in the assay.

The rates of reaction were measured on a Vmax Kinetic Microplate Reader (Molecular Devices Corporation) at 450nm over a period of five minutes. Sets of graphs of optical density (OD) over time for all 96 wells were obtained with an optical density limit of 0.1, and rates of reaction calculated by linear regression. Data were stored on a computer.

The same procedure was repeated for RS mosquitoes.

8.3 RESULTS

8.3.1 Bioassay results

Table 8.1 shows the results of mass selection for a dieldrin and propoxur resistant colony of the FEST strain An. albimanus. After five generations of selection with 4% dieldrin 100% survival was obtained. This should indicate that the strain was homozygous for dieldrin (DLN) but it was not necessarily homozygous resistant to propoxur because RS and RR cannot be discriminated. Selection with propoxur was then applied between the families of individual females in

Table : 8.1

Mass selection for a dieldrin and propoxur resistant colony of the FEST strain of An. albimanus. The figures indicate the percent mortality on exposure to 4% dieldrin and then, after a 24 hour interval to 0.1% propoxur for one hour (no. tested in parentheses).

Generation	Percent mortality	
	4% dieldrin	0.1% propoxur
P	20.2 (495)	33.8 (395)
F1	10.3 (329)	26.4 (295)
F2	5.2 (346)	21.0 (328)
F3	2.0 (350)	17.2 (343)
F4	0.0 (325)	12.6 (325)

order to try to obtain a strain which was homozygous resistant to propoxur (Fig. 8.2). This was obtained after 8 generations of single family selection.

Fig. 8.3 shows single family selection for a DLN and propoxur susceptible homozygote colony of the PANAMA strain of An. albimanus. After 3 generations of selection with both insecticides, a strain which was considered to be homozygous susceptible to both 0.4% DLN and 0.1% propoxur was obtained.

Fig. 8.4 shows the methods of determining whether or not each selected FEST family was pure homozygous for resistance. If the family is pure homozygous resistant, a test cross to the pure homozygous susceptible family would give a progeny that was entirely heterozygous and would therefore survive the discriminating dose. If on the other hand the family is impure, a test cross would result in a progeny including SS homozygotes so the progeny would show some mortality on exposure to the discriminating dose.

The F1 individuals used for backcrossing had survived both dieldrin and propoxur treatment, and are therefore expected to be all doubly heterozygous for resistance to the two insecticides. As mentioned in the introduction to this chapter both dieldrin and propoxur resistance have been shown to be monofactorially inherited: 50% of the backcross progeny should therefore be susceptible to the first

Fig : 8.2

Single family selection for a propoxur resistant colony of the FEST strain An. albimanus after 5 generations of mass selection. The figures indicate no. killed/no. tested on exposure to 0.1% propoxur for one hour. The black dots indicate the families which were selected because they had the lowest mortality.

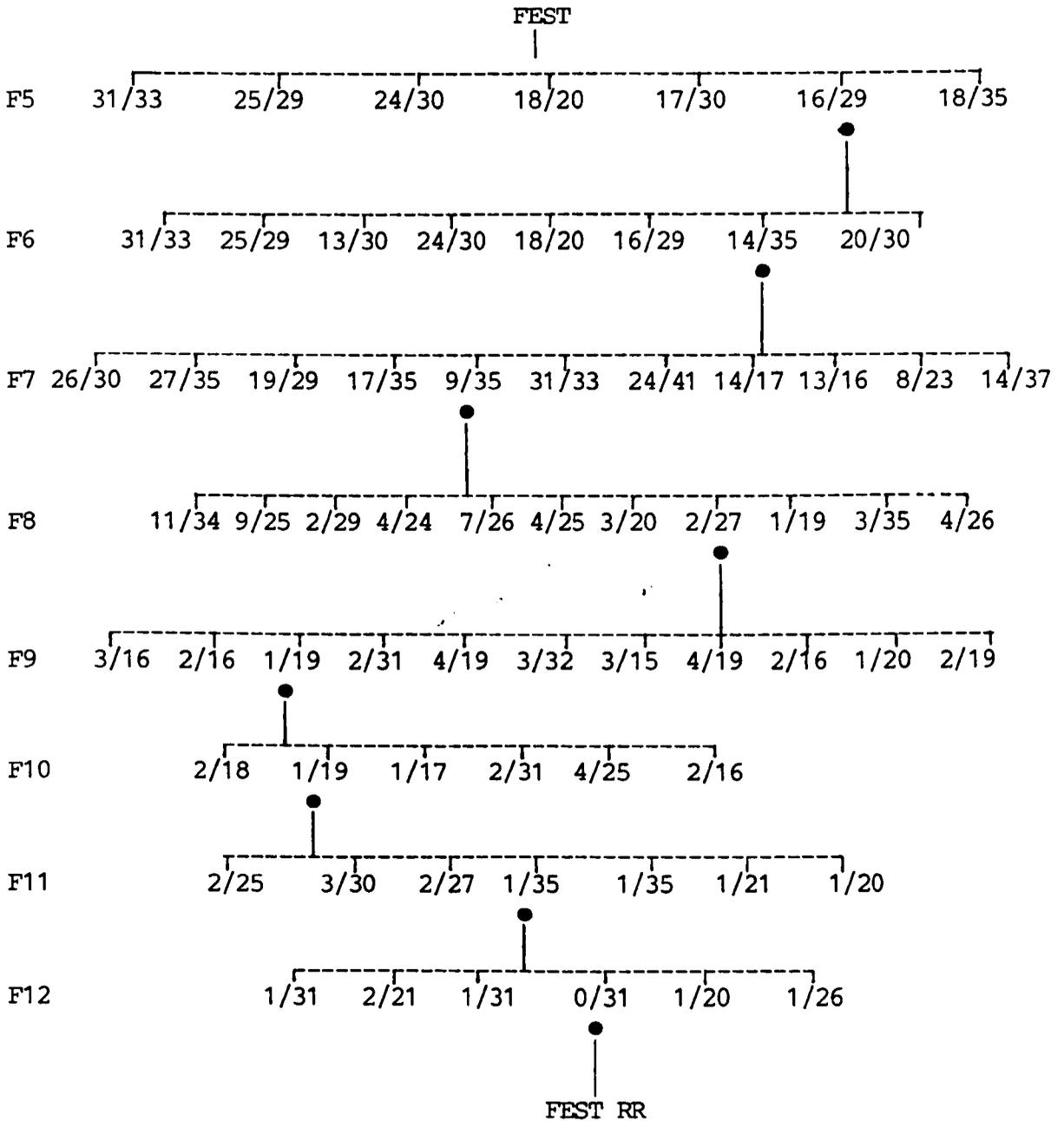


Fig : 8.3

Single family selection for a dieldrin and propoxur susceptible homozygote colony of the PANAMA strain of An. albimanus. The figure show no. killed/no. tested on exposure of a sample to 0.4% dieldrin and 0.1% propoxur for one hour each.

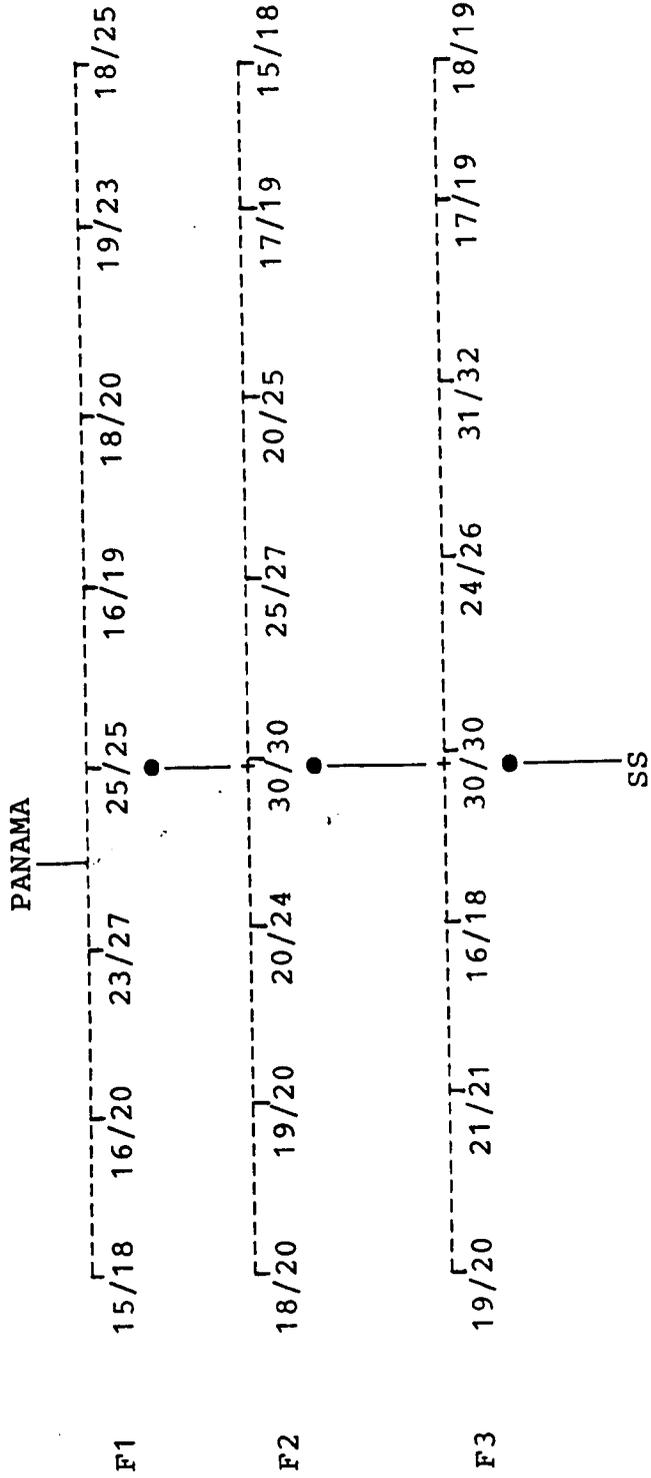


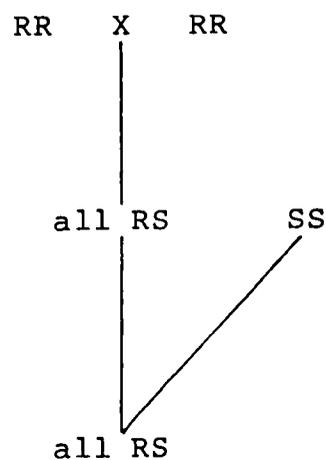
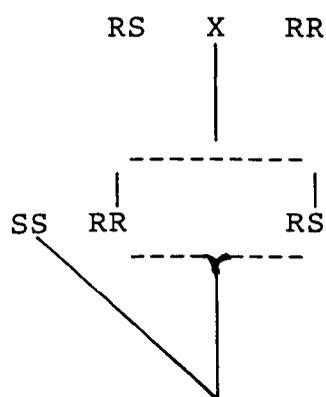
Fig : 8.4

The method used to determine whether or not each family is pure homozygous for resistance.

Impure family including RS

Pure family RR

mating among themselves



test cross to SS

some RS and some SS

all RS

expose to discriminating dose of 0.4% dieldrin and 0.1% propoxur for one hour.

some die on discriminating dose.

all survive discriminating dose.

insecticide treatment, and 50% of the survivors are expected to be susceptible to the second insecticide if the two resistance genes are unlinked and inherited independently. The results of the backcross test are shown in table 8.2. In most tests rather more than 50% mortality was observed from the exposure to the first and the second insecticide and in several cases there was significant deviation from the 50% expectation. However, only in one case out of four (test 2 of experiment 1) was the deviation in the second test towards less than 50% mortality. It thus appears that dieldrin and propoxur resistance genes are probably not linked.

8.3.2 Biochemical results

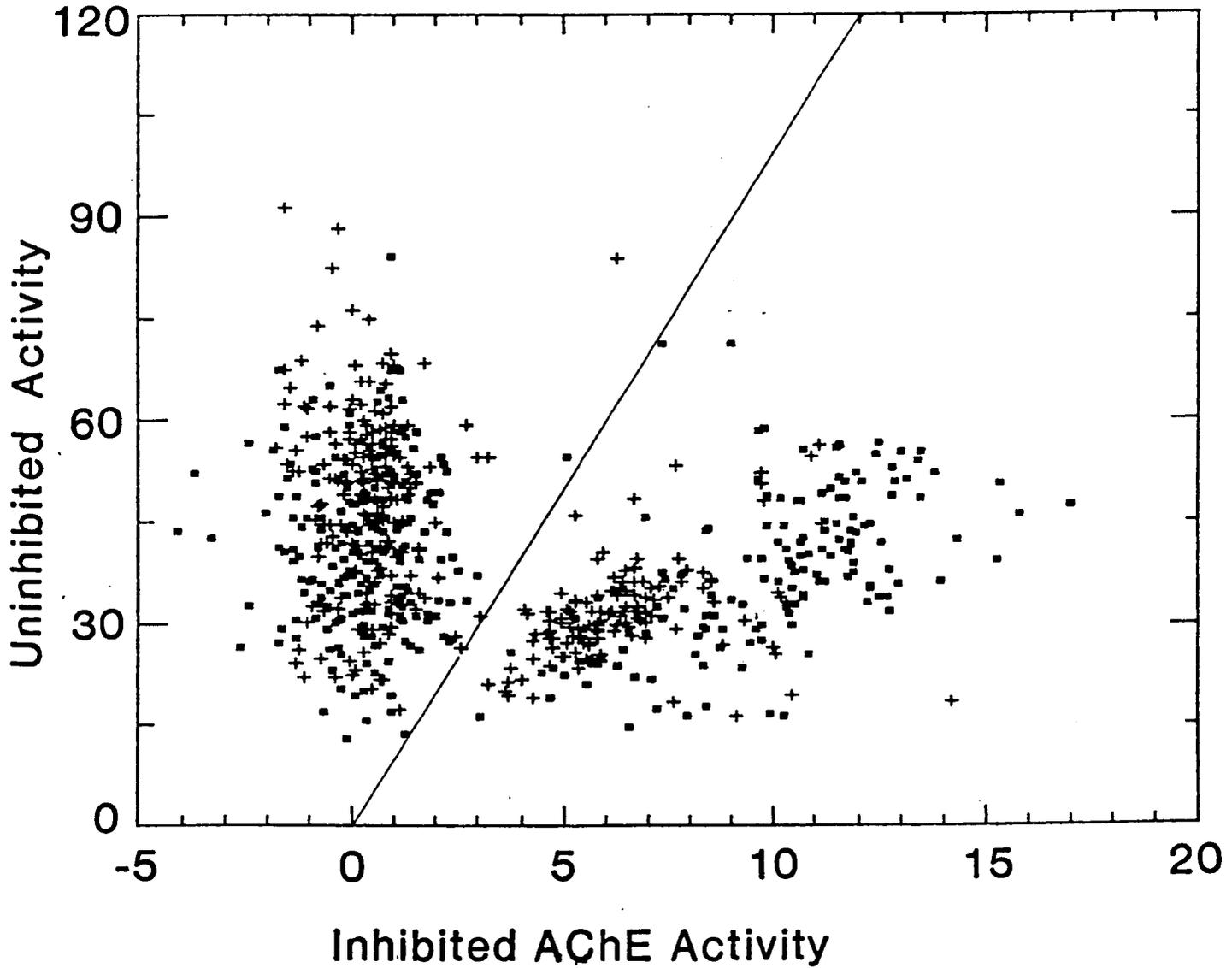
To check this conclusion a bioassay was combined with a biochemical method for testing genetic linkage of the two resistance genes. In the biochemical method altered AChE genotype frequencies were determined by the method of French-Constant and Bonning (1989). Results were represented graphically as scatter plots. Fig. 8.5 shows the inhibited AChE activities plotted against the uninhibited activities of the corresponding individual mosquito extract from the cross FPxP. The dots and crosses in Fig. 8.5 represents the dead and live mosquitoes respectively after exposure to 0.4% dieldrin for one hour. The line in Fig. 8.5 divides the points into two clear clusters, one to the left of the line and one to the right,

Table: 8.2 Results of testing backcross progenies of (F_xP)_♀ x P_♂ and P_♀ x (F_xP)_♂ on dieldrin and propoxur in turn.

		1st experiment (F _x P) _♀ x P _♂				χ ² test (1:1) expectation
		No. tested	Dead	% mort.	Alive	
Test 1	[DAY	1	0.4% dieldrin		
		257	106	41.2	151	7.88 p < 0.01
	DAY	2	0.1% propoxur			
	151	92	60.8	59	7.21 p < 0.01	
Test 2	[DAY	1	0.1% propoxur		
		201	93	46.3	108	1.12 n.s.
	DAY	2	0.4% dieldrin			
	108	43	39.8	65	4.48 p < 0.05	
		2nd. experiment P _♀ x (F _x P) _♂				
Test 1	[DAY	1	0.4% dieldrin		
		191	119	62.5	72	11.56 p < 0.001
	DAY	2	0.1% propoxur			
	72	40	56.0	32	0.88 n.s.	
Test 2	[DAY	1	0.1% propoxur		
		197	107	54.6	90	1.46 n.s.
	DAY	2	0.4% dieldrin			
	90	51	56.8	39	1.6 n.s.	

Fig: 8.5

**FPxP cross: uninhibited versus inhibited AChE activity
as a measure of propoxur-resistance**



**Squares and crosses represent insects
which had survived or been killed by
exposure to dieldrin, respectively**

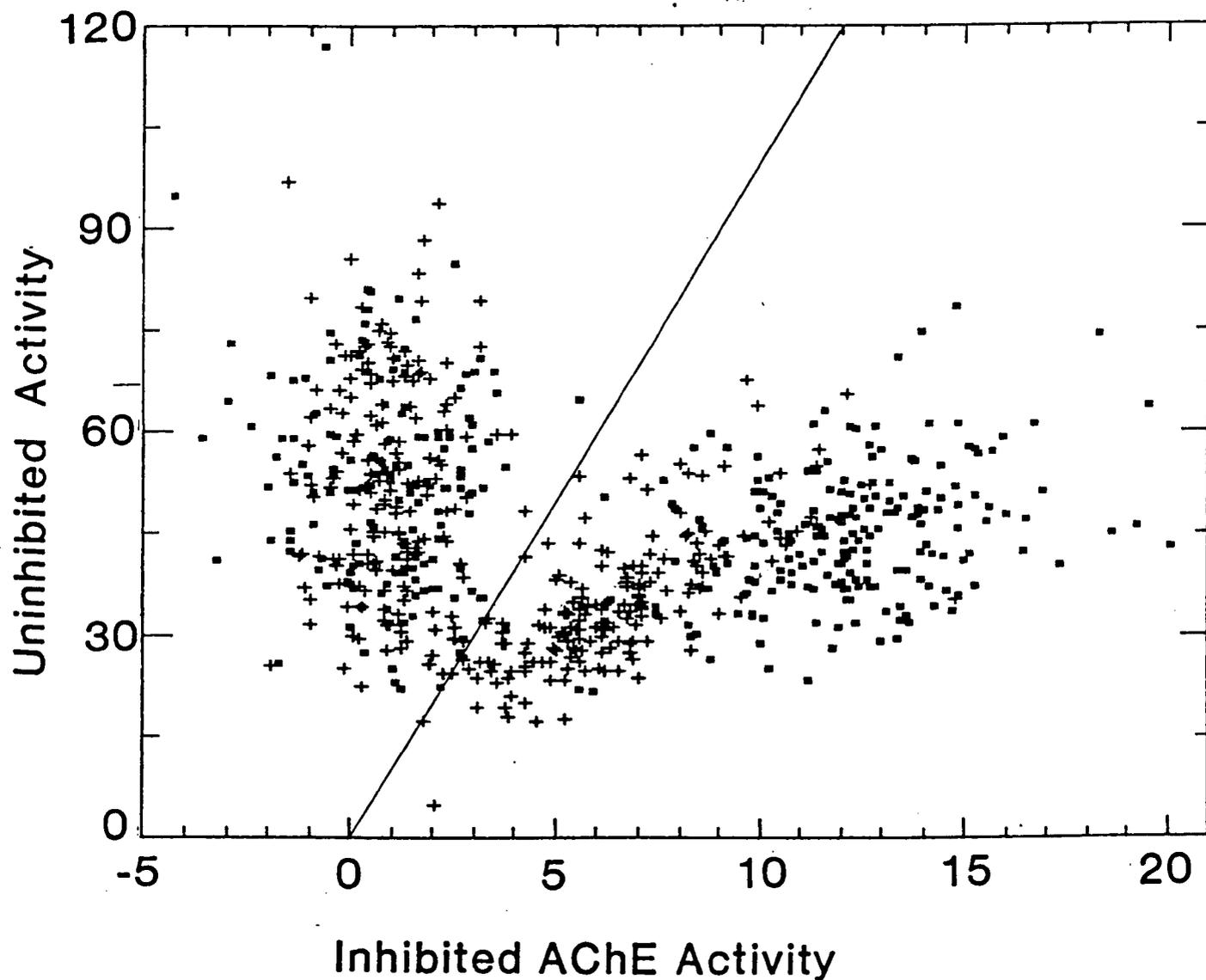
Enzyme activity expressed in milli-O.D. per minute

which correspond to the susceptible homozygotes (SS) and heterozygotes (RS) at the AChE locus respectively (French-Constant and Bonning 1989). Those points on the left have a ratio of inhibited to uninhibited activity (I/U) of less than 10%, and those to the right have a ratio of more than 10%. There seemed to be no difference in the AChE activity among the dead and live mosquitoes in the $\begin{matrix} S S \\ P P \end{matrix}$ cluster, both with and without inhibitor. However among the $\begin{matrix} R S \\ P P \end{matrix}$ cluster there did appear to be lower AChE activity in the dead mosquitoes compared to the live ones. This may indicate that after death resistant AChE enzyme is subject to faster decay than the susceptible enzyme but this did not cause confusion between $\begin{matrix} R S \\ P P \end{matrix}$ and $\begin{matrix} S S \\ P P \end{matrix}$ insects.

Fig. 8.6 shows the results of the reciprocal P x FP cross. The points still form two clear clusters but were more scattered than in fig. 8.5. In both figures there are very few borderline cases - only 10-20 out of more than 600 points.

As mentioned earlier, four genotypes are expected among the progeny of each backcross and these are represented by the dots and crosses to the left and right of the dividing line in fig. 8.5 and fig. 8.6 respectively. Table 8.3 gives the number of these different genotypes from both crosses. To test whether there is any linkage between the two genes, χ^2 tests were done on the figures in table 8.3, the results being 0.24 (P=0.6) and 1.71 (P=0.2) respectively. Genotype

Fig: 8.6
PxFP cross: uninhibited versus inhibited AChE activity
as a measure of propoxur-resistance



Squares and crosses represent insects which had survived or been killed by dieldrin, respectively.

Enzyme activity expressed in milli-O.D. per minute

Table: 8.3 Classification of the backcross progeny into heterozygous $\begin{matrix} R & S \\ (P & P) \end{matrix}$ and homozygous susceptible $\begin{matrix} S & S \\ (P & P) \end{matrix}$ genotypes at the propoxur (P) and dieldrin (D) resistance loci by the biochemical and bioassay methods, respectively. In neither the FPxP ([FESTxPANAMA]xPANAMA), nor the reciprocal PxFP cross, is there evidence for linkage. Totals marked with asterisks deviate significantly from expected 1:1 ratio.

Cross	Dieldrin genotypes	Propoxur genotypes		Total
		$\begin{matrix} R & S \\ P & P \end{matrix}$	$\begin{matrix} S & S \\ P & P \end{matrix}$	
FPxP:	$\begin{matrix} R & S \\ D & D \end{matrix}$	144	197	341
	$\begin{matrix} S & S \\ D & D \end{matrix}$	133	199	332
	Total	277*	396*	673
PxFP:	$\begin{matrix} R & S \\ D & D \end{matrix}$	196	153	349
	$\begin{matrix} S & S \\ D & D \end{matrix}$	177	171	348
	Total	373	324	679

N.B. adapted from Lines et al. (1990)

ratios at the two loci are therefore independent and it may be concluded that the two genes are unlinked.

8.4 DISCUSSION

It is concluded from the study that the dieldrin and propoxur resistance genes in An. albimanus are not linked. This is consistent with the study of Davidson and Sawyer (1975). Knowledge of linkage between resistance genes is important because it may affect their behaviour in populations in two ways. Firstly, selection for one resistance gene could affect the frequency of a closely linked gene by the "hitch-hiking" effect, if there is linkage disequilibrium (Lines and Curtis 1984). Secondly, modelling studies have shown that linkage disequilibrium would greatly reduce the effectiveness of using a mixture of insecticide to slow down the evolution of resistance (Curtis 1985). Insecticide mixtures tend to select for linkage disequilibrium, even with genes on different chromosomes, and for linked genes this effect would be even stronger (Mani 1985).

The linkage procedure followed in the first part of this study was the same as those followed by Haridi (1974), Lines and Curtis (1984) and Rowland (1985). This procedure required the backcross progeny to be tested on each insecticide in turn. Two problems might arise from this

method. The first is that only half of the offspring are scored at both loci, because the others have been killed during the first exposure, thus they are no longer available for testing at the other locus. Another problem with this procedure is that the insects could have been weakened by the first exposure or simply by ageing, and as such might not survive the second exposure. Rowland (1985) found that resistance to malathion declined markedly in the first three days after emergence. This could give a spuriously high kill on the second exposure and thus lead to misclassification as recombinants.

To avoid these problems Lines, ffrench-Constant and Kasim (1990) combined a preliminary test of backcross progeny with dieldrin with a biochemical test where the AChE genotypes could be scored by the AChE microtitre plate assay (ffrench-Constant and Bonning 1989). By combining a bioassay and a biochemical method we were able to score both loci in all the backcross progeny. The results (Table 8.3) clearly showed that there was no linkage between the dieldrin and propoxur resistance genes in An. albimanus. This contrasts with the conclusion of Lines and Curtis (1984) with regard to dieldrin and malathion resistance in An. arabiensis.

CHAPTER 9

FITNESS OF DIELDRIN RESISTANCE GENE IN THE ABSENCE
OF INSECTICIDE

9.1 INTRODUCTION

The development of resistance involves the evolution of the ability to survive treatment and probably also the evolution of the relative fitness of the various genotypes (McEnroe and Neagle 1968). Studies on the fitness of specific resistance genotypes in the presence and absence of treatment started in the 1970's (Roush and McKenzie 1987). Most studies of the fitness of resistance genes have been conducted in the laboratory where two methods have been used. One is the fitness component approach and the other is the use of continuously breeding populations in cages. In the first case, fitness components such as developmental time, fecundity and fertility are measured for each genotype. In the latter method changes in the genotype frequencies are measured for several generations in replicate populations containing a mixture of genotypes (Roush and Daly 1990).

Detailed measurement of genotype fitness in each stage of the life history is a possible means of predicting the

changes in the genetic composition of the population in the absence of insecticidal pressure. It is commonly assumed that in the absence of insecticidal pressure, resistant phenotypes have lower adaptive values (in terms of reproductive potential, viability, developmental period etc.), than do their susceptible counterparts. Examples where this was the case were homozygous organophosphate-resistant Cx quinquefasciatus which exhibited a significantly longer developmental time than did the homozygous susceptibles, while the F1 hybrids were intermediate (Amin and White 1984; Khatib and Georghiou 1985b). Similar findings were also reported by several other workers using the fitness component method (Georghiou 1972a; Georghiou and Taylor 1977a; Ferrari and Georghiou 1981; Roush and Flapp 1982; Flexner et al. 1989).

Arnold and Whitten (cited in McKenzie et al. 1982) conducted population cage studies with Lucilia cuprina to investigate the fitness of the diazinon resistance gene by following the fitness of the susceptible homozygote, heterozygote and resistant homozygote genotypes at each generation. They found that in the absence of insecticide, the R-allele was eliminated at a rate consistent with a constant deleterious effect of the R gene, the RS being intermediate in fitness between RR and SS.

The aim of the present study was to investigate what effect(s) the dieldrin resistance gene in An. albimanus from

El Salvador has on biological fitness in the absence of insecticide. The "population cage" method was chosen for this experiment.

9.2 MATERIALS AND METHODS

9.2.1 MATERIALS

9.2.1.1 Mosquitoes

An. albimanus : FEST strain : homozygous for dieldrin and propoxur resistance. PANAMA strain : susceptible for both dieldrin and propoxur. The history and origin of these strains have been discussed in chapter 8.

9.2.1.2 Insecticides

Dieldrin : 0.4% and 4% dieldrin papers obtained from WHO, Geneva.

9.2.2 METHODS

100 virgin female FEST (homozygous for dieldrin and propoxur resistance) were crossed with 100 male PANAMA (homozygous for susceptibility to dieldrin and propoxur). They were allowed to mate, blood feed and lay eggs. The first batch of eggs was discarded, so as to allow every individual a chance to lay eggs. The second, third and

fourth batches of eggs were collected and reared to adulthood.

A proportion of each batch was tested and the results were pooled. About 100 unexposed females and 100 unexposed males drawn from all three egg batches were used to start the next generation. This procedure was repeated until the F10 generation.

9.2.2.1 Bioassays

A proportion of the adults from each generation were tested first with 0.4% dieldrin and the dead insects were counted 24 hours later. The survivors were tested with 4% dieldrin. Those that died on 0.4% dieldrin were classified as dieldrin susceptible homozygotes, and those that survived 4% were classified as resistance homozygotes. The remainder were classified as heterozygotes (Davidson 1957).

9.3 RESULTS

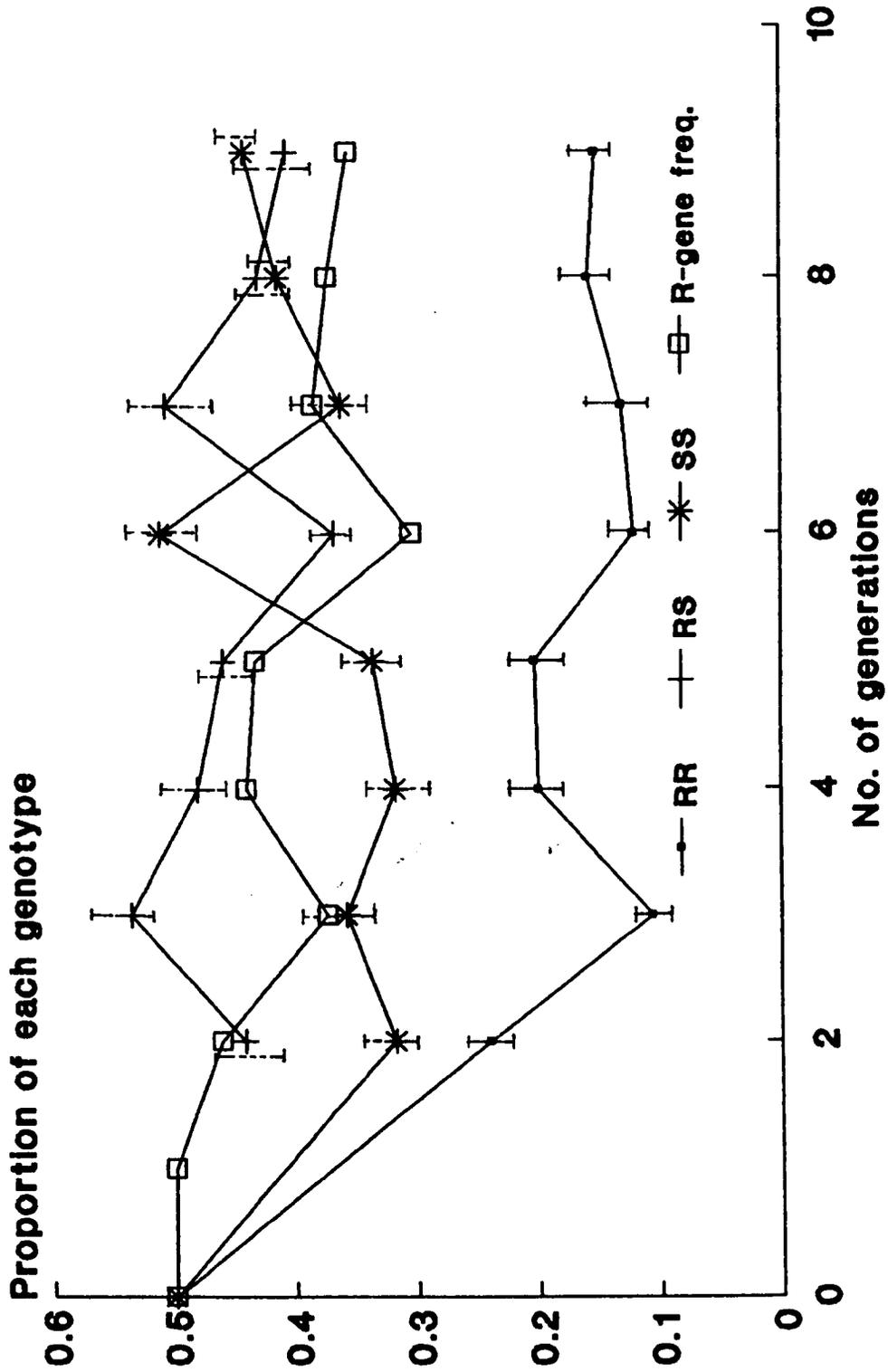
Table 9.1 shows the results obtained by testing with 0.4% and 4% dieldrin and Fig. 9.1 shows the proportion of each genotype (i.e. RR, RS and SS) deduced from these data assuming that the WHO doses perfectly discriminate the three genotypes. The proportion of RR decreased quite rapidly from 0.5 in the parental generation to 0.11 in the F3 generation. It then fluctuated between 0.1 and 0.2 until F9.

Table: 9.1

Mortalities of An. albimanus on exposure to 0.4% dieldrin (on 1st day) and 4% dieldrin (on the 2nd day) for one hour.

Generation	0.4% dieldrin			4% dieldrin		Survival of both doses	
	Total no. tested	No. died	Mortality (= frequency of SS)	No. died	Mort. as % of initial total (= frequency of RS)	No. survived	Survival as % of initial total (= frequency of RR)
F1	450	0		450	100		
F2	1656	526	31.8	732	44.2	398	24.0
F3	1174	420	35.8	630	53.7	124	10.6
F4	1357	431	31.8	654	48.2	272	20.0
F5	1597	537	33.6	735	46.0	325	20.3
F6	2375	1207	51.2	865	36.7	285	12.1
F7	771	279	36.1	391	50.7	101	13.1
F8	1551	640	41.3	666	42.9	245	15.8
F9	1720	759	44.1	699	40.6	262	15.2

Fig : 9.1
Population cage study: proportion of each diedrln resistance /
susceptibility genotype and R gene frequency.



N.B. no measurement at F1

The proportion of SS dropped from 0.5 in the parental generation and fluctuated around 0.35 until F5. It then increased to 0.51 in F6 and fluctuated around 0.45 until F9. The frequency of the RS genotype dropped from 1.0 in F1 to between 0.45 and 0.85 until F5, after which it was below 0.4 for 3 generations of the next 4.

9.4 DISCUSSION

The results of this experiment indicate that though the three genotypes settled down close to a balanced polymorphism, there was a slow but significant decline in the R frequency (Table 9.2). The genotype ratios were close to the Hardy-Weinberg equilibrium, but showed a significant, but unexplained deficit of heterozygotes (Table 9.2). Rowland (1991) reported that heterozygous (RS) larvae of An. gambiae and An. stephensi, developed slightly but significantly faster than their homozygous dieldrin resistant (RR) and susceptible (SS) counterparts. He also showed that the dieldrin resistant (RR) females were less active than SS or RS females and he concluded that the RR was the least fit of the three genotypes. In another study by Rowland (1991b) he reported that RR males were less competitive in mating compared to RS and SS males.

Table: 9.2

Significance tests of regression of R frequency on number of generations and of deviation of genotype frequency from Hardy Weinberg ratio.

No. of generation (x)	Frequency of R (y)
0	0.5
1	0.5
2	0.461
3	0.374
4	0.441
5	0.433
6	0.304
7	0.384
8	0.372
9	0.355

Calculated intercept = 0.483
and regression coefficient = -0.0161

Mean square of Y unexplained by regression = 0.001985
Standard error of regression coefficient = 0.00575

t-test for significance of regression = 2.80 $0.05 > P > 0.01$

Fit to Hardy Weinberg ratio,

Generation	Freq. of R = p	Expected genotype frequency			
		RR	RS	SS	
2	0.461	Expected	0.212	0.496	0.290
		Observed	0.240	0.442	0.318
		Expected	351	821	480
		Observed	398	732	526
$\chi^2 = 20.3, P < 0.001$					
9	0.355	Expected	0.126	0.458	0.416
		Observed	0.152	0.406	0.441
		Expected	217	788	716
		Observed	262	699	759
$\chi^2 = 21.9, P < 0.001$					

Roush and Plapp (1982) mentioned that recessiveness for the effect of R on biotic potential could explain why the rate of reversion of resistance in the absence of pesticides often slows down as the frequency of R decreases. Computer simulations by Rowland (1991b) also showed that the frequency of resistance genes in populations of An. gambiae and An. stephensi decreased in the absence of insecticide at a rate comparable with the field reversion. Recessive adverse effects of the R gene on viability would lower the rate of the evolution of resistance as fixation for resistance was approached (Curtis 1981; Wood and Mani 1981; McKenzie 1984).

Curtis et al. (1978) assumed that the phenotypic effects of R-alleles on biotic potential have the same dominance as resistance. From a range of published data they calculated that the fitness of RR relative to SS was 0.44 - 0.97, depending on the species and insecticide concerned. Georghiou and Taylor (1977a,b) assumed that the fitness of the heterozygote (RS) was intermediate between the two homozygotes (RR and SS). Rowland (1991a) on the other hand, reported that the HCH / dieldrin resistance genes are recessive in their effect on female mosquito activity, thereby reducing the activity of RR but not of RS relative to SS. As already mentioned Arnold and Whitten [cited in McKenzie et al. (1982)] found that in the absence of insecticide, RS was intermediate in fitness between RR and SS.

It had been commonly believed that the fitness of each genotype could be simply defined in the absence or in the presence of insecticide pressure. However, recent studies in Lucilia cuprina have shown specific fitness differences may occur at insecticide concentrations that are sub-lethal for each genotype (McKenzie and Whitten 1984). It is also commonly believed that in a natural population when there is no insecticidal application the fitness of the various genotypes is as follows $SS > RS > RR$. In a populations free of insecticidal pressure R would only be maintained by mutation/ selection equilibrium, RR would hardly exist and the rare R-alleles would virtually only exist in the heterozygous state (RS). Therefore, when insecticide starts to be applied, the fitness difference between SS and RS is critical to the outcome but, as the R frequency increases, appreciable numbers of RR would be produced and its fitness would eventually become an important factor.

CHAPTER 10

COMPARISON OF A LONG-TERM OPPORTUNISTIC (OR REACTIVE)
ROTATION WITH A SHORT-TERM PRE-PLANNED ROTATION USING
COMPUTER SIMULATION

10.1 INTRODUCTION

The use of computer models provides a rapid and low-cost method of simulating what may be happening in the field and provides a means of making an initial study of the variables associated with the evolution of insecticide resistance. Models can also stimulate ideas for future experimental work. Recently the problem of managing insecticide use in relation to resistance has been discussed extensively in the literature (see chapter 1), and these studies have included modelling on the use of multiple pesticide combinations (Curtis 1985, 1987; Curtis and Otoo 1986; Curtis and Lines 1986; Curtis et al. in press; Tabashnik 1986, 1989, 1990b; Roush 1989), in the form of mixtures, rotations and mosaics. Tabashnik (1990b) classified modelling studies of insecticide resistance by four criteria: 1- the basic assumptions, 2- the modelling approach, 3- the factors considered and 4- the problem addressed.

The earlier studies using computer models have been discussed by Taylor et al. (1983). In nearly all these studies it was assumed that resistance is monogenically inherited. Some of these computer simulations studied the effect of incomplete coverage of spraying or immigration of susceptible individuals into the treated population and as emphasised in chapter 1, these have been shown under certain circumstances to slow the build up of resistance especially when mixtures were used (Kable and Jeffery 1979; Skylakakis 1981; Georghiou 1980; Mani 1985; Curtis 1985, 1987).

Roush (1989), discussed critically the pros and cons of using insecticides in alternation, mosaics and mixtures and he concluded that alternation is probably the most viable strategy in most circumstances because of unequal decay rates or incomplete mortality of genetically susceptible types on exposure to mixtures. In the present study, I simulated two types of rotations, i.e. the long-term sequential use (which may be better called "opportunistic" or "reactive" because switches are made in reaction to information from resistance tests), in contrast to short-term, pre-planned, rotations.

Curtis (1987) used a simple computer model to compare a short-term pre-planned rotation with a long-term opportunistic one. He assumed arbitrary values for reduction in fitness due to the resistance genes and he also arbitrarily assumed a modifier gene which, when homozygous,

raised the fitness of the resistance genotypes to that of the susceptibles. He concluded that only if the fitness reduction due to the resistance alleles was large and use of an insecticide continued until high levels of resistance were reached would there be long term advantage for using a pre-planned, as opposed to an opportunistic rotation. McKenzie and Whitten (1984) and McKenzie and Game (1987) give actual values for fitness reduction due to diazinon resistance in Lucilia cuprina and give evidence for a fitness modifier gene which eliminates this fitness reduction. It was decided to test how an opportunistic and a pre-planned rotation would compare using the Lucilia values for fitness but unavoidably arbitrary values for some other parameters. By the time this work was started Curtis's program had been lost, and I therefore had to re-construct it.

10.2 MATERIALS AND METHODS

10.2.1 Computer model

This model was based on that of Curtis (1987) and the programming was done with the assistance of R. Page. A flow chart diagram of the model used in this study is given in fig. 10.1.

10.2.2 Description of the model

The model assumes that resistance to each pesticide is controlled by a single locus with two alleles (R = resistant and S = susceptible). This program simulates two loci in a diploid organism such as mosquitoes. Depending on the settings of the general fitness values (H) and the fitness on exposure to insecticide (C) the two loci may be used to represent two different resistance genes without modifiers or a resistance gene and a modifier (N) with its neutral allele (M). In the simulation to be shown here the model was used to simulate one resistance gene (A^R) with its modifier. Resistance to another compound (B^R) was assumed to behave exactly like A^R when compound B was in use. In the table of young larvae at the top right of fig 10.1 are listed the various genotypes that could exist in the population. There are 10 different genotypes (SSMM to RRNN) including the coupling and repulsion forms of the double heterozygote represented by RSMN and RSNM.

The 10 different genotypes of larvae, L1 to L10, can each be assigned different general fitness values of H1, H2 up to H10 which allow for effects of the genes on general fitness in the absence of insecticide.

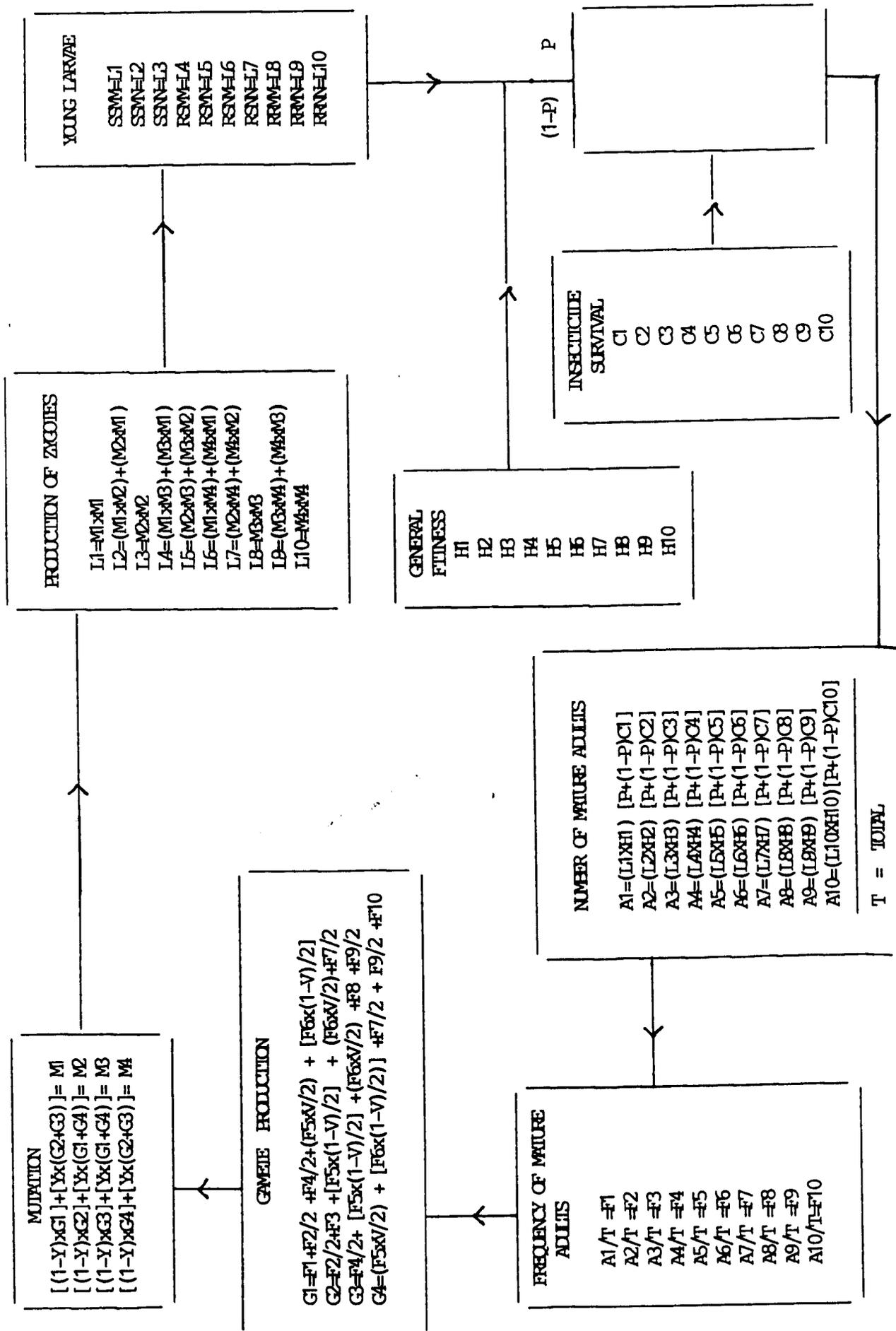
The diagram also show a proportion, P, of the mosquitoes unexposed to insecticide ; this can be varied to simulate "refugia" where some insects avoid exposure to

insecticide and also can be varied between generations to simulate intermittent use of an insecticide as when a rotation between two insecticide is being used. (1 - P) represents the number that is exposed to the insecticide.

The values C1 - C10 represent the survival of insecticide exposure of genotypes 1-10 after selective mortality due to general fitness factors and insecticide selection. After such selection the total number of adults, A1 - A10, does not equal 1.0 but is represented by T. This value T, was divided into the value A1 - A10 to obtain the frequencies of each adult type which thus total 1.0.

It would be very laborious to simulate all possible matings. Instead the contributions of each genotype of adult to the pool of gametes were computed and the contributions of the pool of gametes to the zygotes of the next generation were then computed. During gamete and zygote production, the normal Mendelian genetic rules were applied to calculate the frequency of the four types of gametes G1 (type SM), G2 (type RM), G3 (type SN) and G4 (type RN) and then to calculate the types of zygote (L1 - L10) which would arise if they fertilise at random. No distinction is made in this version of the model between males and females (but this has been made in a later version of the model to be published by Curtis et al. in press). The gamete production section of the model also allows for crossing-over between the loci. The cross-over value is represented by V.

Figure 10.1 : Flow chart of computer programme



Crossing over only has any effect in the double heterozygotes which, as already mentioned, are considered to exist in two forms RSMN (type 5) and RSNM (type 6).

At the gamete production stage some gamete types arise from a cross-over in type 5, i.e. (F5 x V) and others arise from non-cross-over in type 6, i.e. {F6 x (1-V)}. Conversely other gamete types arise from a cross-over in type 6 or a non-cross-over in type 5.

The parameter Y allows for mutation to and from the alternative alleles, so that low frequencies of the resistance and modifier alleles can exist stably in the initial untreated population, as a result of equilibrium between mutation to resistance or modifier alleles and selective elimination of these alleles because of their adverse effect on general fitness.

The following assumptions were also made in the present study :-

- 1 The general fitness values were as shown in table 10.1, based on the case of the R gene of McKenzie and Game (1987), the reason for assuming a slight fitness reduction due to the N gene is explained below in point 6.
- 2 Table 10.1 also shows the survival values in the presence of insecticidal exposure of McKenzie and Whitten (1984), i.e. there is intermediate dominance of the

Table : 10.1 Parameters used in the computer simulations including the general fitness values where R gene causes moderate fitness reduction, conditional on the absence of a modifier as in Lucilia cuprina diazinon resistance (McKenzie and Game 1987, Fig. 4 and Tables 2 and 3) and the insecticide survival values of McKenzie and Whitten (1984, Table 2, data for weeks 6-20).

Genotype	Initial proportions among larvae	H (general fitness)	C (insecticide survival)
SSMM	0.98986	1.0	0.49
SSMN	0.00995	0.998	0.49
SSNN	0.00002	0.996	0.49
RSMN	0.00017	0.883	0.68
RSMN	0	0.998	0.68
RSNM	0	0.998	0.68
RSNN	0	0.996	0.68
RRMM	0	0.883	1.00
RRMN	0	0.998	1.00
RRNN	0	0.996	1.00

V (cross-over value) = 0.5

Y (mutation rate) = 0.00001

P (Proportion which escape exposure),
= either 0.01 or 1.0 i.e. no insecticide applied.

effect of R gene in protecting against the insecticide, not unlike that found for DDT in chapter 6.

- 3 - There is a forward and backward mutation rate of 10^{-5} per gene per generation.
- 4 - 1% of the population escape exposure to the insecticide ($P = 0.01$).
- 5 - There is free recombination, i.e. a cross over value of 0.5 between the R/S and M/N loci in the double heterozygous genotypes.
- 6 - The initial frequency of the resistance genes is taken as 0.000085 which is near equilibrium due to the production of resistance genes by mutation and their selective elimination due to their reduced general fitness in the absence of insecticide. The modifier allele, N, has a slight fitness disadvantage and is initially close to its mutation/selection equilibrium at a frequency of 0.005.

The program was first run using the values tested by Curtis (1987) and the same results were obtained; this constitutes a check that there were no serious errors in either of our programs.

In the opportunistic rotation, $P = 0.01$ was used until the frequency of A^R reached slightly more than 0.5, i.e. if it took T generations to reach this frequency the value of P was switched to 1.0 for the next T generations during which time it was assumed that insecticide B was in use. The

value of P was then switched back to $P=0.01$ until the frequency of A^R again exceeded 0.5 and so on until both A^R and B^R exceeded 0.5 simultaneously.

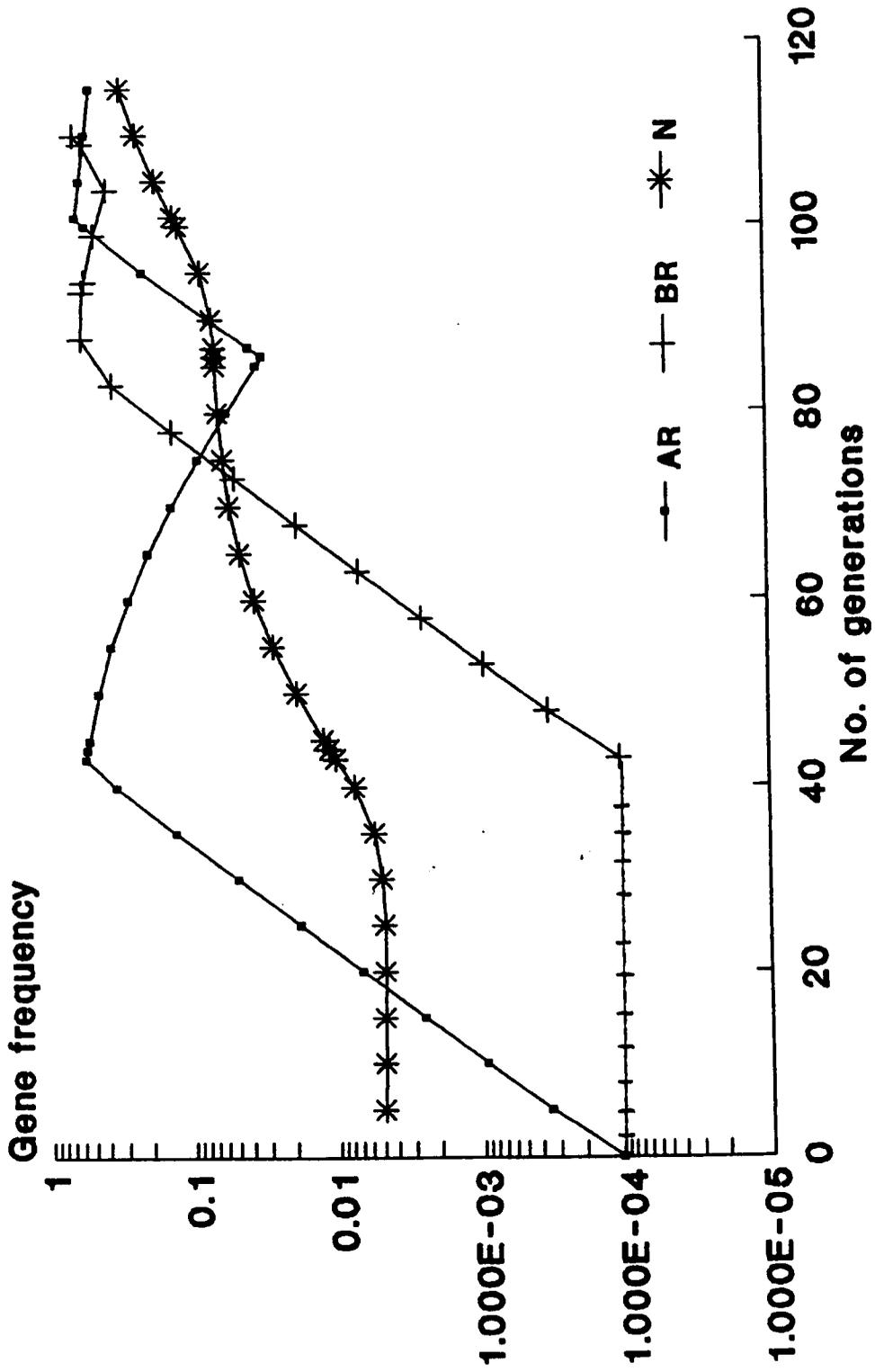
In the short-term pre-planned rotations, values of $P = 0.01$ and $P = 1.0$ were alternated every five generations until the frequency of $A^R > 0.5$.

10.3 RESULTS

Fig.10.2 shows the result of a computer simulation of a opportunistic rotation using a simple genetic model of a single gene, A^R , conferring resistance to compound A and another single gene, B^R , conferring resistance to compound B.

From generation 1 to 43 the frequency of A^R rose to 0.5 because insecticide A was in use, but B^R remained at mutation/selection equilibrium. Because A^R had exceeded 0.5 a switch was made to B at generation 43 and B^R followed the same course as A^R had previously. Selection against the A^R gene when A was not used (see fitness values in Table 10.1) resulted in a decline in the frequency of the A^R gene from 0.5 at generation 43 to 0.03 at generation 86. However the frequency of A^R increased again when A had to be used again because by then B^R had exceeded 0.5. A^R exceeded 0.5 at generation 100 by which time B^R was still 0.3, and the whole system was considered to have failed by generation 105 because there was high resistance to both compounds.

Fig : 10.2
Long-term opportunistic rotation

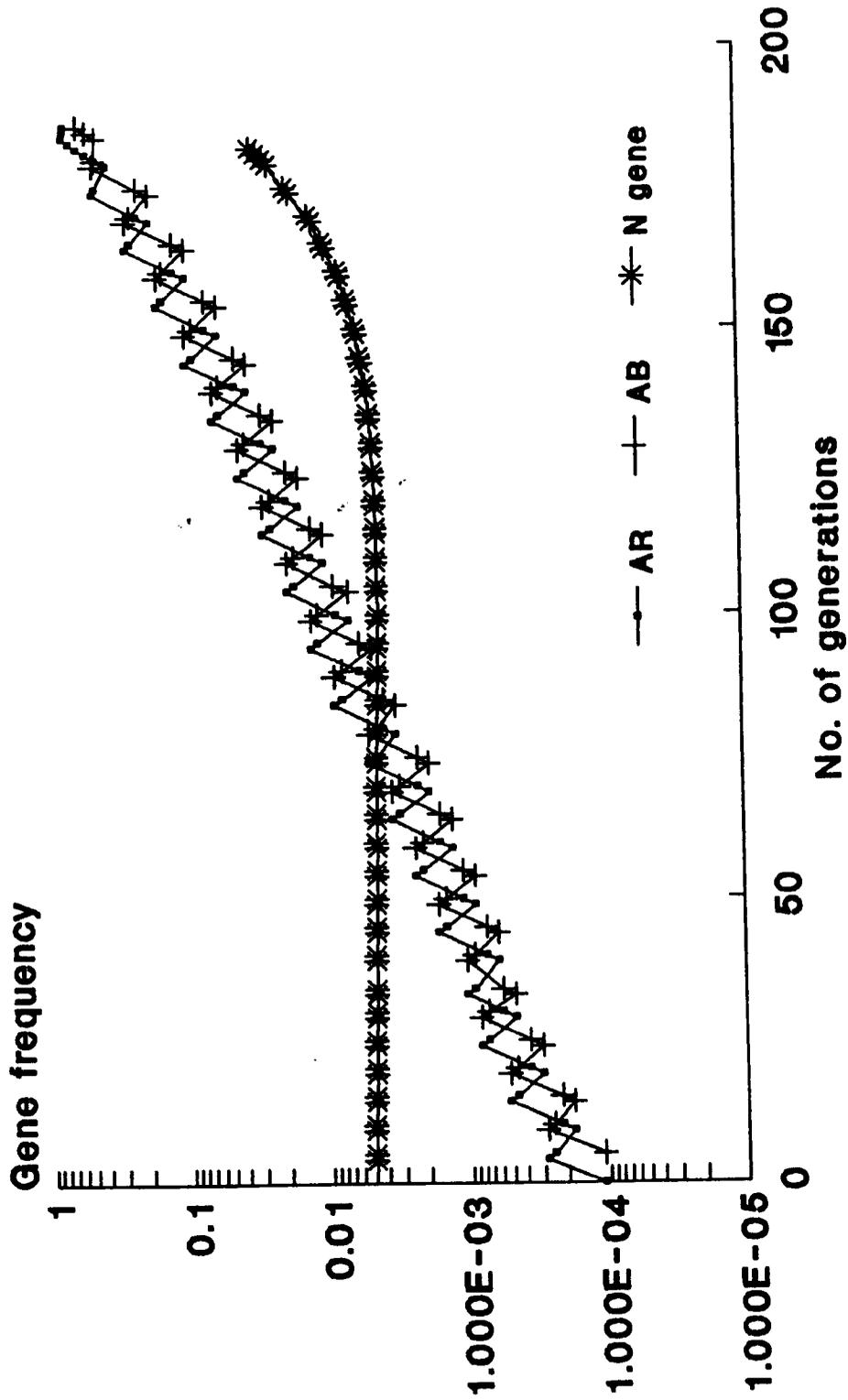


AR - freq. of AR gene, BR - freq. of BR gene, N - freq. of modifier gene which gives normal fitness to AR or BR

Fig. 10.3 shows the result of a computer simulation of a pre-planned short-term rotation. The frequency of A^R increased in the five generations in which A was used but dropped in the next five generations in which A was not used. The increase in resistance frequency was more than the drop at each cycle such that a "saw-tooth" pattern with an upward trend was produced and reached 0.5 after 182 generations of alternation. The same pattern (5 generations out of phase) was observed for gene B^R .

Fig. 10.2 also shows the frequency of the modifier gene, N , of the fitness of A^R . It can be seen that with the opportunistic rotation the frequency of the N allele hardly changed for the first 45 generation until the frequency of the A^R allele was about 0.5. From this time onward the N frequency increased quite rapidly because of the relatively high A^R frequency, and it had reached a frequency of about 0.2 when double resistance was reached. From fig. 10.3, it can be seen that with the short-term rotation the frequency of N (the modifier) allele hardly changed until generation 145 when the frequency of the A^R allele reached about 0.1. It then increased at an accelerating rate and had reached 0.03 by the time that double resistance was reached. Fig. 10.2 and 10.3 do not show the modifier gene which is assumed to act on the fitness of B^R in exactly the same way as N does on A . This second modifier would not start to increase in frequency until B^R became relatively frequent.

Fig : 10.3
Short-term, pre-planned, rotation
of insecticide every 5 generations



AR - freq. of AR gene, BR - freq. of BR gene, N - frequency of modifier gene which gives normal fitness to AR or BR

10.4 DISCUSSION

From the simple computer model of Curtis (1987) with two resistance genes, one affecting compound A and another affecting compound B, it was found that there was no long term difference between pre-planned rotation and long-term opportunistic use, i.e. intolerable levels of resistance to both insecticides concerned would be reached after the same number of generations regardless of the switching policy adopted. But when there are fitness modifiers in the genetic background that can eliminate the reduction in general fitness there could be some long term advantage in a policy of early switching of insecticides (Curtis and Lines 1986, Curtis 1987, Curtis et al. in press). The present study emphasised that fitness modifiers would be at an appreciable advantage only when in the presence of a high frequency of the resistance allele. There was therefore some advantage in this case in adopting the short-term rotation policy, as the fitness modifier genes would not exert their effect until the frequency of the resistance genes was high, after about generation 145 in fig. 10.3. If the resistance gene frequency is allowed to become very high before switching, general fitness of the resistance genes may be improved by modifier genes which eliminate fitness disadvantages of resistance genes (Roush and McKenzie 1987, Roush 1989) and no decline in the resistance level would then be observed on withdrawing the use of the insecticide concerned. It may thus be a better policy to switch to

another insecticide when the resistance has reached a measurable level and avoid the resistance genes reaching a very high level (Curtis et al. in press).

Frequent switching could be disadvantageous if residual insecticides are used. The decaying residue of the recently withdrawn insecticide may allow the resistance heterozygotes to survive (Curtis et al. in press), thus increasing the chances of the resistance genes being selected (Georghiou and Taylor 1977b; Curtis, Cook and Wood 1978).

Direct evidence for the occurrence of modifiers of fitness associated with insecticide resistance is scarce (McKenzie et al. 1982; Clarke and McKenzie 1987; Roush and McKenzie 1987). So far, there is only one case in which a fitness modifier gene has been proved to be present and has been quantitatively studied in the laboratory. This is the case of diazinon resistance in the Australian sheep blow fly Lucilia cuprina (McKenzie et al. 1982; McKenzie and Purvis 1984; McKenzie 1984; McKenzie and Game 1987). With prolonged selection pressure, the fitness of the homozygous resistant genotype increased (McKenzie et al. 1982) so that finally there was no difference in fitness between RR and SS genotypes when selection was relaxed (McKenzie and Whitten 1984). It was concluded that this was due to the selection of modifier genes which enhanced the relative fitness of the R allele following modification of genetic background (McKenzie et al. 1982). The modifier caused a decrease in

developmental time of the RS and RR genotypes, but the developmental time of the SS genotype was unaffected by the modifier gene (McKenzie and Game 1987).

Although this seems to be a much less common phenomenon in the field than many people suppose, (Roush and McKenzie 1987), it requires further investigation, especially as Uyenoyoma (1986) suggested that evolution of insecticide resistance involves changes in at least two loci : a regulatory locus controlling the level of synthesis of a key catabolic enzyme and a modifier locus that reduces the fitness disadvantage associated with the resistance gene.

Figs.10.2 and 10.3 and Curtis (1987) showed no great advantages or disadvantages for either switching policy. However, it should be noted that the use a pre-planned rotation conflicts with the usual advice not to switch pesticides until evidence of operational impact of resistance is obtained. There may be valid practical reasons for adopting the pre-planned rotations such as efficiency of ordering and storing of pesticides and where there are seasonal environmental restrictions on the use of certain insecticides such as in the Onchocerciasis Control Programme, where as detailed in chapter 1, short-term pre-planned rotations are dictated by the seasonal factors which limit the use of Bti, permethrin and carbosulfan. However, in programmes which depend on residual insecticide every change of insecticide use will leave decaying residues

and these may be dangerous as they are more likely to give a selective advantage to resistance heterozygotes. In the OCP none of the insecticide used are residual because they are sprayed onto fast flowing rivers.

CHAPTER 11

TEST OF A ROTATION WITH A MALAYSIAN Culex quinquefasciatus
STRAIN

11.1 INTRODUCTION

As discussed in the previous chapter there are at least two types of rotational patterns of insecticide use. The first type is that when the chemical in use fails to control the insect pest due to resistance, one is forced to change to a new more effective chemical. After a period of use, the new chemical may also lose its effectiveness and one may then be able to change back to the original chemical which by then may have regained its effectiveness (Cutright 1959). This is what is normally done in any well-managed control programme (Metcalf 1983) and might be called an "opportunistic" or "reactive" rotation (Curtis et al. in press). The second type of rotation is a pre-planned or short-term rotation (Curtis 1987, Curtis et al. in press). In this type of rotation two or more compounds are rotated alternately over time without regard to the presence of detectable resistance.

There have been conflicting claims, either theoretical or experimental, as to which type of rotation should be adopted in control programmes. It seems important that experiments are done to try to clarify this problem. In this chapter comparisons are described of pre-planned short-term rotations versus the opportunistic type of rotation (or longer-term sequential use of insecticides). The comparisons were carried out on adults and larvae of the urban filariasis vector Cx quinquefasciatus in the laboratory.

11.2 MATERIALS AND METHODS

11.2.1 MATERIALS

11.2.1.1 Mosquitoes

Culex quinquefasciatus

The origin of the strain from which the adults came was explained in chapter 2. The larvae used in this experiment were first selected with temephos for several generations (as explained in chapter 3). When the resistance level to temephos was about 15%, a sub-colony was set up and used for this study.

11.2.1.2 Insecticides

5% malathion papers, 0.25% permethrin papers and

standard temephos solutions supplied by WHO.

Bti as explained in chapter 2.

11.2.2 METHODS

11.2.2.1 Insecticide rotation with adult Culex quinquefasciatus.

Batches of 25, 2-3 day old adults were exposed to 0.25% permethrin papers at each generation until about 40% resistance had been reached and then there was a switch to malathion exposure at each generation, until high resistance had been reached. The survivors at each generation were collected and put into empty cages. They were fed and allowed to lay eggs for the next generation. This kind of rotation will be referred to as a long-term or reactive rotation. This procedure was repeated with malathion as the first selecting agent after which there was a switch to permethrin exposure for the next several generations. Batches of 25 adults were also exposed to permethrin and malathion at alternate generations. Breeding for the next generations was initiated from the survivors at each generation. This type of rotations is referred to as the short-term, pre-planned, rotation. Each time, apart from the exposures to the insecticide currently being used for selection, a sub-sample was also tested with the other compound to monitor the resistance level to it.

11.2.2.2 Insecticide rotation with Culex quinquefasciatus larvae

Batches of 25 healthy larvae were exposed to temephos at the diagnostic dosage of 0.02 mg/l for 24 hours during the late third or early fourth instar, according to WHO standard procedures (WHO 1970). Survivors were collected and transferred to clean water and reared to the adult stage to produce the next generation. This was repeated until 50% resistance was reached, after which a change to Bti at a dose of 0.015mg/l was made. Bti selection was then carried on for an equal number of generations. The whole procedure was repeated but using Bti as the first selecting agent. This type of rotation is referred to as the long-term rotation.

Another line was exposed as larvae to temephos and Bti at alternate generations. At each generation the survivors were collected and reared to the adult stage to produce the next generation. This constitutes a short-term, pre-planned, rotation.

The % survival on each insecticide was checked on sub-samples at each generation so that any change in resistance level to either chemical, whether it was currently being used for selection or not, could be followed.

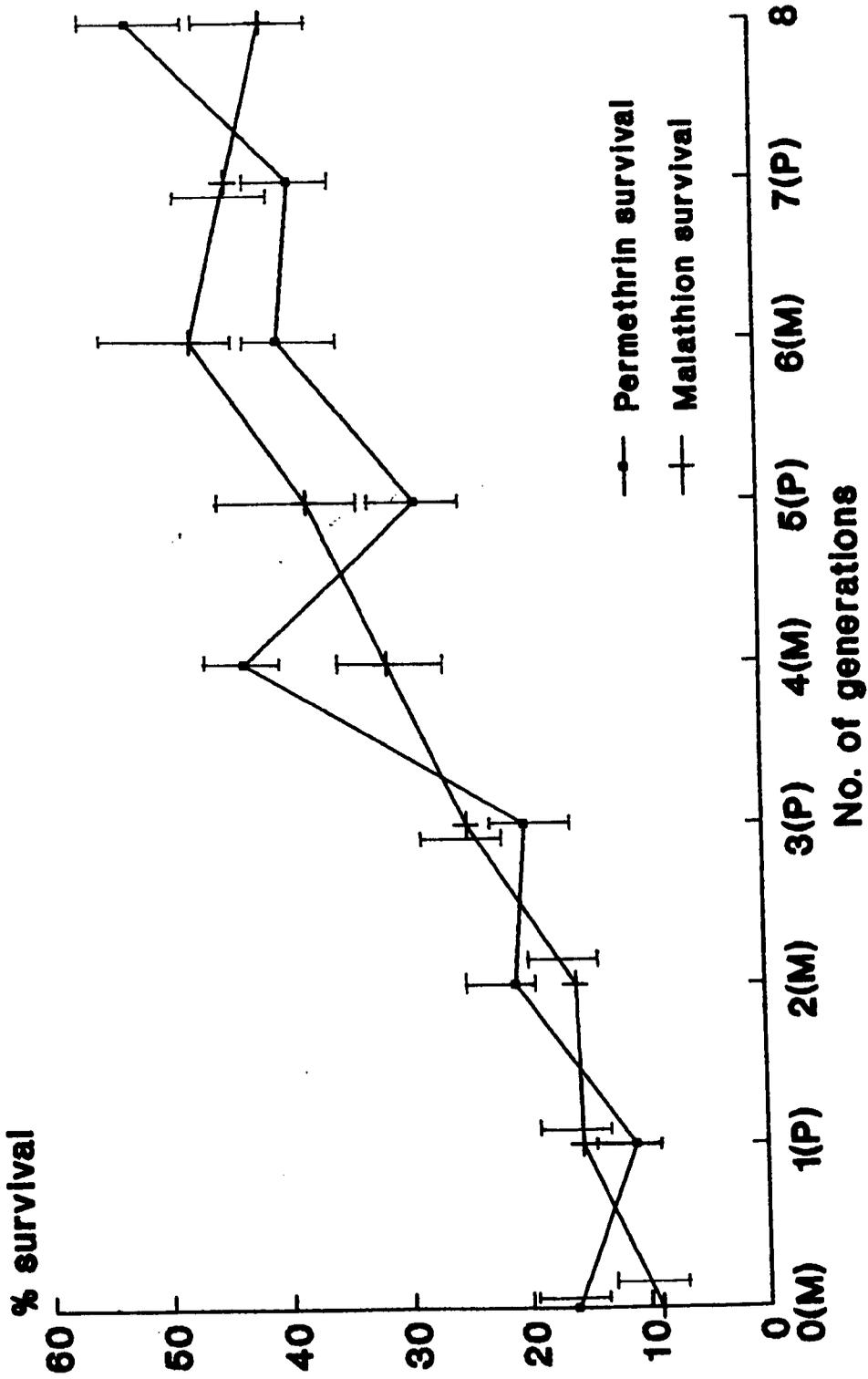
11.3 RESULTS

11.3.1 Insecticide rotation with adult Culex quinquefasciatus

In the short-term pre-planned rotation (Fig 11.1), resistance to permethrin rose somewhat in the generation in which it was applied and then declined slightly in the generation it was not applied. It rose once more in the next generation when permethrin was again applied, but dropped slightly in the 4th generation. The rise when permethrin was in use was greater than the fall when it was out of use so that each cycle of the rotation led to a net increase in resistance. This pattern almost repeated itself every generation throughout the experiment, until F8 when the level of permethrin resistance was found to be about 52%. With malathion there was not an obvious "saw tooth" pattern but there was a net increase at each cycle of the rotation and the resistance level was found to be 41% at F8.

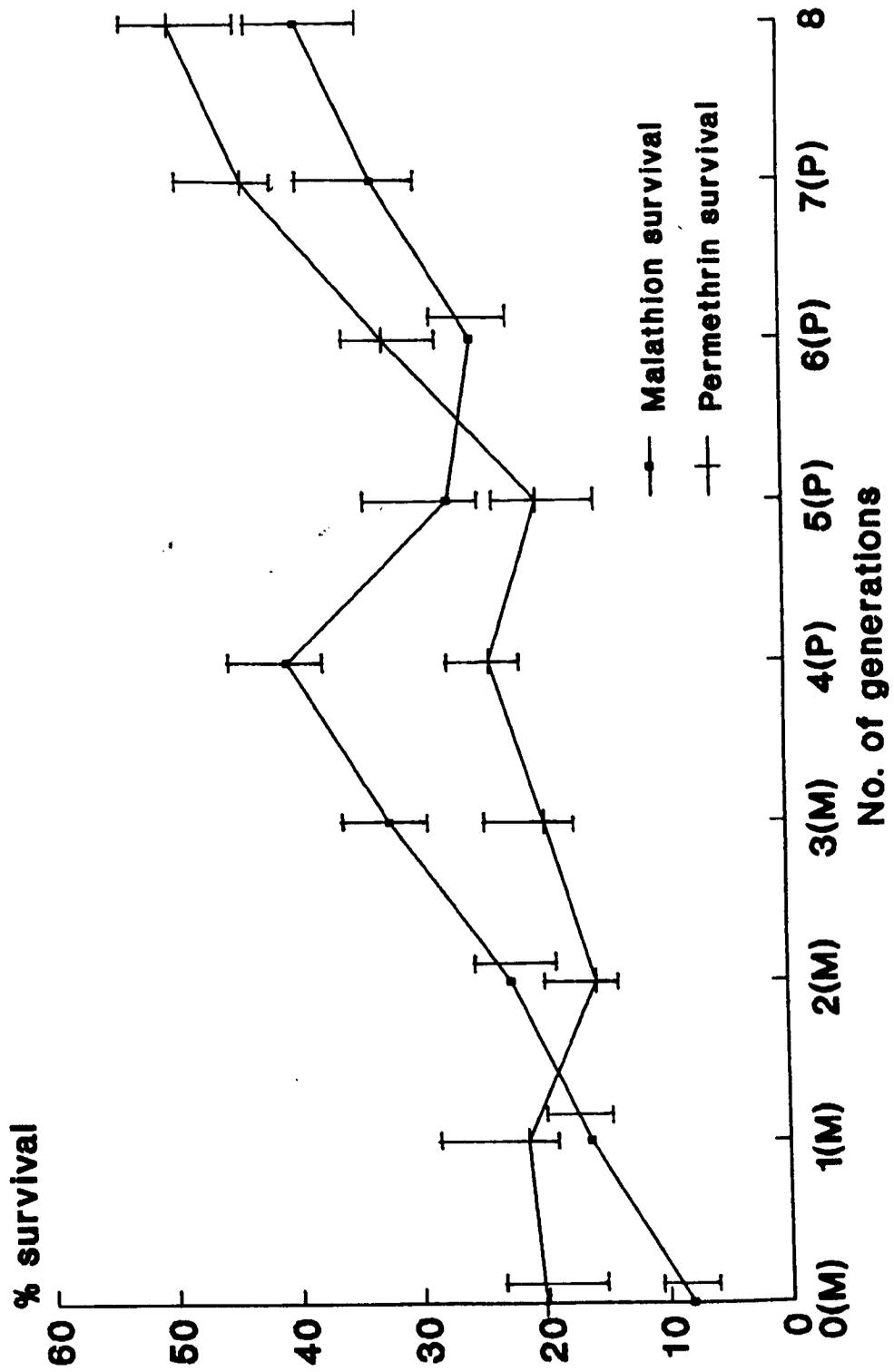
In the long-term rotation (Fig. 11.2) the permethrin resistance level fluctuated very little when it was not in use but it increased steadily to about 50% between generation F4 and F8 when permethrin was applied. The malathion resistance level increased steadily from an initial level of 8% to about 40% at generation F4 when malathion was used but the level dropped to about 28% at F5 when malathion was replaced by permethrin as the selecting

Fig : 11.1
Short-term pre-planned rotation of malathion and permethrin
(MPMP...) with Cx quinquefasciatus adults



Note : exposure to 0.25 % permethrin and 5.0% malathion for one hour.

Fig : 11.2
Long term rotation of malathion and permethrin (MMM...PPP)
with adult Cx quinquefasciatus



agent. However, by F8 the malathion level had gone back to the level of 40% which it had reached at F4; there was insufficient decline in malathion resistance to warrant re-using it. Fig 11.3 is a repetition of the long-term rotation but carried out in the reversed order (permethrin first). The permethrin and malathion levels at F8 were similar or slightly lower (about 37% and 30% respectively) compared with fig. 11.2. It can thus be seen that the final result was not much affected by which pattern of insecticide usage was adopted.

11.3.2. Insecticide rotation with Cx quinquefasciatus larvae

Fig. 11.4 shows the short-term pre-planned rotation with Cx quinquefasciatus larvae using temephos and Bti alternately for 8 generations. There was fluctuation in the development of temephos resistance level, but it showed an overall increase from 6% to about 32% by F8. The survival on Bti showed less change but there was an overall rise from about 20% in the first three generations to about 30% in the last three with non-overlap of the confidence limits. Figs 11.5 and 11.6 show the long-term rotation of Bti and temephos. In fig 11.5 the larvae were first selected with temephos until 50% resistance was reached, then switched to Bti, whereas in fig. 11.6 Bti was used for the first four generations. In both cases the final Bti survival was about

Fig : 11.3
Long term rotation of permethrin and malathion (PPP...MMM)
with adult Cx quinquefasciatus

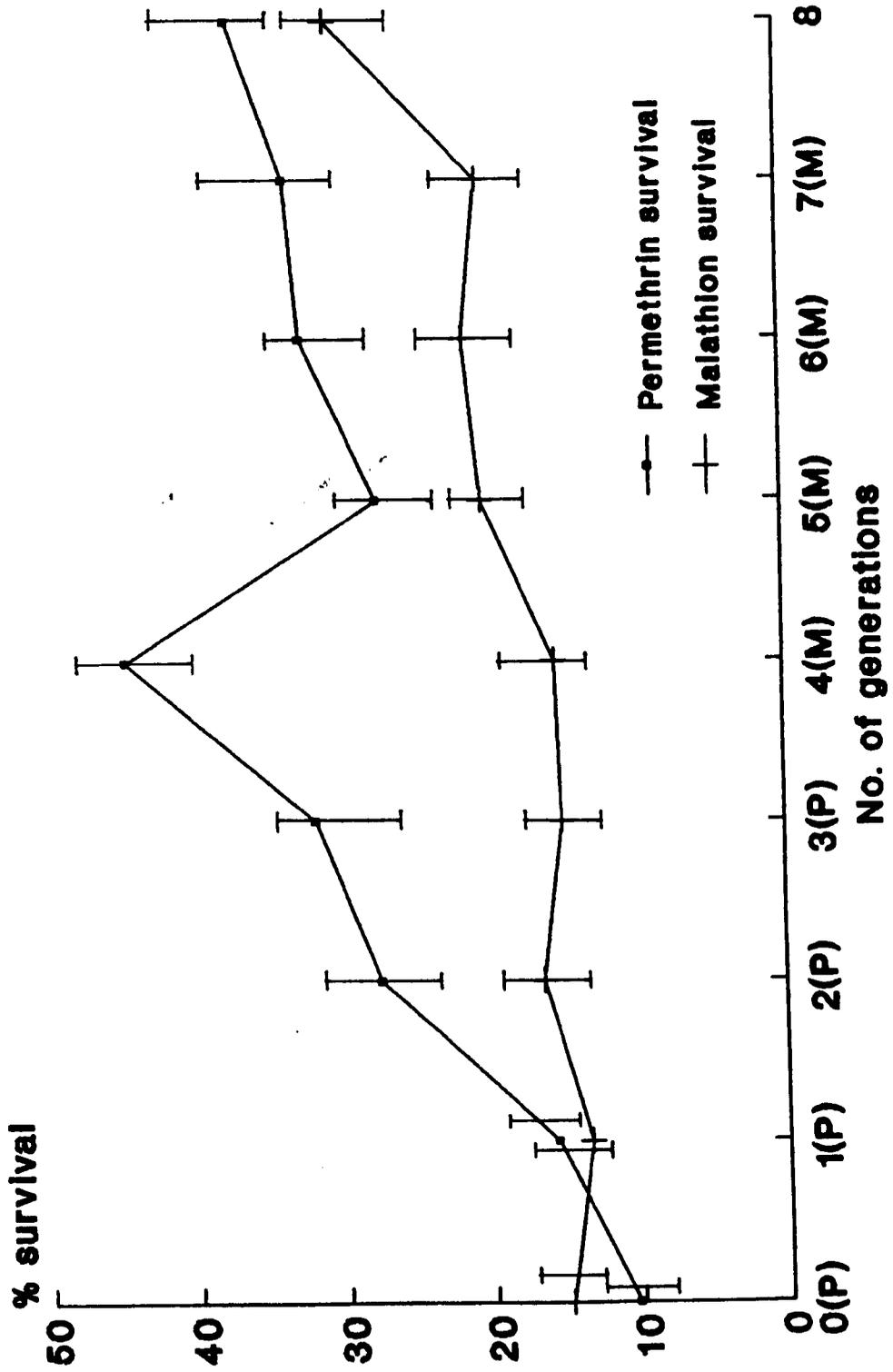
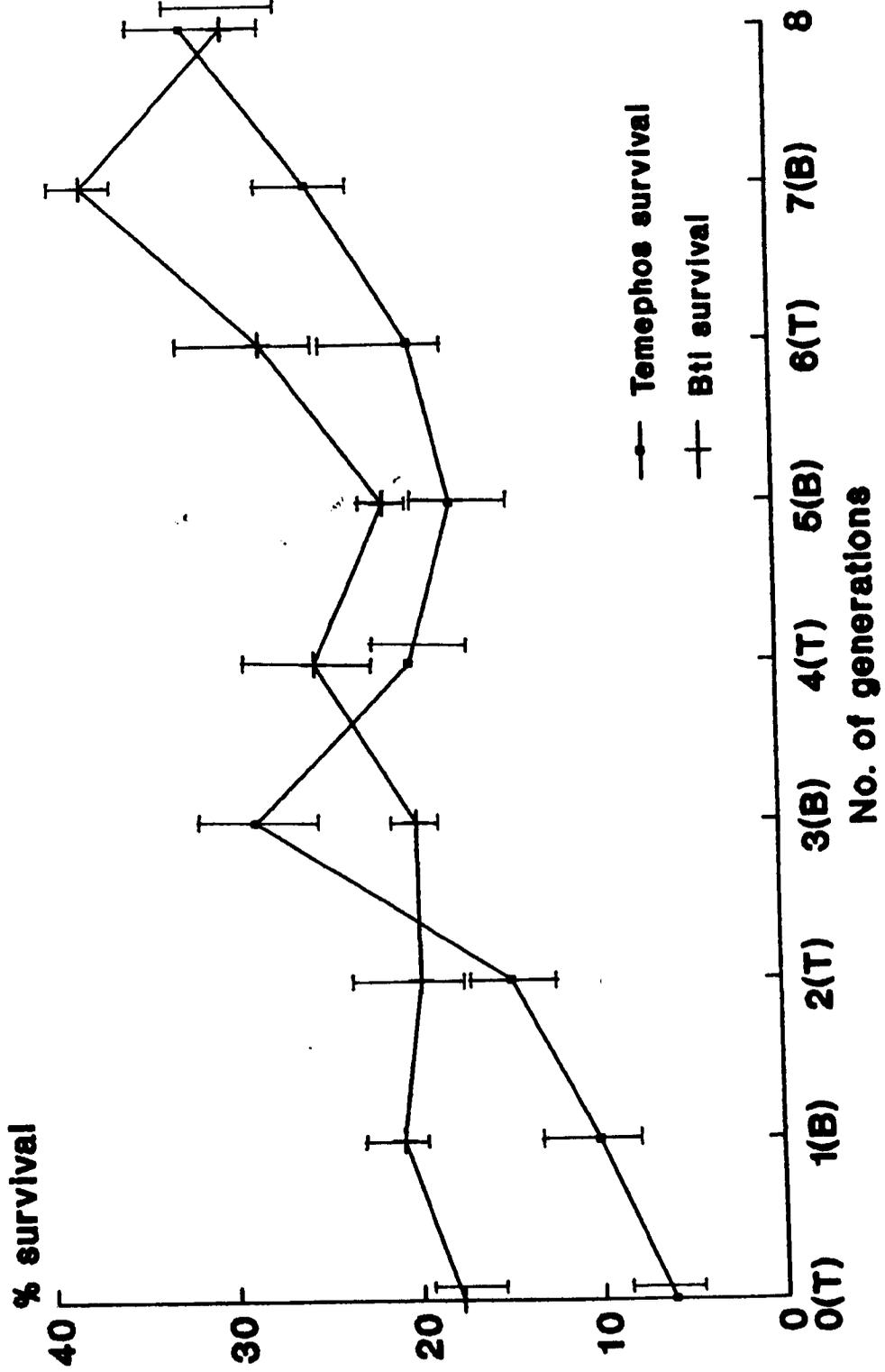


Fig : 11.4
Short term pre-planned rotation of temephos and Btl (TBTB..)
with Cx quinquefasciatus larvae



25%. The initial Bti survival in fig. 11.6 of only 10% seems to have been anomalously low. The temephos level increased faster and higher when it was the first selecting agent used but more slowly when Bti was used first (Fig.11.6). However in figs. 11.4, 11.5 and 11.6 the final level of temephos survival was between 30 to 37% in all cases.

11.4 DISCUSSION

The final results obtained with rotations applied to adult Cx quinquefasciatus were quite similar irrespective of whether a pre-planned alternation or the longer term sequential use was employed. In the short-term pre-planned rotation, generally a slight decline in the permethrin resistance level was observed when it was not used as the selecting chemical. The decline was presumably due to the reduced fitness of the resistance gene(s) in the absence of the insecticide concerned. In the long-term rotations, the expression of reduced fitness and/ or genetic instability of resistance sometimes gave a sudden drop in the resistance level when a change to another compound was made but in general in the second phases of the experiments illustrated in figs 11.1 to 11.3 the resistance selected in the first phase remained fairly stable.

The population cage simulations in the present study cannot be claimed to be conclusive because it was only

Fig : 11.5
Long term rotation of temephos & Btl
(TTT...BBB) with Cx quinquefasciatus

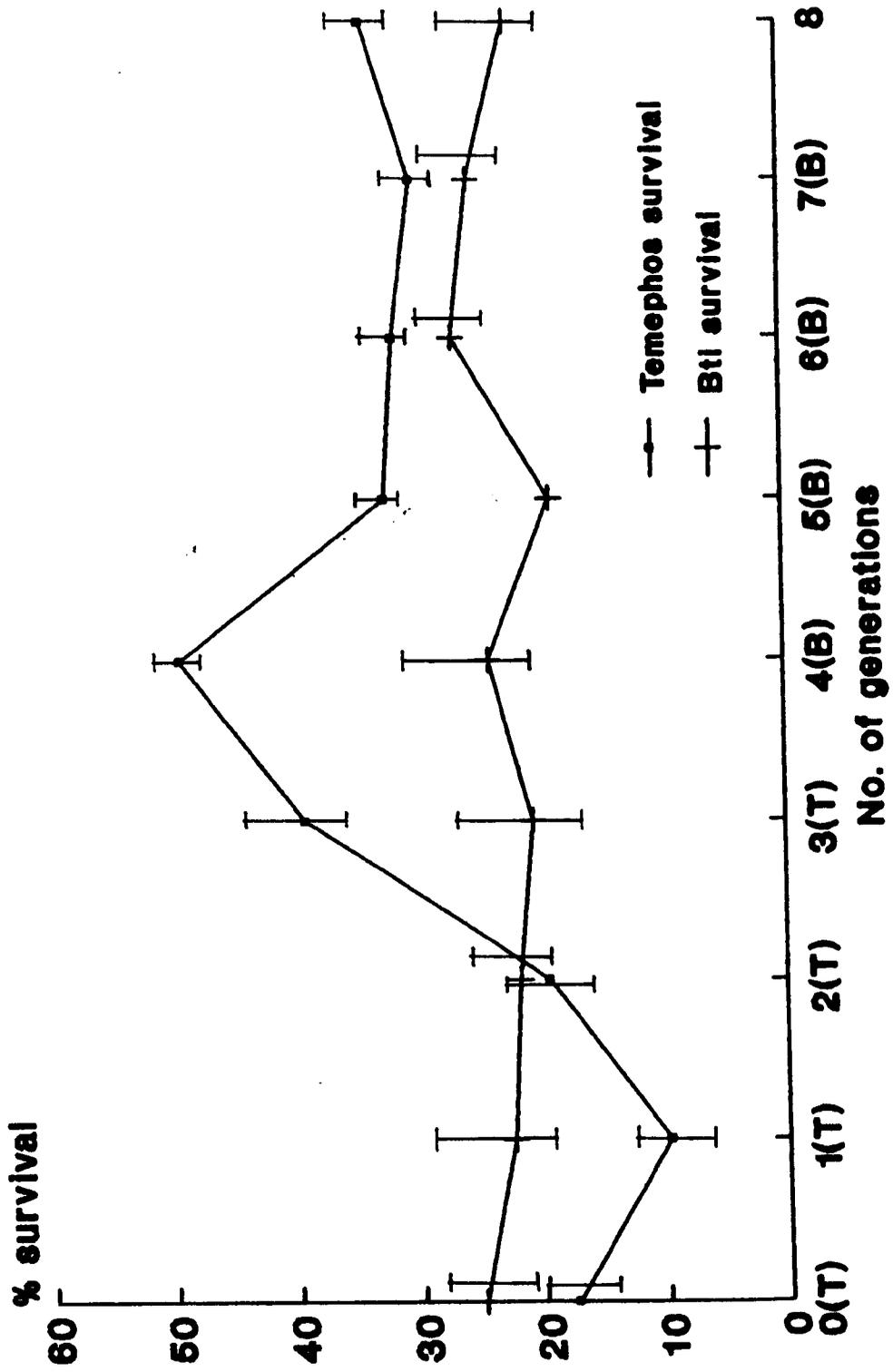
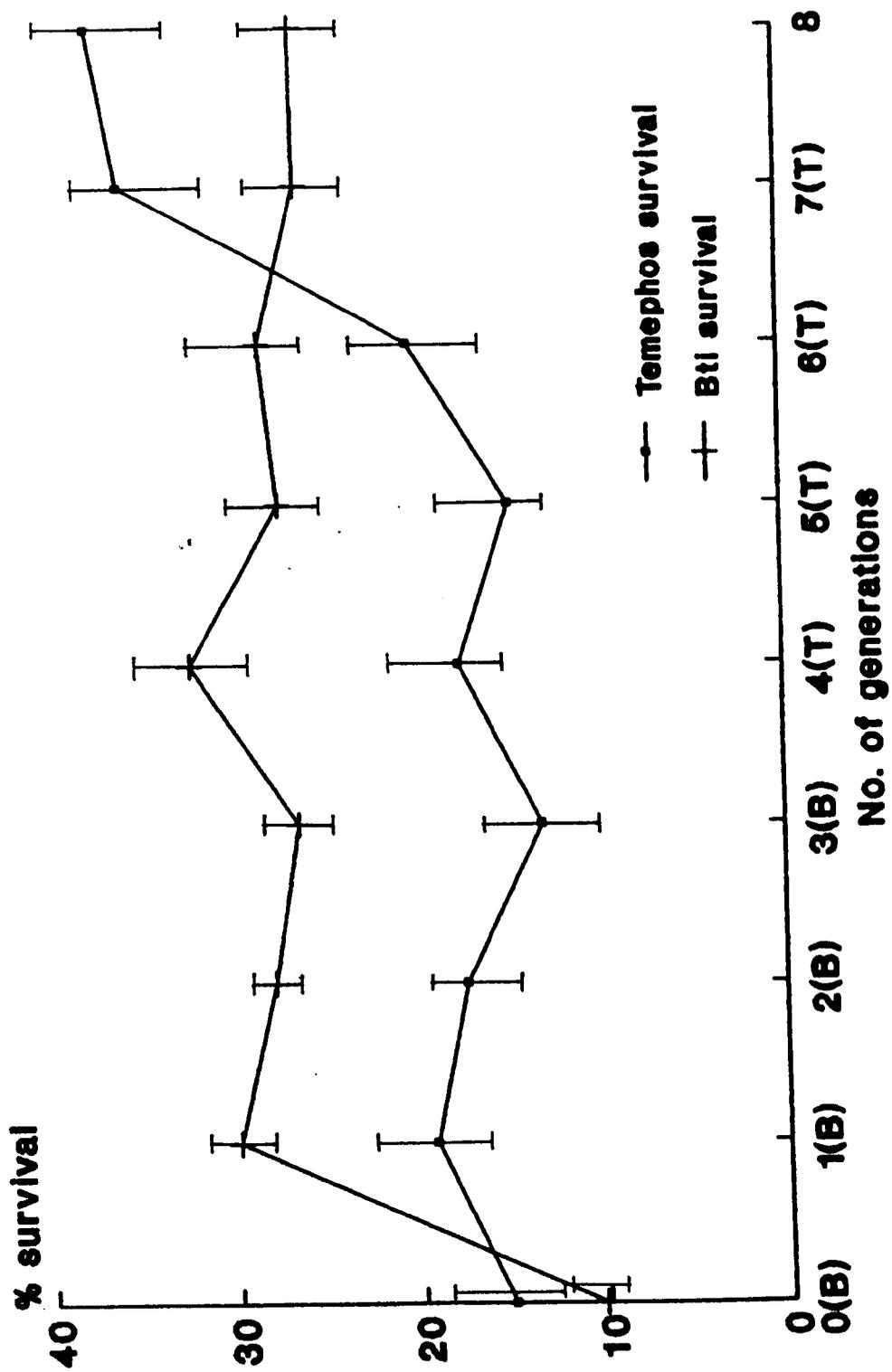


Fig : 11.6
Long term rotation of Btl and temephos
(BBB...TTT) with Cx quinquefasciatus



possible to continue them for 8 generations, but the comparisons which could be made of the two types of rotations showed that neither was consistently more beneficial than the other in terms of the gene frequencies finally reached.

Rotations of Bti and temephos were also investigated in Cx quinquefasciatus larvae but the results are not a full test of the rotation concept where both compounds have a severe risk of selecting for resistance. Georghiou (1990) indicated that true Bti resistance in this species is unlikely and in the present study there was only evidence for a slight rise of Bti tolerance in fig 11.4 and in generation one of fig 11.6. However the final temephos resistance level was much the same whatever type of rotation was used. Chapter 2 contained the suggestion that the risk of temephos resistance in Malaysian Ae. aegypti could be reduced by pre-planned rotation of temephos and Bti but the present 8 generation study on Cx quinquefasciatus gives no support to the idea that it would be better to use such a plan rather than wait for measurable temephos resistance and then switch to Bti. It would seem equally helpful to either strategy if temephos resistance were selected against or "diluted" by immigration in the absence of temephos usage and equally harmful to both strategies if temephos resistance genes are neutral to natural selection or if there is no "diluting" effect of immigration.

CHAPTER 12

ROTATION OF TWO INSECTICIDES AGAINST A POPULATION OF

An. albimanus

12.1 INTRODUCTION

It was the aim of the experiments described in this chapter with cage populations of mosquitoes to carry out a more prolonged study of whether any difference could be found between the end results of different types of insecticide rotation in the laboratory, using the malaria vector An. albimanus from Central America. Dieldrin and propoxur were chosen as two unrelated compounds to which resistances are known in this species (Davidson and Sawyer 1975; Ariaratnam and Georghiou 1975, see also chapter 8 of this thesis).

In order to be likely to observe results in a limited number of generations and in a population of manageable size it was necessary to start the rotations from a population with a low but measurable frequency of the two resistance genes. Such a strain was not available as such but was "synthesised" by crossing a double resistance stock to a double susceptible and then backcrossing seven times to the

susceptible without selection. After this backcrossing process there were several generations of breeding without selection to give an opportunity for recombination of the resistance genes so that approximate linkage equilibrium could be expected at the beginning of selection. The linkage study in chapter 8 showed that no restriction on the recombination process was expected.

Included in the design of this experiment was simulation of the presence of "refugia" (Georghiou and Taylor 1977b) so that selection was not as intense as it can be in the laboratory. It has already been emphasised that natural immigration of unselected individuals from "refugia" into treated areas would be expected to slow the evolution of insecticide resistance. Roush and McKenzie (1987) also noted that immigration could reduce the frequency of resistant individuals, thereby enhancing the success of rotation.

12.2 MATERIALS AND METHODS

12.2.1 MATERIALS

12.2.1.1 Mosquitoes

An. albimanus :

FEST strain : The origin of this strain has been discussed in chapter 8. It has moderate levels of dieldrin and

propoxur resistance with only 20% and 30% mortalities on exposure to the WHO recommended discriminating dosages of 0.4% dieldrin and 0.1% propoxur for one hour, respectively.

PANAMA strain : this is fully susceptible to dieldrin and propoxur. The origin of this strain has been discussed in chapter 8.

12.2.1.2 Insecticides

0.4% dieldrin papers supplied by WHO and 0.1% propoxur papers (impregnated by the experimenter as explained in chapter 8).

12.2.2 METHODS

12.2.2.1 Establishment of An. albimanus colonies for the rotation experiment.

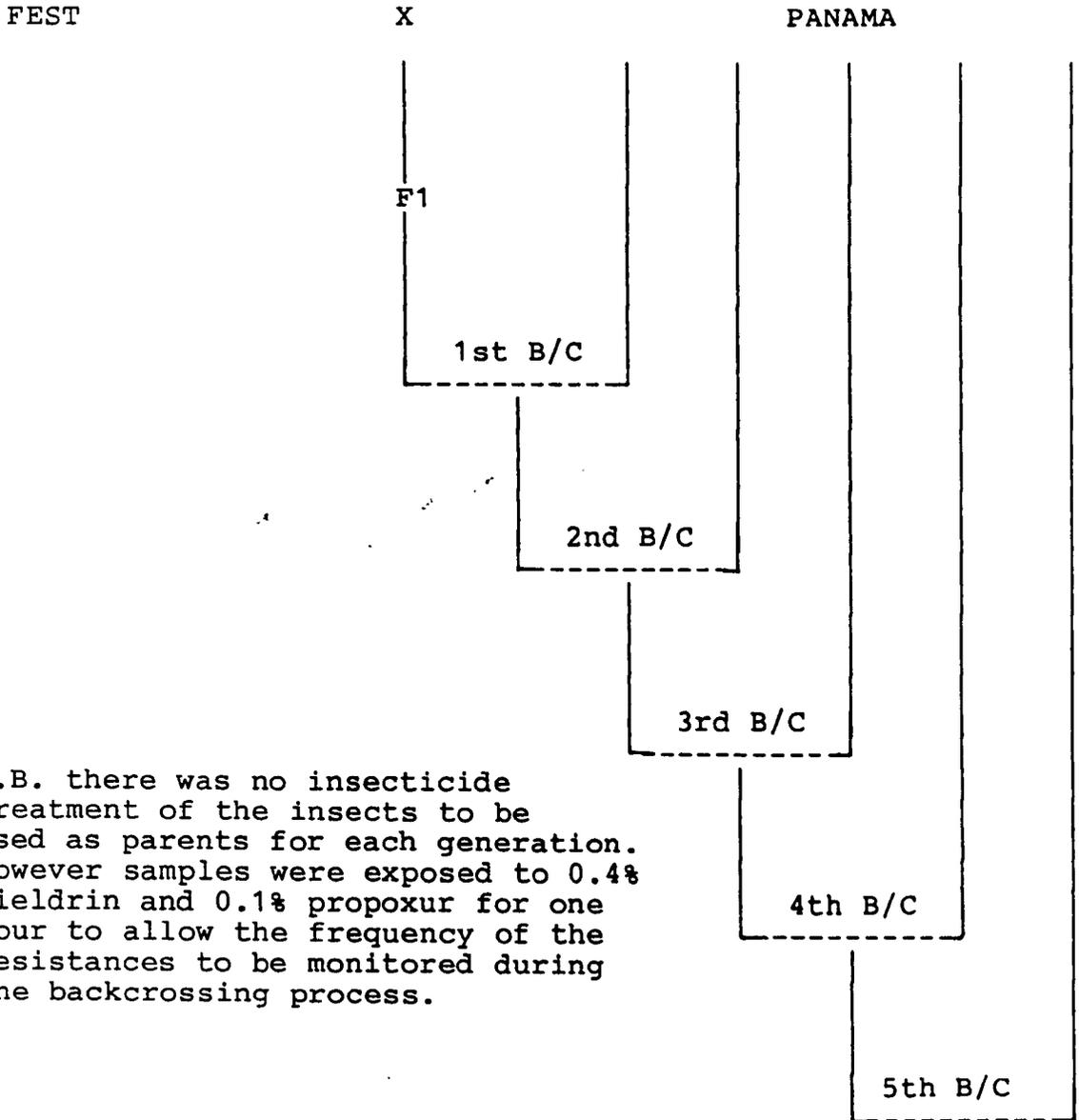
12.2.2.1.1 Backcrossing of FEST X PANAMA strains.

About 100 virgin females of the original FEST stock colony were collected within 24 hours of emergence and transferred to a 20 x 20 x 20 cm cage. About 100 males from the PANAMA susceptible colony were introduced into this cage and mass matings were allowed between them. A blood meal

was offered two days later and, subsequently, twice a week in order to obtain eggs. These eggs were allowed to hatch after 48 hours and larvae were transferred into a plastic bowl 30 cm in diameter. The larvae were fed and allowed to grow into adults. The male and female adults were separated within 24 hours of emergence and were put into separate cages. About 100 virgin F1 females were backcrossed to 100 PANAMA susceptible males. They were allowed to mate and feed in order to obtain eggs for the next generation. Samples of the F1 hybrids were treated with 0.4% dieldrin or 0.1% propoxur for one hour. The mortalities were recorded. The F2 male and female adults were again separated and backcrossed to the PANAMA susceptible males as before. The backcrossing process (without selection) was repeated for 7 generations until the frequency of both resistance genes had been "diluted" to under 5% for both dieldrin and propoxur (Fig. 12.1). When this level was reached the backcrossing process was stopped. The males and females of the population were then allowed to mate among themselves for three generations. This was to try to ensure that the two resistance genes occurred at random with respect to each other (i.e. linkage equilibrium). No treatment was given to this population but a sample of the mosquitoes from each generation, were treated with 0.1% propoxur or 0.4% dieldrin for one hour to determine whether there was any change in the frequency of the resistance genes, since natural

Fig : 12.1

A diagram to show backcrossing to produce a population with a low frequency of each resistance gene.



N.B. there was no insecticide treatment of the insects to be used as parents for each generation. However samples were exposed to 0.4% dieldrin and 0.1% propoxur for one hour to allow the frequency of the resistances to be monitored during the backcrossing process.

and so on until the frequency of both resistance genes reached about 5%.

population allowed to mate among themselves to allow resistance to approach linkage equilibrium.

selection might have favoured the survival of the susceptible individuals.

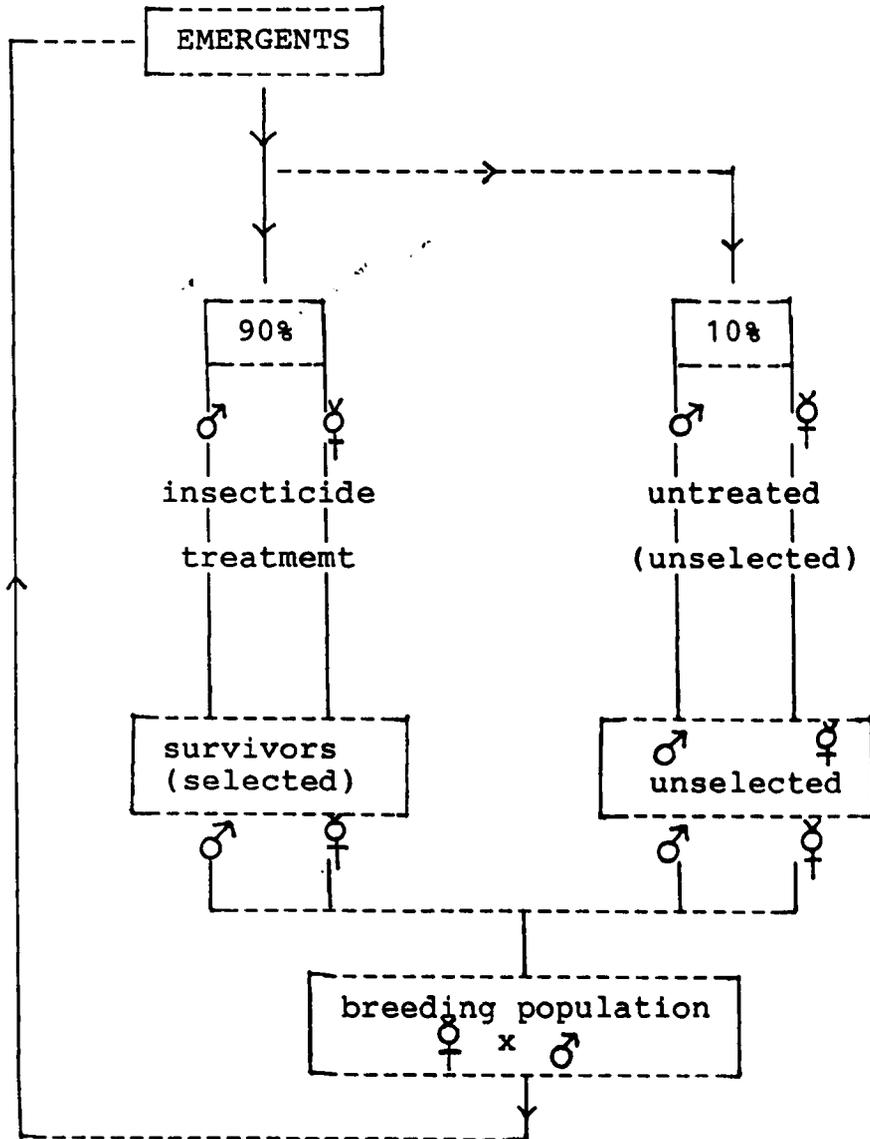
12.2.2.1.2 Long-term "opportunistic" type of rotation

The male and female adults of one sub-colony were counted and separated 24 hours after emergence. In the line to be selected with propoxur about 300 mosquitoes were used for checking the dieldrin resistance level. Of the remainder 90% were treated with 0.1% propoxur for 1 hour at two or three days old. The male and female survivors were then counted and put into different cages marked F1♂ and F1♀. To simulate "refugia" the remaining 10% of the emergents which had not been treated were added to the F1♂ cage and an equivalent addition of unselected females was added to the F1♀ cage (Fig.12.2).

When all adults from one rearing bowl had been collected and handled as described, the F1 males and females were held separately for two or three days more so that the last male to emerge was allowed time to reach maturity so that all had an equal chance of mating. Mass mating was then allowed between the F1 males and females and a blood meal was offered in order to obtain eggs for the next generation. The eggs were allowed to grow into adults as described before.

Figure : 12.2

Diagram of the selection process designed to simulate the situation where part of the population escaped treatment but combined with the survivors of the treatment to form the parents of the next generation (i.e. the existence of "refugia" as defined by Georghiou and Taylor 1977b).



The above method was repeated at each generation until the frequency of the resistance gene reached about 50%, at which point a switch to dieldrin was made, after which dieldrin selection was carried out at each generation.

The whole procedure as described above was repeated but using dieldrin first followed by propoxur.

12.2.2.1.3 Short-term, pre-planned, rotation.

The same method as described above was used in order to observe the effect of a pre-planned programme of rotating propoxur and dieldrin alternately every generation.

12.3 RESULTS

12.3.1 Backcross of FEST X PANAMA

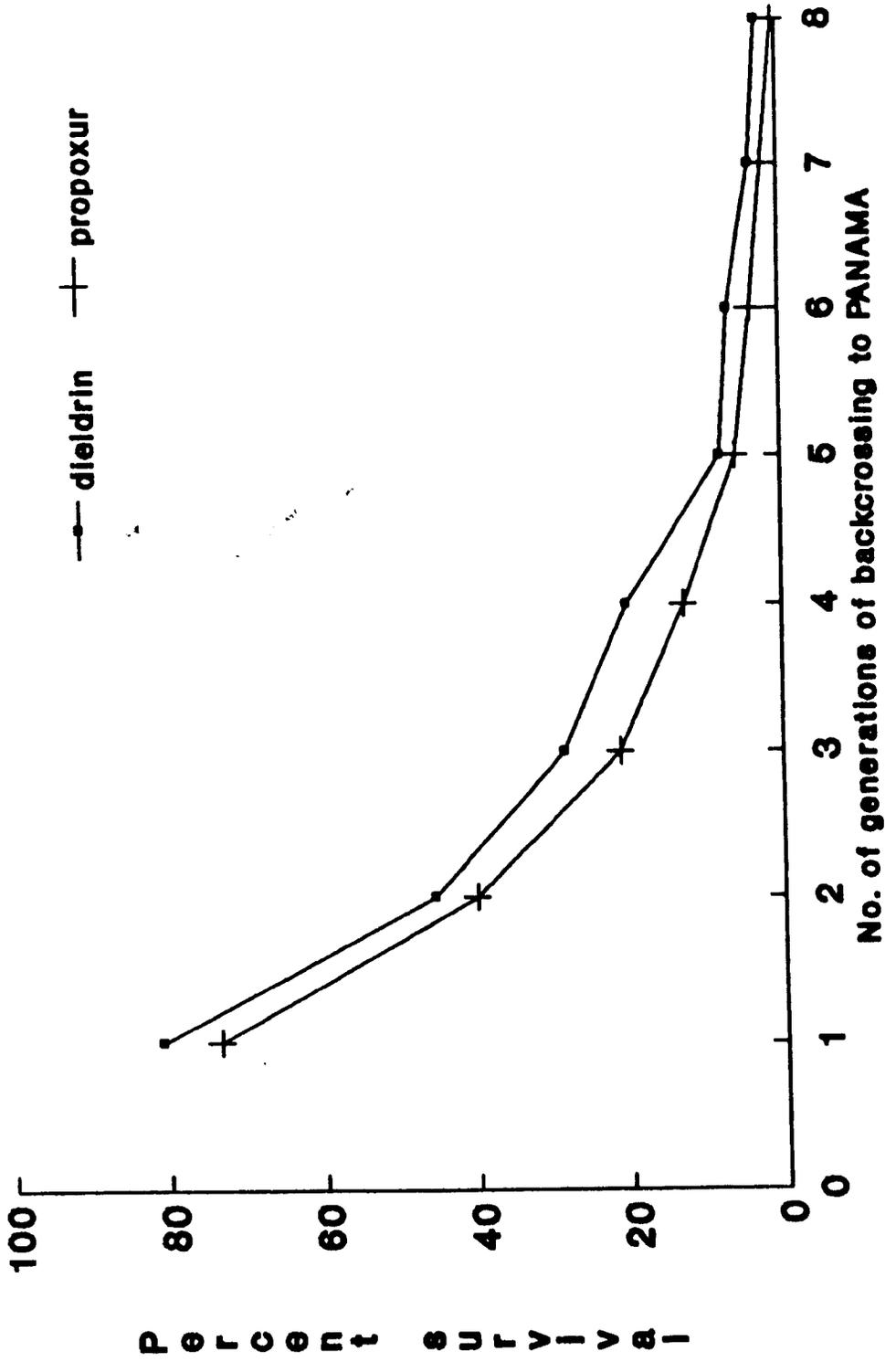
Table 12.1 and Fig. 12.3 show the results of the backcross process. The mortalities on exposure to both compounds increased greatly after the first backcross, from 19% in the F1 to 55% with respect to 0.4% dieldrin. Corresponding figures were 27% and 60% on 0.1% propoxur. It then increased gradually to 97% and 99% (2 survivors from 376 tested) on 0.4% dieldrin and 0.1% propoxur respectively after seven generations of backcrossing. To reduce the risk of losing the rare resistance genes by chance genetic drift,

Table : 12.1

Backcross of FEST x PANAMA strains in preparation for rotation studies. The figures indicate % mortality on exposure of samples of mosquitoes from each generation to 0.4% dieldrin or 0.1 % propoxur for one hour.

No. of generations	0.4% Dieldrin		0.1% Propoxur	
	No. tested	Mortality	No. tested	Mortality
Parental (P)	197	20.1	286	33.9
F1 (FEST X PAN)	198	19.2	128	26.6
1st Backcross (B/C)	403	54.6	401	60.1
2nd (B/C)	305	71.7	496	79.0
3rd (B/C)	437	80.0	315	87.3
4th (B/C)	183	92.2	262	94.3
5th ((B/C)	348	93.4	526	96.4
6th (B/C)	281	96.4	278	97.9
7th (B/C)	246	97.4	376	99.5

Fig: 12.3
[(FEST X PANAMA) X PANAMA] X PANAMA.....
backcross



the populations were maintained at a level of over 1000 females at each generation.

12.3.2 Long-term or "opportunistic" rotation.

The results of the long-term rotations are shown in Figs. 12.4 and 12.5. Dieldrin was used first for 10 generations over which the dieldrin resistance level increased from about 3% to about 50% at F10 (Fig. 12.4). When the switch to propoxur was made the dieldrin level dropped to 37% at F11. However, it picked up again to about 47% at F16 during which propoxur selection continued.

At the same time, the propoxur level in this colony was noted and this was found to remain almost constant as long as propoxur was not in use. Once the propoxur was applied, the propoxur level increased quite steadily from 6% at F10 to about 32% at F16.

Fig. 12.5 shows the long-term rotation where propoxur was used first. The propoxur resistance level seemed to increase more slowly, with 51% survival being reached at F12 when propoxur selection continued but it dropped to 37% at F13 when dieldrin selection started to be applied. However the propoxur survival level had regained its value of 50% by F16. The dieldrin level remained more or less the same (about 5%) when this compound was not in use for selection

Fig : 12.4
Long term rotation of dieldrin and
propoxur (DD...PP) against An. albimanus

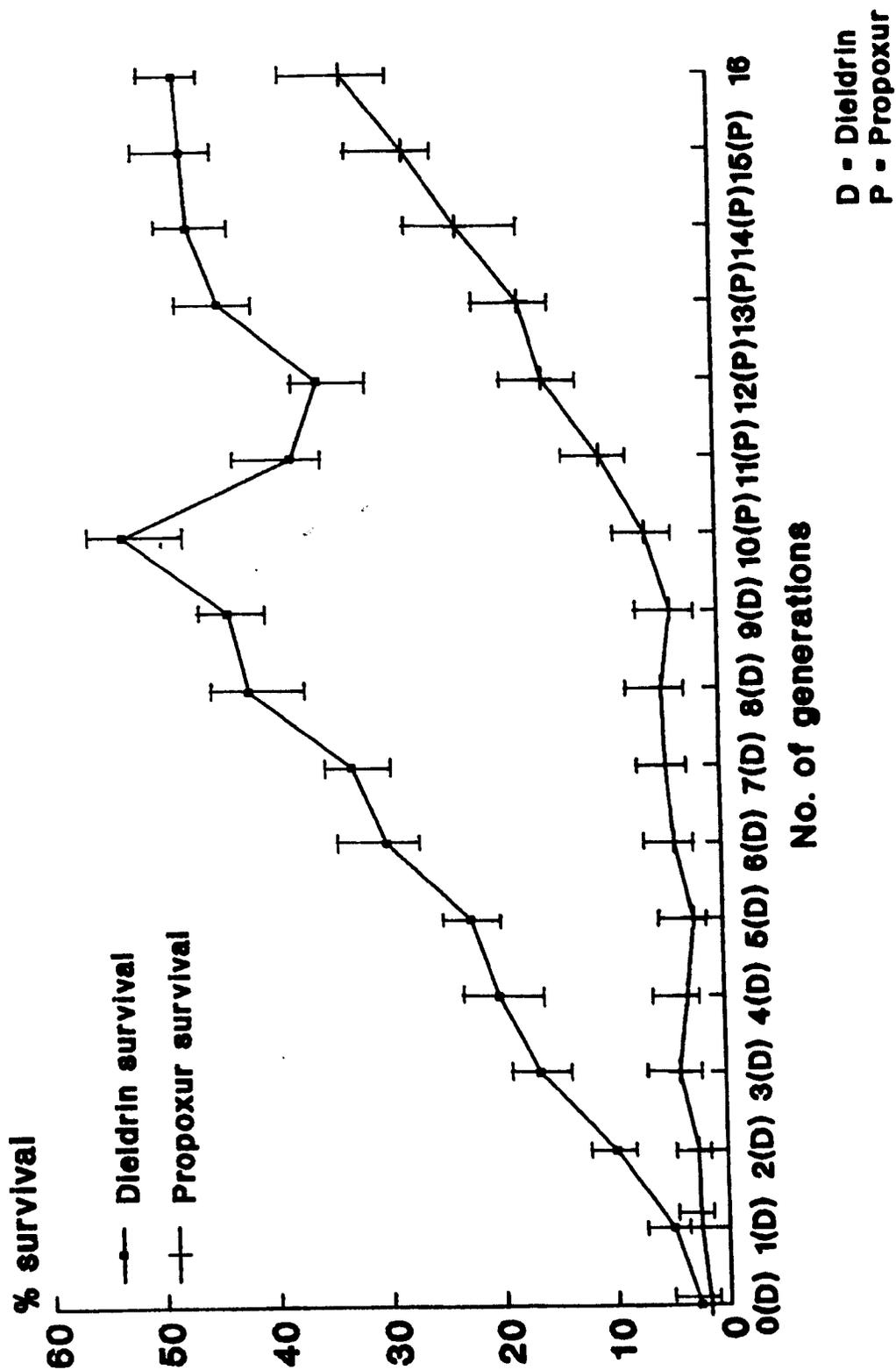
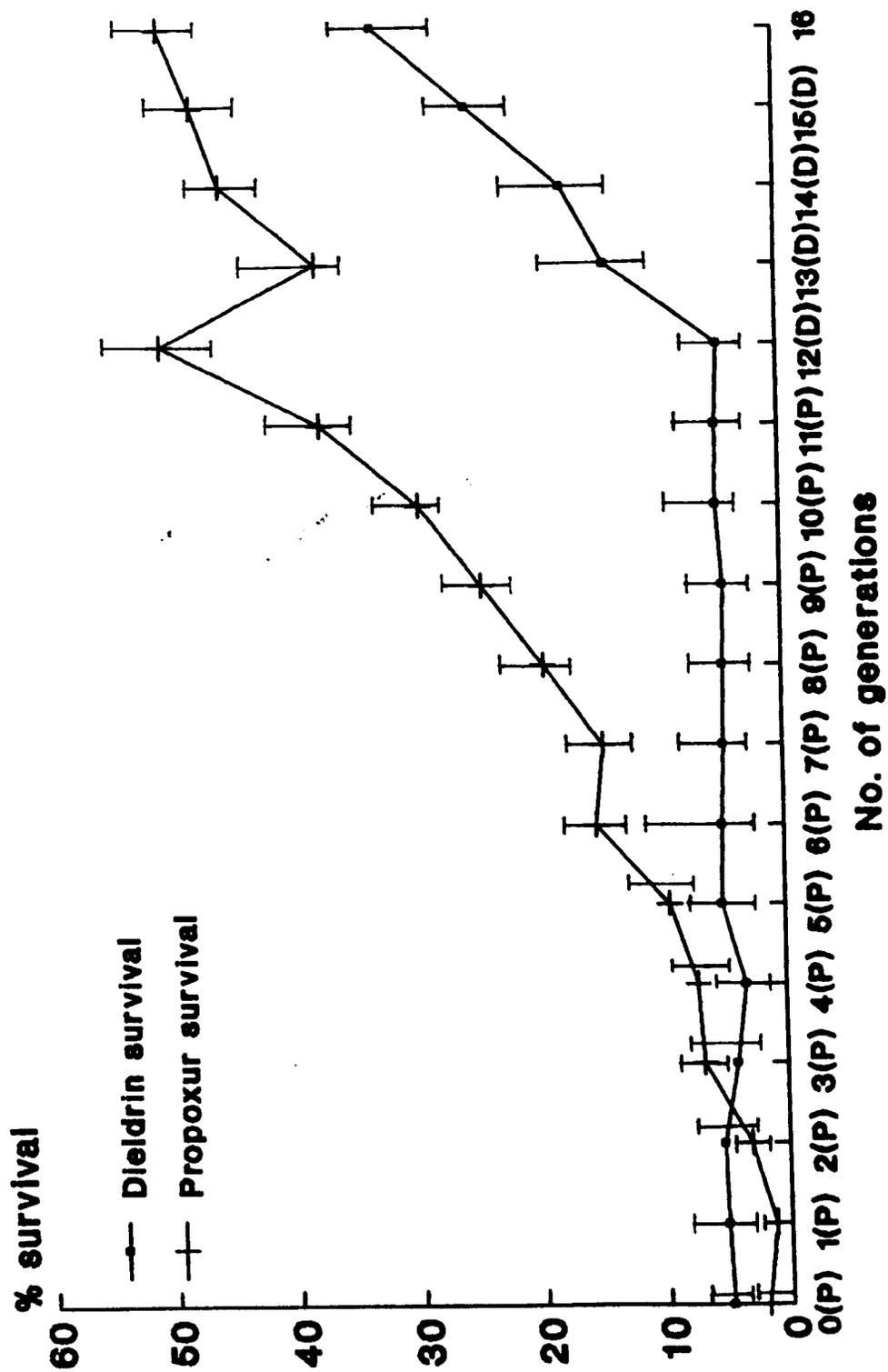


Fig : 12.5
Long term rotation of propoxur and
dieldrin (PP...DD) against An. albimanus



D - Dieldrin & P - Propoxur

but increased from 5% at F12 to 33% at F16 under dieldrin selection.

12.3.3 Short-term, pre-planned, rotation

The results of alternating propoxur and dieldrin for 16 generations are shown in fig. 12.6. Generally the propoxur survival level seemed to increase when it was used for selection and to show no consistent trend when it was out of use. However, the 95% confidence limits of the adjacent generations generally overlapped so one cannot be certain about the details of changes at each generation, but only about the longer term trends. The same trends were observed with dieldrin resistance. The propoxur survival at F16 was found to be 40% and that of dieldrin was 37%.

12.3.4 Simulation of the presence of "refugia" in both types of rotation

Tables, 12.2, 12.3 and 12.4 show the results of adding unselected individuals to the breeding population in numbers equal to 10% of the total submitted to selection. As might be expected the proportion of the breeding population represented by those from the refugia was much larger when few survived the treatment in the early stages of selection than when many survived it towards the end.

Fig : 12.6
Short term rotation of dieldrin and
propoxur against An. albimanus

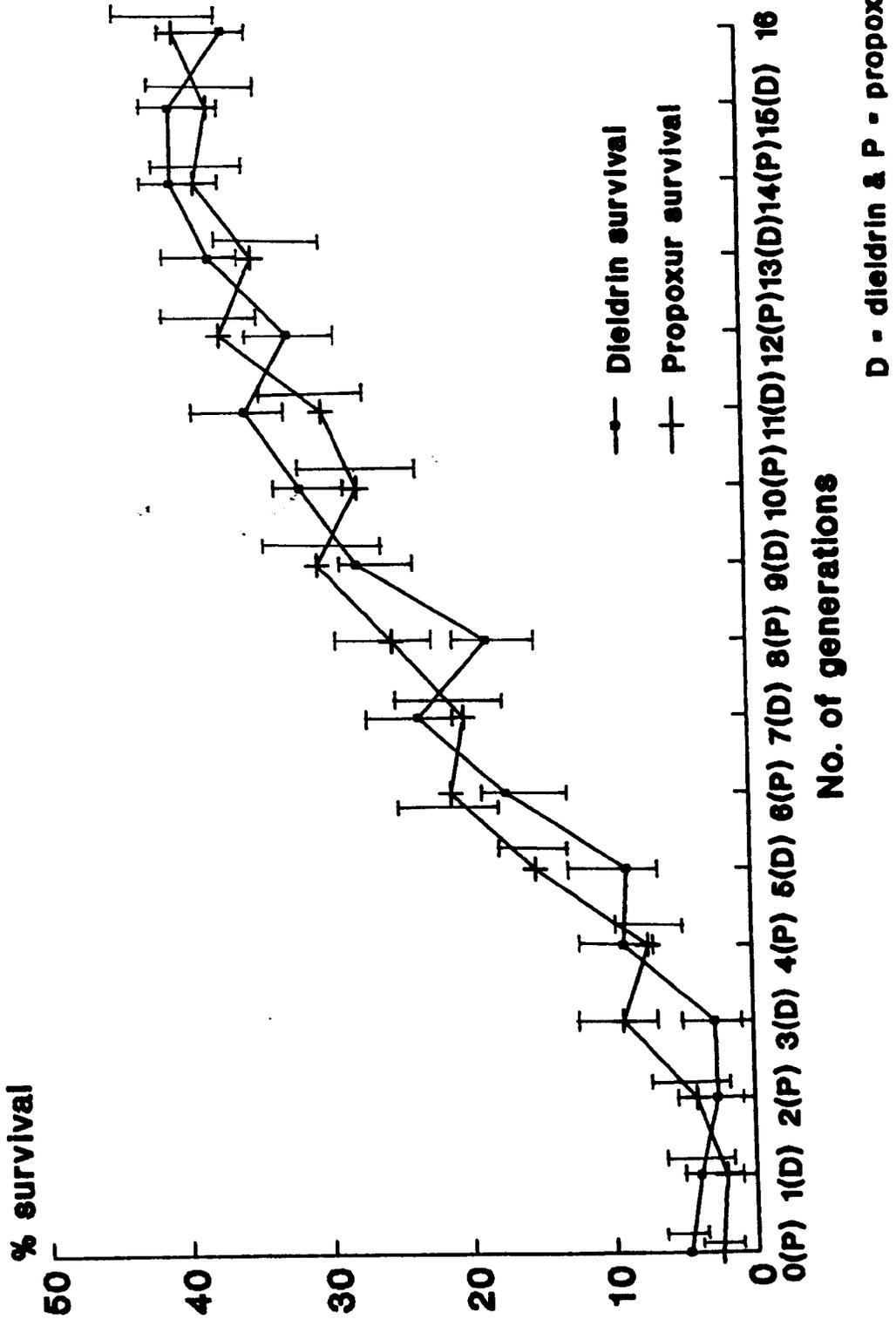


Table: 12.2 Long-term rotation of dieldrin followed by propoxur for 16 generations with 10% of the total number selected, added as unselected individuals to represent those from "refugia".

No. of generations	selecting chemical	Dieldrin tests			Propoxur tests			No. un - selected added
		No. Tested	Morta- lity	No. Survd	No. tested	No. Survd	% Morta- lity	
P	Dieldrin	914	96.5	32	349	8	97.3	91
F1	- Do -	955	95.0	48	373	10	97.3	94
F2	- Do -	906	90.0	91	325	9	97.1	90
F3	- Do -	871	83.5	144	315	13	95.9	87
F4	- Do -	850	79.0	171	295	12	95.9	85
F5	- Do -	884	77.5	199	316	9	97.2	88
F6	- Do -	834	70.5	246	285	13	95.4	83
F7	- Do -	867	68.4	274	279	14	95.0	86
F8	- Do -	753	59.0	309	325	17	94.8	74
F9	- Do -	749	56.5	326	346	14	96.0	74
F10	Propoxur	319	47.3	168	672	37	94.5	67
F11	- Do -	295	62.7	110	734	72	90.0	73
F12	- Do -	325	65.2	113	784	118	84.9	78
F13	- Do -	285	56.1	125	735	127	82.7	72
F14	- Do -	285	54.0	131	746	173	76.8	74
F15	- Do -	291	52.2	139	729	195	73.3	72
F16	-	321	47.0	170	732	239	67.3	73

Table : 12.3 Long-term rotation of propoxur followed by dieldrin for 16 generations with 10% of the total number selected, added as unselected individuals to simulate "refugia".

No. of generations	selecting chemical	Propoxur tests			Dieldrin tests			No. un - selected added
		No. tested	Mortality	No. survd	No. tested	No. survd	% Mortality	
P	Propoxur	962	97.9	20	295	14	95.2	96
F1	- Do -	936	98.5	14	296	15	94.9	93
F2	- Do -	923	96.5	32	301	16	94.7	92
F3	- Do -	914	93.5	59	315	13	95.9	91
F4	- Do -	906	92.3	70	300	10	96.7	90
F5	- Do -	894	90.4	86	305	16	94.8	89
F6	- Do -	875	84.9	132	296	15	94.9	86
F7	- Do -	857	85.1	128	287	14	95.1	85
F8	- Do -	876	80.5	171	312	15	95.2	86
F9	- Do -	835	75.4	205	295	14	95.3	83
F10	- Do -	846	70.4	250	286	15	94.8	84
F11	- Do -	849	62.5	318	276	14	94.9	84
F12	Dieldrin	308	49.7	155	755	38	95.0	75
F13	- Do -	310	62.6	116	763	111	85.5	76
F14	- Do -	311	55.3	139	746	129	82.7	74
F15	- Do -	299	52.2	143	764	189	75.3	75
F16	-	307	50.2	153	754	249	67.0	75

Table : 12.4

Short-term rotation of propoxur and dieldrin every generation for 16 generations with 10% of the total number selected, added as unselected individuals to simulate "refugia".

No. of generations	selecting chemical	Dieldrin tests			Propoxur tests			No. un - selected added
		No. tested	Mortality	No. survd	No. tested	No. survd	% Mortality	
P	Propoxur	249	95.2	12	864	21	97.6	81
F1	Dieldrin	734	96.5	26	235	4	98.3	71
F2	Propoxur	261	97.3	7	824	27	95.5	82
F3	Dieldrin	749	97.1	22	301	27	91.0	74
F4	Propoxur	291	90.4	28	618	47	92.4	61
F5	Dieldrin	753	91.0	68	289	43	85.1	74
F6	Propoxur	254	83.1	43	604	130	78.5	59
F7	Dieldrin	850	76.0	204	262	52	80.2	84
F8	Propoxur	251	81.3	47	888	222	75.0	88
F9	Dieldrin	884	72.5	243	249	75	69.9	87
F10	Propoxur	246	68.7	77	850	234	72.5	84
F11	Dieldrin	834	65.0	292	260	77	70.4	83
F12	Propoxur	298	67.8	96	825	307	62.8	82
F13	Dieldrin	792	62.5	297	300	99	67.0	79
F14	Propoxur	263	60.1	105	695	267	61.6	69
F15	Dieldrin	680	60.0	272	291	107	63.2	68
F16	-	255	63.1	94	720	432	60.0	71

12.4 DISCUSSION

The comparative study carried out on the long-term ("reactive" or "opportunistic") and short-term, pre-planned rotations on An. albimanus for 16 generations indicated similar end results of about 50% survival of exposure to each compound in each case. Knipling and Klassen (1984) and Comins (1986) predicted from two-locus models, assuming no substantial fitness disadvantage and no cross-resistance, that the development a resistance to insecticides used in short-term rotations would not be slower than insecticides used sequentially. Since in the present experiments there was no attempt to bring the first chemicals back into use when resistance to the second reached a high level the long-term (reactive) rotation was equivalent to simple sequential use. Experimental studies by Georghiou et al. (1983) also showed that the rate of increase in resistance per application was similar in sequential and in short-term rotational use.

The tendency in some cases for decline in the resistance levels to either propoxur or dieldrin when the compounds were out of use could be attributed to reduced general fitness of the resistance genes which was reported in the case of the dieldrin resistance in chapter 9. The present selection experiments did not continue long enough for the effects of any co-adaptation of the genetic background on the fitness of resistance to be detectable.

Several theoretical studies have shown that immigration of susceptible individuals into a treated population can have an important impact on the rate of resistance development (Comins 1977a; Georghiou and Taylor 1977a; Taylor and Georghiou 1979) and enhance the benefit of the use of mixture or rotation in managing resistance problems (Curtis 1985, 1987). However, to date, the relative effect of immigration or the presence of "refugia" on various types of rotation versus sequences has not been tested experimentally. In the present study it was found that with the simulation of the presence of "refugia" there was a similar end result irrespective of whether there was a short-term pre-planned rotations or long-term sequential use of propoxur and dieldrin on An. albimanus. As described in chapter 11 a similar experiment on rotation without simulated "refugia" was carried out with Cx. quinquefasciatus and similar conclusions were reached.

CHAPTER 13

GENERAL CONCLUSIONS

Temephos and malathion are the two principal insecticides used to control Ae. aegypti and Ae. albopictus which are the main vectors of DF/DHF in Malaysia. Organophosphate resistance had earlier been reported in Ae. aegypti from Malaysia. However in the present study no resistance was found to malathion and only a very slight response to temephos selection. This suggests that it will be a long time before resistance to these compounds could cause a control failure in the field.

There was no response to selection for resistance to the bacterial agent Bti in either Ae. aegypti or Cx quinquefasciatus.

Malathion resistance was found in a Malaysian strain of Cx quinquefasciatus and this strain responded to selection for temephos resistance. However, this limited level of organophosphate resistance did not prevent the mosquitoes from being killed by malathion and Resigen thermal fogging. However, this fogging did not reach the inner rooms of houses when the sprayman stayed near the front door. Thus at present, poor spraying technique seems a more important obstacle to control than does resistance.

Malaysian Cx quinquefasciatus and Ae. aegypti were used in bioassays of temephos treated water storage containers. Initially all were killed but after several weeks, as the insecticide was diluted, larval survival was observed, first in resistant Cx quinquefasciatus, then in susceptible Cx quinquefasciatus and finally in susceptible Ae. aegypti.

The protective effect of DDT resistance in An. gambiae was tested in simulated mud huts in which the mosquitoes could fly freely and choose their own resting sites. It was concluded that as the deposits aged there was increasing survival of resistant homozygotes and, to a lesser extent, of heterozygotes. The latter survived much better than the susceptible homozygotes and there seems no possibility that one could use a dose which would render the resistance gene effectively recessive. Observations on the blood feeding rate and location of the mosquitoes indicated that DDT resistance did not much affect the feeding rate or tendency of the mosquitoes to be driven out of the hut by the DDT.

Chemical analysis of DDT in the mud bricks of the hut showed that the insecticide penetrated deeply into the mud. Survival of susceptible mosquitoes began when the deposit density in the upper layers of mud dropped below about 2 gm/sq m.

Genetic linkage of resistance genes may be an important

factor in determining the outcome of multiple pesticide use for resistance management. Such linkage is difficult to measure accurately by bioassays with two insecticides and it was shown that a better method is bioassay with one compound and testing the killed and surviving mosquitoes for the enzyme type which causes resistance to the other compound.

Two insecticides might be rotated either in a short term, pre-planned, way or in the longer term, only in response to detection of high levels of resistance. A computer simulation was set up incorporating partly arbitrarily assumed values, but with a value for reduced fitness of the resistance gene and its co-adaptation by modifier genes as reported for Lucilia cuprina. The simulations showed some advantage in adopting the short-term rotation policy as compared to the long term opportunistic rotation.

To test this conclusion with real insects a comparison was made of a long-term, "opportunistic", rotation with a short-term, pre-planned, rotation using caged An. albimanus from El Salvador, with low levels of dieldrin and propoxur resistance. This showed no apparent advantage of one method over the other, as both methods led to about 50% resistance to each compound at almost the same time. The addition of simulated "refugia" to the breeding population did not change this conclusion.

The same results were obtained with permethrin and malathion against adult Cx quinquefasciatus from Malaysia and temephos and Bti against the larvae, though these experiments were not carried out for as long as those with An. albimanus.

Many entomologists believe that short-term, pre-planned rotations, as in the Onchocerciasis Control Programme in West Africa and the Heliothis programme in Australia, have been a success. However, the spraying policies adopted by these programmes, are dictated by seasonal factors only relevant to the particular pest populations concerned.

In the absence of controlled field experiments, success or failure of a resistance management programme cannot at present be attributed to any particular tactic adopted and general recommendations are not yet possible.

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