Genetics and Biochemistry of Insecticide Resistance

in <u>Anopheles</u> <u>stephensi</u>

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By

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Abstract

The genetics and mechanism of DDT resistance were studied in the larvae of the DUB-S strain, a wild strain of _An. stephensi_, originating from Dubai (U. A. E). Crossing experiments suggest that DDT resistance is inherited as an autosomal recessive character. The results from back-crosses, the F2 generations and repeated back-crosses with selection suggest the involvement of more than one genetic factor in DDT resistant larvae. Synergist studies suggest that neither mixed function oxidase nor dehydrochlorinase are involved. Pirimiphos-methyl selection was carried out on the adults and larvae of this strain. Pirimiphos-methyl selection resulted in a modest increase in tolerance of about 3.7-fold in the adults and larvae. The tolerance reverted to susceptibility after a few generations of withdrawing the insecticide. The activity of mixed function oxidases in the selected and parental stock was determined. Piperonyl butoxide (PB), a mixed function oxidase (MFO) inhibitor, produced a continuous antagonism at all the doses tested, suggesting that mixed function oxidases are involved in activation of pirimiphos-methyl to a more toxic compound. Permethrin selection was carried out on the adults and larvae of two sub-strains derived from the DUB-S strain. Eight generations of selection on the adults resulted in an increase in resistance of about 10-fold and resistance to knock-down 7.8-fold compared with the IND-S strain, permethrin susceptible adults and larvae . The selected adults showed a 3.8-fold increase in cross-tolerance to lambdacyhalothrin. Adult selection also increased cross-tolerance in larvae, 36.6-fold that of the IND-S strain and 3.6-fold that of the parental stock. Crossing experiments suggested that adult permethrin resistance was inherited as a polyfactorial partially recessive character with no indication of sex linkage. Relationships between DDT and permethrin resistance and the role of the kdr type resistance mechanism were studied in the permethrin selected larvae. Piperonyl butoxide (PB), a mixed function oxidase inhibitor, and chlorofenethol (DMC), a dehydrochlorinase inhibitor, had no synergistic effects on DDT in DDT resistant larvae, but a synergistic ratio of 1.9 with PB was recorded for permethrin in adult females of the selected line. This indicates that oxidative detoxication of permethrin by mixed function oxidase is not the major resistance mechanism in the adults of the selected line. These results raise the possibility of involvement of a kdr type resistance mechanism as the primary mechanism for permethrin resistance in the adults of _An. stephensi_. Irritability of adult females of the selected strains and parental stocks was determined with permethrin. The stock strains showed no significant differences in their irritability to permethrin, but the selected strains showed significantly reduced irritability to permethrin compared with stock strains. The larvae of the DUB-S strain were highly heterogeneous for permethrin resistance. After only 3 generations of permethrin selection, resistance was fully developed in the larvae 1030-fold compared with the IND-S strain and 138-fold compared with the parental stock. The selected larvae showed cross-resistance to lambdacyhalothrin and deltamethrin. PB produced a strong synergistic effect on permethrin and lambdacyhalothrin in the larvae of selected line. This suggests an oxidase-based resistance mechanism for observed resistance in the larvae. Crossing experiments suggest that larval permethrin resistance is inherited as a monofactorial semidominant character with no indication of sex linkage.

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Chapter 1. Introduction

Malaria is one of the major vector-borne diseases throughout the tropics and sub-tropics, and it has been estimated that, excluding Africa, the number of malaria cases might be of the order of 92 million in each year (WHO, 1986).

Among the vectors, 58 species of Anopheles have been reported to be resistant to one or more insecticides. Most of them are resistant to organochlorines, organophosphates and even in some cases to carbamates and pyrethroids. In Anopheles albimanus and An.sacharovi, resistance has been developed to most insecticides currently available for indoor spraying. Multiple resistance has been developed in a number of species, almost certainly, in part, as a result of widespread use of large quantities of pesticides in agricultural areas. Use of agricultural pesticides presents a serious problem in increasing the resistance in mosquito species.

An.stephensi is known to be a major malaria vector in the Persian Gulf, the Middle-East and Indian subcontinent areas. It has been recorded as one of the five most important malaria vectors in the world (WHO, 1986). As result of the continuous application of insecticides in the Eastern Mediterranean region, 14 species out of 18 have become resistant to one or more insecticides, and in most areas An.stephensi is resistant to DDT, dieldrin and malathion. The insecticides currently used for control of this species are organophosphates applied as residual sprays, or propoxur and pyrethroids as larvicides.

With the wide use of insecticides both in mosquito control and

agricultural control programmes, and the increasing number of cases of resistance, it is necessary to use our available insecticides in the most effective way, both to achieve higher efficiency and to avoid further development of resistance in the field.

Among the large number of resistant species reported, the genetics and mechanisms of resistance in a number of species are still unknown. The purpose of this project was to determine the mode of inheritance and mechanisms of resistance in <u>An. stephensi</u> to a number of insecticides currently used in public health programmes.

Chapter 2: Literature Review.

2.1 History and Classification of Insecticides

The use of chemical, insecticides essentially began with readily available materials such as arsenical compounds, sulphates, petroleum oils and botanical insecticides (nicotine, pyrethrum and rotenone).

The 1930s represent the beginning of the modern era of synthetic organic insecticides, such as dinitro and thiocyanate compounds (see Matsumura, 1975). The development of new insecticides in the past four decades has been rapid. Perhaps the most significant discovery leading to the proliferation of new synthetic insecticides was that of DDT. This insecticide was first synthesized by Zeidler in 1874, but its insecticidal properties were not discovered until 1939 by Paul Muller of Geigy Company in Switzerland. DDT was manufactured in 1943 and soon become the single most widely used insecticide in the world (Cremlyn, 1978).

In this section, insecticides have been classified into four main groups, according to their chemical nature and origins. Particular compounds have been chosen for mention because of their use in public health, notably in mosquito control programmes.

2.1.1 Chlorinated Hydrocarbons

There are three major kinds of chlorinated hydrocarbon insecticides, which are often considered as contact insecticides.

(a) DDT and DDT analogues: The effectiveness of DDT has led to numerous

attempts to synthesize effective analogues, none of which have exceeded the overall value of DDT. The important DDT analogue insecticides include (2,4-dichlorophenoxy) acetic acid (DDD), methoxychlor, dicofol (Kelthane), chlorobenzilate and chlorofenethol (DMC), which has also been used as a dehydrochlorinase inhibitor.

(b) **Benzene hexachloride (BHC):** This insecticide was first prepared in 1825 by Michael Faraday, but its insecticidal properties were not discovered until 1942. BHC was used in malaria control programmes, and in some cases replaced DDT where resistance to DDT appeared.

(c) **Cyclodiene compounds:** These compounds include important insecticides such as chlordane, heptachlor, aldrin, endrin, endosulfan and dieldrin. Among the chlorinated hydrocarbon insecticides, after DDT, dieldrin has been the most extensively used in mosquito control programmes. It is one of the most persistent insecticides, with a long residual effect. Because of the appearance of dieldrin resistance in DDT resistant strains, after a few insecticide applications, the use of this insecticide was abandoned.

2.1.2 Organophosphates

The organophosphorus compounds represent another extremely important and very large class of organic insecticides. Serious investigations into the synthesis of toxic organophosphorous compounds began during the second world war by Gerhard Schrader in Germany. Early examples included in 1941 the powerful insecticide, schradan, which acts as a systemic insecticide; tetraethyl pyrophosphate (TEPP) in 1942, the first widely marketed organophosphate insecticide; and then in 1944 parathion.

Many OP compounds are excellent inhibitors of cholinesterases. OP compounds are currently used as stomach and contact poisons, fumigants, and systemic insecticides, for nearly every type of insect.

The organophosphorous compound can be divided into five classes according to their phosphorous moiety (Eto, 1974). Among them, two classes, phosphorothionates and phosphorothiolothionate esters contain important insecticides which have been widely used in mosquito control programmes.

Phosphorothionates: These compounds have been one of the most important classes of organophosphorus insecticides since the discovery of parathion. Many compounds in this class have been developed into commercial insecticides. These compounds have no anticholinesterase activity, until bioactivated in the body. The important compounds in this class are as follows:

(a)Fenitrothion (Sumithion): Fenitrothion was introduced in 1959 by Sumitomo Chemical Company as an experimental insecticide, then became a widely used insecticide for the control of flies and mosquitoes.

(b)Fenthion (Baytex): Fenthion was developed in 1958 by Bayer. It is a highly persistent insecticide.

(c)Temephos (Abate): Temephos was introduced in 1965 by American Cyanamid Company as a mosquito larvicide. This insecticide has been widely used as a larvicide in mosquito control programmes.

(d)Chloropyrifos (Dursban): Dursban was discovered by Dow Chemical Company in 1965 as a larvicide with a moderately persistent property.

(e)Pirimiphos-methyl (Actellic): Pirimiphos-methyl was introduced by

ICI in 1972. It is a contact insecticide with some fumigant activity. This insecticide has been used as a larvicide and adulticide.

Phosphorothiolothionates: This class includes many useful insecticides, particularly for use in agricultural pest control. These compounds also have no anticholinesterase activity, until bioactivated. Malathion, one of the most important insecticides in this class, was introduced in 1950 by American Cyanamid Company. Malathion is a safe insecticide with a low mammalian toxicity and a high insecticidal activity. It has been used on a large scale in malaria control programmes in different areas.

2.1.3 Carbamate Insecticides

The carbamate esters were first discovered by Geigy Company in Switzerland in 1947, although the most generally effective members of the group, such as carbaryl and sevin, were not introduced until about a decade later.

Propoxur (Baygon) is one of the most important members of this group. It is a contact insecticide with fumigant activity and a long residual life. This insecticide has been widely used in malaria control programmes, particularly where resistance to DDT and organophosphorous insecticides has appeared.

2.1.4 Pyrethroid Insecticides

Pyrethrum is a contact insecticide obtained from the flower heads of <u>Chrysanthemum cinerariaefolium</u>. The time and place of discovery of the insecticidal activity of pyrethrum are unknown. It is likely that

it was discovered in the Caucasus-Iran region of Asia, the region between the Black and Caspian seas, and in Dalmatia, now part of the Adriatic coast of Yugoslavia, where C. cinerariaefolium is a native plant.

The elucidation of the structures of the pyrethrins was a process which continued over a period of about 60 years. The active insecticidal constituents of pyrethrum extract are known collectively as the pyrethrins. They are esters of two carboxylic acids, chrysanthemic and pyrethric acids, and three cyclopentenolones; pyrethrolone, cinerolone and jasmolone. In naming these six types of esters the alcohol component is distinguished by the name and the acid by the number. Among the six esters, pyrethrins 1 and 2 have the most insecticidal activity. Pyrethrin 1 is generally considered to be the most potent, while pyrethrin 2 has the greatest knockdown activity (see Leahey, 1985).

Synthetic pyrethroids

The synthesis of chrysanthemic acid by Campbell and Harper (1945) and cyclopentenolones by Schecter et al., 1949, led to the synthesis of allethrin, the first man-made pyrethroid, by esterification of synthetic chrysanthemic acid with allethrolone (Schecter et al., 1949). Allethrin had just 40% of the activity of pyrethrins in synergised formulations. Subsequently bioallethrin and S-bioallethrin were produced; they also had poorer kill but greater knock-down activity than the pyrethrins (Gersdorff & Mitlin, 1953). The first synthetic pyrethroids to show higher or equal killing activity to pyrethrins were resmethrin and bioresmethrin, which were developed by Elliott et al.

(1967). Higher activity, but with a higher mammalian toxicity, was obtained with the development of K-othrin by Martel *et al.* in 1971 (see Malcolm, 1981). All of the above mentioned synthetic pyrethroids show marked instability in air and sunlight. These are characteristics of natural pyrethrins. Further investigation led to the discovery of some photostable pyrethroids, such as permethrin and decamethrin. Permethrin showed much more stability to light than the previous synthesised pyrethroids, having activity similar to, or greater than bioresmethrin, and possessing low mammalian toxicity (Elliot *et al.*, 1973). Decamethrin, now renamed deltamethrin, is another photostable pyrethroid with higher insecticidal activity and higher mammalian toxicity when compared with permethrin (Elliot *et al.*, 1974). Both insecticides have been commercially produced.

Pyrethroid insecticides are receiving increased attention for use in public health programmes. The most important advantages of these insecticides are their rapid action and high activity against a wide range of species. They also have low mammalian toxicity and resemble the natural pyrethrins, being easily biodegraded to a harmless product, and they do not accumulate in biological systems. Pyrethroids have also low volatility and low polarity, properties which restrict their movement in the air or soil from the site of application .

2.2. History and Development of Resistance in Mosquitoes

The first case of resistance was recorded in 1908, in the plant scale insect, <u>Aspidiotus perniciosus,</u> towards lime sulphur (see Bishop

& Cook, 1981). About 30 years later, after the discovery and application of DDT, the first insecticide to be used worldwide, a substantial rise in the number of resistant species became apparent. Following the development of WHO standard tests for elucidation of resistance in various vectors, the number of confirmed cases of resistance has increased.

This section reviews the development of resistance in mosquitoes to the four main groups of insecticides, according to their application in the field, based on reports in the WHO Technical Report Series.

The first evidence of resistance in mosquito species to a compound used in their control in the field was observed in Aedes taeniorhynchus, Ae. sollicitans, and Culex pipiens in 1947, and in An. sacharovi in 1951 with DDT (WHO, 1963; Brown, 1986).

Until 1953, about 9 species of mosquitoes (2 anophelines and 7 culicines) had developed resistance to either DDT or dieldrin (WHO, 1963).

By 1957, the total number of cases of resistance in mosquitoes to one or more insecticides was reported in 16 species (8 anophelines and 8 culicines), to DDT or dieldrin/BHC (WHO, 1963). Evidence of resistance to an OP compound (malathion) was observed in Culex tarsalis near Fresno, California in 1956 (Brown, 1986).

By 1962, among the anopheline mosquitoes, 32 species had developed resistance to 1 or more insecticides; 30 species had developed resistance to dieldrin/BHC, and 12 to DDT. Among culicine mosquitoes, 17 species had developed resistance to one or more insecticides, 10 species to dieldrin/BHC, 15 to DDT, and 3 to organophosphorus compounds (WHO, 1963).

By 1968, in anopheline mosquitoes, 38 species had developed resistance to one or more insecticides, 36 to dieldrin/BHC, 15 to DDT, and an increase in tolerance to malathion in An. albimanus. Among the culicine mosquitoes, a total of 19 species had developed resistance to one or more insecticides, 16 species to DDT, 12 to dieldrin/BHC and 9 to organophosphates (WHO, 1970).

By 1975 a total of 42 anopheline species had developed resistance. 24 were resistant to DDT, 41 to dieldrin/BHC and 6 to organophosphorus insecticides. Among the organophosphorus resistance cases, An. albimanus and An. sacharovi showed multiple resistance to OP and carbamate compounds, and resistance to one or more insecticides was reported for most species. In culicine mosquitoes, as with anophelines, there had been a large increase in the number of cases of insecticide resistance, compared with 1968. Among the 41 species exhibiting resistance, 35 were resistant to DDT, 26 to dieldrin, and 16 to organophosphorus and carbamate compounds. Resistance to bioresmethrin, a pyrethroid compound, was reported in Ae. aegypti in Thailand after a short period of insecticide application (WHO, 1976).

By 1980, among anopheline mosquitoes, 51 species were reported to be resistant to one or more insecticides, 34 were resistant to DDT, 47 to dieldrin and 30 to both DDT and dieldrin. Organophosphate resistance was recorded in 10 species and resistance to carbamates in 4 species. Resistance to pyrethroids was not recorded, but permethrin resistance was selected out in the laboratory in a number of strains. In culicine mosquitoes, 42 species had been reported to be resistant to one or more insecticides. Of these, 37 were resistant to DDT, 27 to dieldrin/BHC, 23 to organophosphates and one to pyrethroid insecticides. (WHO, 1980).

Table 2.1 Development of resistance to four main groups of insecticides in mosquitoes during the period 1953-1985.

Years	Total No resistance		Chlorinated DDT		Hydrocarbon BHC/ DLD		OPs		Carbamates		Pyrethroids	
	Cu	An	Cu	An	Cu	An	Cu	An	Cu	An	Cu	An
Up to 1953	7	2	7	1	2	0	0	0	0	0	0	0
1957	8	8	7	4	0	6	1	0	0	0	0	0
1962	17	32	15	12	10	30	3	0	0	0	0	0
1968	19	38	16	15	12	36	9	1	0	0	0	0
1975	41	42	35	24	26	41	16	6	2	2	1	0
1980	42	51	37	34	27	47	23	10	2	4	1	1
1985	45	58	39	56	31	50	28	31	5	14	2	2

An = Anopheline mosquitoes, Cu = Culicine mosquitoes, DLD = dieldrin,

Fig 2.1 Development of resistance to four main groups of insecticides

in mosquitoes during the period 1953-1985.
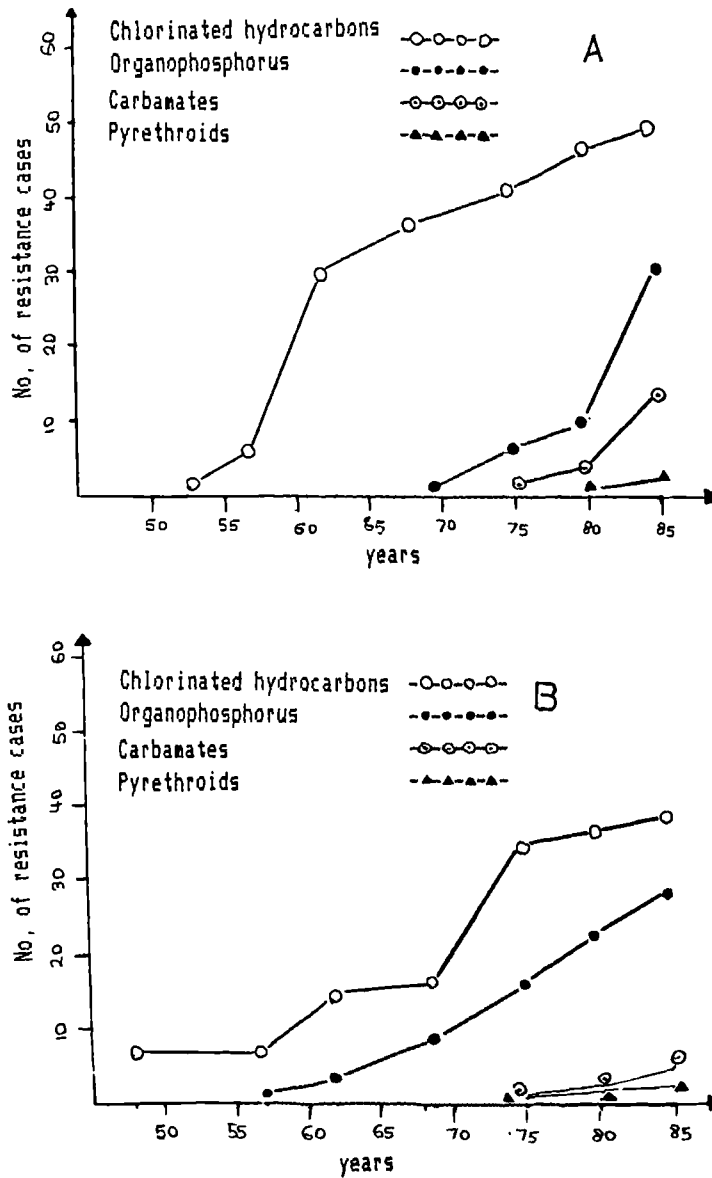
A = Anopheline mosquitoes

B = Culicine mosquitoes

By 1985, among the anophelines, 58 species had developed resistance to different insecticides. Of these, 56 species had developed DDT resistance, 50 species developed resistance to dieldrin/BHC, 31 to OP compounds, 14 to carbamates, often as a direct result of the use of carbamates in agriculture, and 10 species to pyrethroids. In culicines. a total of 45 species had developed resistance to one or more insecticides; 39 species were recorded as resistant to DDT, 31 to dieldrin, 28 to OP, 5 to carbamates, and two species to pyrethroids (Brown, 1986; WHO, 1985). The development of resistance to four main groups of insecticides are shown in table 2.1 and Fig 2.1.

In short, among the anophelines, a great increase in the number of species resistant to chlorinated hydrocarbon insecticides, from 8 to 32, was recorded between 1957 and 1962. Similar changes with respect to OP compounds were recorded some twenty years later (from 1980 to 1985), from 10 to 31 species.

In culicines, between 1968 and 1975, the major changes observed were increases from 19 to 41 in the number of resistant species, and from 37 to 80 in the total number of cases of resistance to both chlorinated hydrocarbon and OP compounds. During this time similar changes were reported among agricultural pests.

Pyrethroid and carbamate resistance in the anopheline and culicine mosquitoes is still at low level, probably because of the limited use of these insecticides in mosquito control programmes (see table 2.1 and Fig 2.1).

Brown, (1986) and WHO, (1985) quote 10 cases of pyrethroid resistance

in *Anopheles*, whereas pyrethroid resistance has so far been reported in only two species in the field (see Malcolm, 1988).

2.3 Factors Influencing Development of Resistance

Development of resistance in the field is multidimensional. It depends on the interaction of several important factors, such as ecological and biological factors, species, population, type of insecticide and treatment, selection pressure, use of agricultural pesticides, and genetic factors (frequency of resistance genes and their dominance). Therefore, investigations on the development of resistance, should ideally take account of all these factors.

The four main types of insecticide differ in the speed with which resistance develops. In principle, insecticides with a long residual activity, for example in the form of wall deposit, exert selection for a long time. By contrast, with non-residual applications, such as space-spraying, resistance takes longer to emerge.

It is often assumed that more intense selection pressure will cause more rapid development of resistance, provided that the number of survivors is large enough to maintain genetic variability. When resistance is due to one gene, higher selection pressure will normally cause a rapid development of resistance. A moderate level of mortality (50-75%), subsequently raised to higher levels, may be the most favourable for the development of resistance, particularly when resistance is dependent on the interaction of two or more factors. An increase in the frequency of insecticide application, and total coverage of areas with insecticide, could select resistance gene(s) faster (WHO,

1980).

The application of larvicides is more liable to induce resistance in the field than adulticides. An. sacharovi and An. sundaicus were among the earliest anophelines to develop DDT resistance, as the result of larvicide applied by aircraft (see Brown & Pal, 1971). The most severe problems of resistance have developed in agricultural areas where large volumes of OPs such as parathion-methyl, and certain carbamates, such as carbaryl and propoxur, are applied to crops; for example, the development of resistance to carbamate and OP compounds in An. sacharovi in Turkey and in An. albimanus in El Salvador, resulting from the use of agricultural insecticides in the field (Brown, 1986).

Some species have been extremely slow to develop resistance. This might suggest that a principal gene for resistance is absent, or its frequency in the population is very low. On the other hand, if the genetic potential for development of resistance to a given insecticide is present, selection will ultimately lead to the development of resistance. For some time it was thought that DDT resistance might not develop in An. culicifacies in India. In fact, the first sign of resistance was observed after 11-12 years of spraying (Rahman et al., 1959). Resistance in this species is now widespread from Burma to Iran. In An. stephensi from southern Iran, DDT resistance was detected after 5-6 years, dieldrin resistance after 1-2 years, and malathion resistance 9-10 years after the commencement of residual spraying (Manouchehri et al., 1976; Zaim, 1987). However, An. superpictus and An. dthali (the second malaria vector in southern Iran) are both still susceptible to all types of insecticides, and yet have long histories of insecticide application, including DDT, dieldrin, malathion and propoxur (Zaim, 1987). Behaviouristic

resistance, "the increased ability to escape from sprayed houses", and exophilic habit, are the most important characters which could prevent the development of resistance in the mosquitoes.

The factors influencing development of resistance to insecticides in a population have been classified by Georghiou & Taylor (1977) and WHO (1980) as follows.

(a) Genetical factors

Presence of resistance (R) genes and ancillary genes (genetic potential); frequency of R genes; number and combination of R genes; the degree of resistance due to an R gene or combination of R genes; dominance or recessiveness of R genes; fitness of R genotypes.

(b) Operational factors

Selection pressure; Stages exposed; Insecticide used; Type and background of insecticide application.

(c) Biological and ecological factors

Generations per year; Relative isolation of the population; Size; Growth-rate and breeding; Structure of the population; Variance of ecological conditions.

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2.4 Genetics and Biochemistry of Insecticide Resistance

Organophosphorus and carbamate compounds are known to act by inhibiting acetylcholinesterase, whereas pyrethroids and organochlorine insecticides act on the nervous system. After the penetration of insecticide into the pest, subsequent biological modification of the

compound results in a great change in the toxicity of the insecticide.

According to the mode of entry of insecticides into the body, an insecticide must first pass through the natural barriers and finally reach the target site. The exact mechanism of penetration of insecticide through the cuticle is still debated (Devonshire, 1973), but there is considerable evidence that the insecticide moves from the cuticle to the nervous tissue via the tracheal system (Burt, 1970). The insecticides may accumulate in non-sensitive tissues or be excreted, but biochemical mechanisms of resistance rely on the metabolism of the insecticide in the body. Only a small proportion of the insecticide applied to the surface of an insect need reach the target site in order to kill the insect. Therefore a small change in the complex interaction between penetration, detoxication, activation, excretion and inert storage could have a large effect on the toxicity of the insecticide.

2.4.1 DDT

2.4.1.1 Mode of Action and Metabolism

Although large quantities of DDT have been used since the second world war, comparatively little is known about its precise mode of action. DDT acts on the nervous system and produces toxic effects in nervous tissue at a much lower concentration than that needed to induce toxic effects in other tissues and enzyme systems. DDT apparently exerts its toxicity by binding to the nerve membrane and interfering with the transmission of nervous impulses, possibly by upsetting the sodium or potassium ion balance across nerve membranes.

The metabolism of DDT occurs by a number of different pathways, but

the most important in insects appears to be the dehydrochlorination of DDT to dichloroethylene (DDE) by the non-microsomal enzyme, DDT-dehydrochlorinase.

DDE is a highly persistent metabolite, with slight insecticide activity, but it is a major environmental pollutant. The metabolic conversion of DDT to DDE is the major detoxication mechanism in a number of DDT resistant strains, in adults as well as in larvae (see Brown and Pal, 1971). Chlorofenethol (DMC) and WARF (N-di-n-butyl-p-chloro-benzene sulphonamide) act as DDT synergists against DDT resistance strains. Such compounds inhibit the in vivo activity of the dehydrochlorinase.
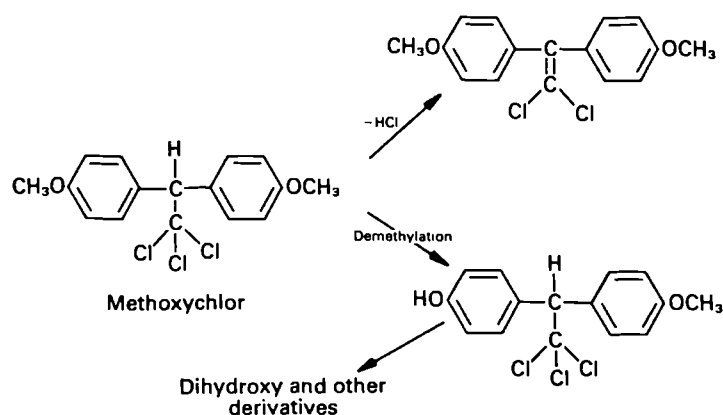
(From Hassal, 1982)

Fig 2.2 Metabolism of methoxychlor

Dehydroxylation of DDT to dicofol by microsomal enzymes is another metabolic pathway, which has rarely been identified in DDT resistance strains. In houseflies it has been shown that microsomal oxidation is involved in the metabolism of DDT, but the products were not identified (see Devonshire, 1973).

(a) p,p'-DDT

(b) Methoxychlor

(c) Dicofol
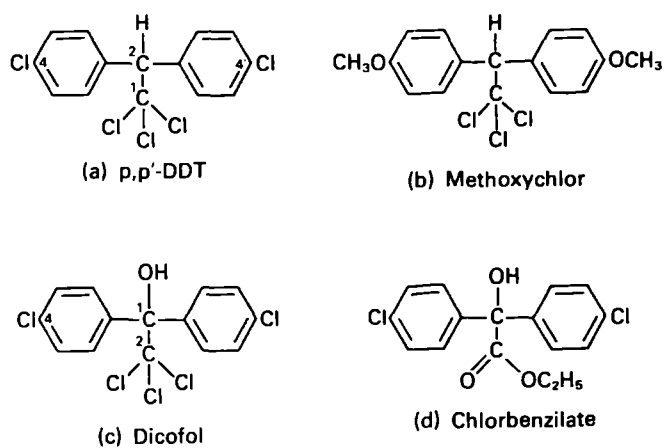
(d) Chlorbenzilate

(From Hassal, 1982)

Fig 2.3 DDT and its analogues. ((a) 1,1,1-trichloro-2,2-bis(4'-chlorophenyl) ethane; (b) 1,1,1-trichloro-2,2-bis(4'-methoxyphenyl)ethane; (c) 2,2,2-trichloro-1, 1-bis(4'-chlorophenyl)ethanol; (d) ethyl p,p'-dichloro-benzilate)

2.4.1.2 Genetics of DDT Resistance in An. stephensi

An. stephensi is a major malaria vector in the Persian Gulf area, the Indian subcontinent and the Middle-East. The first evidence of resistance to DDT was reported in 1955 in Saudi Arabia, and it subsequently appeared in Iraq and Iran in 1957 (Brown & Pal, 1971). By 1963, DDT resistance was distributed in India, Pakistan and Afghanistan (Davidson & Mason, 1963). In spite of the development of DDT resistance in a number of anopheline mosquitoes in different areas, DDT is still widely used as a residual insecticide for malaria control and in eradication programmes in the developing world (Brown, 1986).

Adults of An. stephensi from Iraq, which were resistant to DDT, showed cross-resistance to DDT analogues but were susceptible to dieldrin. Crosses between this resistant strain and an Indian susceptible strain indicated that a major gene was involved, and that resistance was inherited as a partially recessive character. In DDT resistant larvae a major gene was also involved and resistance behaved as an intermediate character, suggesting that DDT resistance in adults and larvae was dependent for its expression on the genetic background (Davidson & Jackson, 1961). In strains which were doubly resistant to DDT and dieldrin, expression of DDT resistance proved to be almost semi-dominant, while in strains resistant to DDT alone, it was found to vary from recessive to partially recessive (Davidson & Jackson, 1961).

The mechanism of DDT resistance has been studied in different strains of An. stephensi. In a DDT resistant strain from Iraq, less DDT was converted to DDE than in the unselected Delhi strain, and the presence of DDT dehydrochlorinase could not be demonstrated. WARF (N-di-n-butyl-p-chloro-benzene sulphonamide), a dehydrochlorinase inhibitor, and

piperonyl butoxide, a dehydroxylation inhibitor, did not make the resistant strain much more susceptible (see Brown & Pal, 1971).

In a strain of An. stephensi from Pakistan, DDT selection on larvae with 8-fold resistance to DDT, produced 144-fold resistance to DDT, and 12 and 18-fold resistance to trans and cis permethrin respectively after 6 generations of selection compared with parental stock. DMC and piperonyl butoxide had little or no synergistic effect on the DDT or pyrethroid in resistant larvae. This suggested that DDT and pyrethroid resistance was due to non-metabolic factors, and it was postulated that reduced sensitivity of the active site was the major factor responsible for the observed resistance (Omer et al., 1980).

2.4.2 Organophosphorus Insecticides

2.4.2.1 Mode of Action

The organophosphorus compounds apparently inhibit the action of several enzymes, but the major action in vivo is against the enzyme acetylcholinesterase, which controls the hydrolysis of acetylcholine, generated at nerve junctions, into choline. Acetylcholine acts as a transmitter in the synapses within the central nervous system and also at neuromuscular junctions. In the absence of effective acetylcholinesterase, the liberated acetylcholine accumulates and prevents the smooth transmission of nervous impulses across the synaptic gap. This causes loss of muscular coordinations, and ultimately death (see Eto, 1974). √

2.4.2.2 Mechanisms of Action of insecticide on acetylcholinesterase

Acetylcholinesterase is an essential component of the nervous systems

of both insects anc mammals, so the basic mechanism of toxic action of the organophosphorus compound is considered to be essentially the same in insects and mammals. The active centre of the enzyme acetylcholinesterase contains two main reactive sites: an anionic site which is negatively charged and binds to the cationic part of the substrate (acetylcholine), and the esteratic site, containing the primary hydroxyl group of the amino acid serine which attacks the electrophilic carbonyl carbon atom of the substrate. The reaction of acetylcholine with AchE can be shown as follows.

$$EH + AX \rightleftharpoons EH{\cdot}AX \underset{-HX}{\longrightarrow} EA \underset{+H_2O}{\longrightarrow} EH + AOH$$

$$(CH_3)_3\overset{+}{N}CH_2CH_2O\overset{O}{\overset{\|}{C}}CH_3 + ECH_2OH \overset{(1)}{\rightleftharpoons} (CH_3)_3\overset{+}{N}CH_2CH_2O\overset{O}{\overset{\|}{C}}CH_3{\cdot}ECH_2OH \overset{(2)}{\longrightarrow}$$

$$H_2O \uparrow (3)$$

$$CH_3COOCH_2E + HOCH_2CH_2\overset{+}{N}(CH_3)_3$$
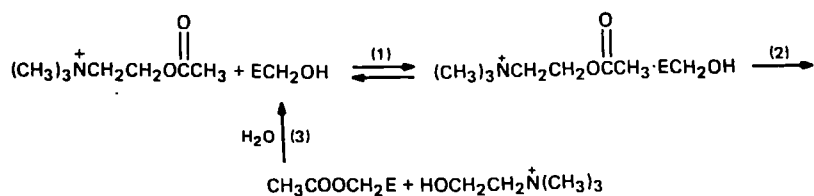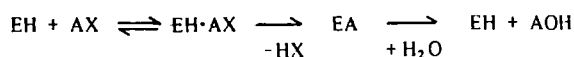
(Modified from Cremlyn. 1978)Where:

Where:

EH = Acetylcholinesterase = ECH₂OH

AX = Acetylcholine (substrate) = $(CH_3)_3NCH_2CH_2O\overset{O}{\overset{\|}{C}}CH_3$

EH. AX = Enzyme-substrate complex = $(CH_3)_3NCH_2CH_2O\overset{O}{\overset{\|}{C}}CH_3{\cdot}ECH_2OH$

EH = Free enzyme = ECH₂OH

AOH = Coline = $HOCH_2CH_2\overset{+}{N}(CH_3)_3$

23

Initially a complex (the enzyme-substrate complex) is formed between the enzyme (AchE) and acetylcholine, by the orientation of the active centres of acetylcholinesterase to the substrate acetylcholine (step 1). Subsequently, in step 2, a hydrogen atom is transferred from the esteratic site of the enzyme to the choline moiety of acetylcholine. This process is called acetylation. In step 3, the acetylated enzyme is rapidly hydrolysed by water to choline and acetic acid, so that the active enzyme is quickly regenerated, permitting it to repeat the enzymic hydrolytic process on further substrate molecules (enzyme recovery). This process occurs very rapidly, whereas the reaction of enzyme with OP compounds takes place at an extremely slow pace.

Some organophosphorus compounds mimic the natural substrate acetylcholine by binding to the esteratic site of acetylcholinesterase. The subsequent reactions mirror the three normal reactions between the enzyme and acetylcholine mentioned above. The reaction of enzyme with OP compound can be expressed as follows.

$$EH + PX \rightleftharpoons EH \cdot PX \longrightarrow EP + HX$$
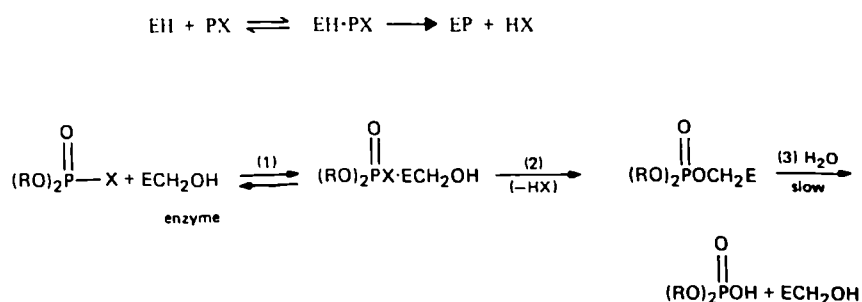
(Modified from Cremlyn, 1978)

Where: PX = Organophosphorus inhibitor

EX = Acethylcholinesterase

ECH2OH

$$(RO)_2 \overset{\overset{\text{O}}{\|}}{P} X \cdot ECH_2OH$$

EX. PX = Enzyme—substrate complex

EP = Phosphorylated enzyme

HX = Leaving product $(RO)_2 \overset{\overset{\text{O}}{\|}}{P} OH$ ·

In the presence of an OP compound and with the formation of phosphorylated enzyme the P=O bond is much stronger than the C=O of the acetylated enzyme, and the hydrolysis rate is much slower than with the normal substrate, so that the OP effectively poisons the enzyme by phosphorylation and thus blocks efficient hydrolysis of acetylcholine into choline.

2.4.2.3 Metabolism

The majority of organophosphorus insecticides, except phosphates and phosphorothioates, show little capacity to inhibit acetylcholinesterase unless they are activated; their effective anti-cholinesterase activity in vivo is the net result of competing biochemical processes, activation and detoxication.

(a) Activation

Many useful insecticides such as phosphorothionates and phosphoro thiolothionates contain P=S groups and show little in vivo anticholinesterase activity, the in vivo activity being the result of metabolic activation of P=S to P=O (oxo-analogues).

Activation means the metabolic conversion of inactive compounds to active compounds or the conversion of one active compound to another.

Metabolism and biological modification of organophosphorous insecticides often causes a change to a more toxic product. The bioactivation of OP compounds is mainly due to oxidative reactions and microsomal oxidation.

Microsomal Mixed Function Oxidase System

Microsomes are derived by homogenation from the endoplasmic reticulum, which is a tubular network of lipoprotein extending throughout the cytoplasm. There are two types of endoplasmic reticula: rough and smooth. The former is studded with ribosomes which serve an important role in biosynthesis of proteins, but the latter is free of these particles. The smooth endoplasmic reticulum contains enzymes of high activity to oxidase a great variety of lipophilic substrates including steroids, lipids and foreign organic compounds (Eto, 1974). Such oxidation is catalysed by microsomal oxidases, some related to activation and others to detoxication. These reactions require molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) or NADH as a cofactor. One of the atoms of molecular oxygen is incorporated into the substrate and the other is reduced to water. The reaction may be generalized as follows.

$$RH + NADPH + H^* + O_2 \longrightarrow ROH + NADP^* + H_2O$$
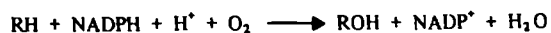
The mixed function oxidase (MFO) enzymes are abundant in the vertebrate liver, and in the fat body, Malpighian tubules and digestive tract of insects (Haydishi, 1969). The microsomal hydroxylation of foreign compounds depends on the participation of an enzyme belonging to

the iron-containing enzymes in the cytochrome P-450 group (Omural & Sato, 1964).

Oxidative Desulphuration

Oxidative desulphuration of the thiophosphoryl sulphur atom has been demonstrated with a wide variety of insecticides such as fenthion, fenitrothion, chloropyrifos, temephos, pirimiphos-methyl and malathion (phosphorothiolothioate and phosphorothionate esters). These compounds are poor inhibitors of cholinesterases, whereas the corresponding oxo-analogues are highly potent anticholinesterase agents. The toxic action of thiono compounds is attributable to their oxo-analogs formed *in vivo* by oxidative desulphuration of the thiophosphoryl group (Gage, 1953). The formation of the oxo form requires NADPH and molecular oxygen in the presence of mixed function oxidase systems (Fig 2.4).
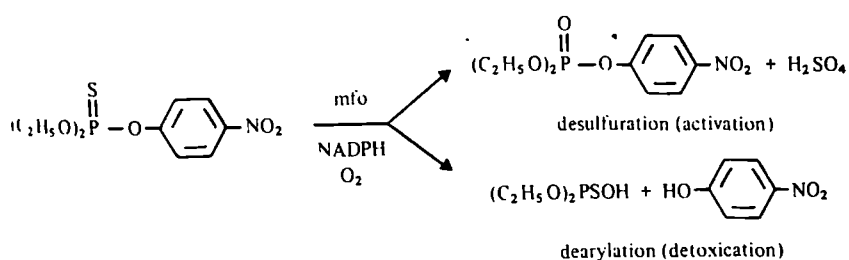
(From Eto, 1974)

Fig 2.4 Oxidative detoxification and dearylation of parathion by mixed function oxidases.

(b) Detoxication

A number of different enzymes are involved in the detoxication of organophosphates. Metabolic detoxication is mainly due to the cleavage of a phosphorus ester bond which results in the formation of a negative charge on the molecule. The negative charge causes the phosphorus compound to behave as a phosphorylation agent or an anticholinesterase agent. The products are also much more soluble in water and can be readily excreted (Eto, 1974). The leaving moiety of OP's usually possesses a hydroxy, amino, or thiolo group. The enzymatic disruption of any of these bonds results in inactivation or detoxication of the compound.

Two different types of ester bond in OP insecticides can be cleaved: anhydride and alkyl ester bonds. The phosphorus ester bond cleavages have been attributed exclusively to the action of hydrolytic enzymes. The biotransformation of a nonphosphorus functional group, including hydrolysis of carboxylester and carboxyamide linkages and nitro groups is another important detoxication mechanism in some—OP compounds. The important reactions resulting in detoxication of OP compounds are as follows.

1- Anhydride Bond Cleavage

Oxidative Dearylation

The cleavage of an ester bond was first found in parathion in 1962 by Knaak. Five years later it was reported by Nakatsugawa & Dahm (1967) and Neal (1967) that the mammalian liver microsomes catalyse both the desulphuration of parathion to yield paraoxon, and cleavage of the aryl ester bond to yield nitrophenol and phosphorothioic acid. Both reactions

require the presence of NADPH as a cofactor, molecular oxygen and mixed function oxidase systems, and are strongly inhibited by carbon monoxide, and benzodioxoles.

Oxidative dearylation by MFO systems from mammalian liver or housefly abdomens has been demonstrated with aryl phosphorothionates, such as parathion, parathion methyl and fenitrothion (Nakatsugawa *et al.*, 1968). The P-S (aryl) bond in phosphorothiolothionates such as malathion also appeared to be disrupted oxidatively by MFO systems in resistant houseflies (Motoyama, 1972). The metabolism of insecticides usually depends on the oxidative desulphuration and oxidative dearylation reaction in resistant strains.

Glutathion-S-aryltransferase

The important reaction of this enzyme with OP compounds is usually attributed to the combination of glutathion with the leaving group, leading to detoxication of organophosphorus compounds.

$$(C_2H_5O)_2\overset{\displaystyle S}{\overset{\displaystyle \|}{P}}{-}O{-}\!\!\left\langle\!\!\bigcirc\!\!\right\rangle\!\!{-}NO_2 \xrightarrow[\text{aryltransferase}]{\text{GSH}} (C_2H_5O)_2\overset{\displaystyle S}{\overset{\displaystyle \|}{P}}OH + GS{-}\!\!\left\langle\!\!\bigcirc\!\!\right\rangle\!\!{-}NO_2$$
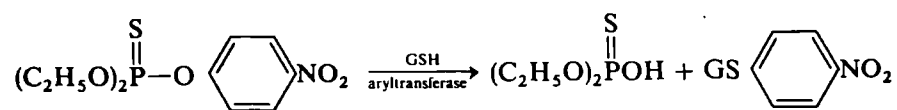
(From Matsummua, 1975)

Fig 2.5 Detoxification of parathion by gluthion-S-aryltransferase in houseflies.

Glutathion-S-aryltransferase activity has been demonstrated in rat liver, cockroach fat body, and houseflies (Nakatsugawa *et al,*. 1969; Yang *et al.,* 1971). S,S,S- tributyl phosphorothioate appeared to inhibit glutathione dependent dearylation of diazinon in resistant houseflies (Lewis and Sawicki, 1971). The most likely case of glutathion-S-aryltransferase action has been observed with parathion, which probably degrades in the housefly, (Dahm, 1970; Oppenoorth *et al.,* 1972) Fig. 2.5.

2- Alkylester Bond Cleavage

The biodegradation reaction and cleavage of P-O-alkyl bonds was first found in 1958 by Plapp and Casida, who examined the metabolism of a variety of OP insecticides. The degradation reaction has been regarded as being catalysed by hydrolytic enzymes, oxidation mechanisms and glutathion-s-alkyltransferase mechanisms, which are important biochemical mechanisms responsible for the formation of dealkylated metabolites.

Oxidative-O-dealkylation

The de-ethylation of chlorofenvinphos by liver microsomes in the presence of NADPH and oxygen has been demonstrated by Donninger *et al.* (1971). Similar dealkylation occurs with paraoxon by a microsomal preparation from resistant strains of housefly in the presence of NADPH and oxygen (Oppenoorth, 1972; Lewis and Sawicki, 1971).

Glutathion-S-alkyltranferase

The important reaction of glutathion-S-alkyltransferase with OP compounds is the removal of a methyl group. Demethylation of dichlorvos with a soluble enzyme fraction from rat liver was demonstrated by Hodgson

and Casida (1962). The enzyme catalysing the demethylation of parathion-methyl and fenitrothion in the supernatant fraction of rat liver homogenates was greatly increased by the addition of glutathion (GSH), suggesting that glutathion may act as a methyl group acceptor (Fukunaga *et al.*, 1969). The enzymes present in resistant strains of insects appear to be more specific to diethyl phosphorous esters. The glutathion dependent O-de-ethylation of diazinon, diazoxon and parathion occurs in certain organophosphorus resistant strains of houseflies, which show greater resistance to diethoxy phosphorus insecticides than to dimethoxy insecticides (Lewis and Sawicki 1971; Oppenoorth, 1972). Both phosphorothionates and phosphate esters can be substrates, but only one of two o-methyl groups in the molecule is removed by this reaction. The mechanism could be proposed as follows (Fig 2.6).
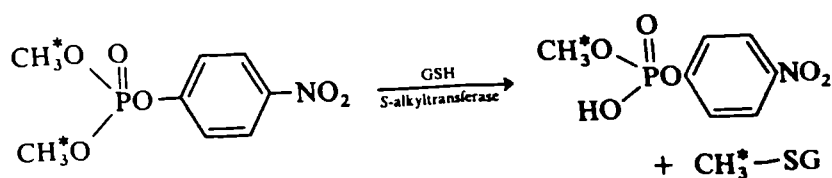
(From Matsummura, 1975)

Fig 2.6 Detoxification of mythyl parathion by gluthion-S-alkyltransferase

3- Hydrolysis of Carboxylester Linkage

A number of organophosphorus insecticides, such as malathion, contain a carboxy ester linkage in the molecule. The hydrolysis of the ester linkage results in detoxication. The carboxylesterases are also called ali-esterases or B-esterases, and are widely distributed in mammals, having been found in the liver, kidney, serum, lung, and spleen. Carboxylesterase activity is low in susceptible insects. This is responsible, at least in part, for the highly selective toxicity of malathion. Certain strains of arthropods which are resistant to malathion show elevated levels of carboxylesterase activity which is responsible, at least partly, for the malathion resistance of mosquitoes and houseflies (Matsumura & Brown, 1961) (Fig 2.7).
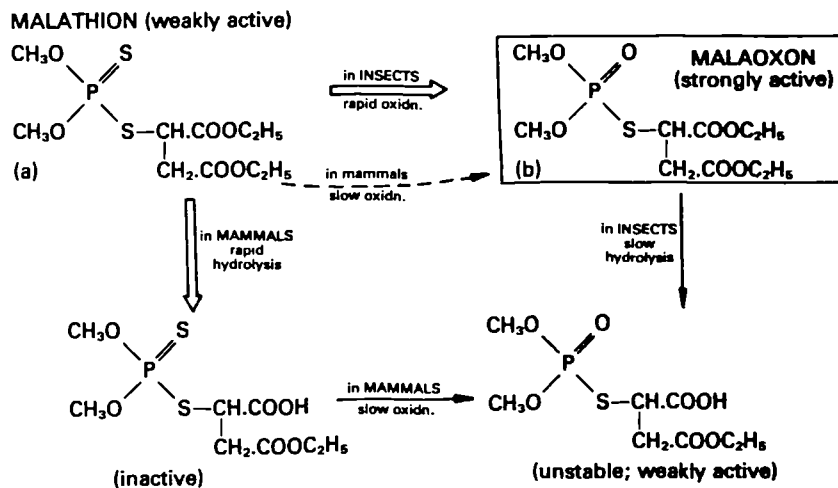
(From Hassal, 1982)

Fig 2.7 Metabolism of malathion in mammals and insects.

The carboxylesterases are inhibited by synergists, such as N-propyl paraoxon and phenyl-phosphonate (Takahashi *et al.*, 1973; Okawa *et al.*, 1968).

Carboxylesterases are important in the inactivation of OPs such as malathion, phenthoate, malaoxon and acethion. This detoxication involves the hydrolysis of carboxylester bonds resulting in a non-toxic ionic product.

2. 4. 2. 4 Genetics of Organophosphorus Resistance

A total of 59 species of anopheline mosquito have been recorded as resistant to one or more insecticides, of which 31 species are resistant to organophosphorus insecticides (Brown, 1986). Among these, 26 species are malathion resistant, 20 fenitrothion resistant, 10 fenthion resistant, 6 chloropyrifos resistant and 5 temephos resistant in either the larvae or adults, in some cases as a result of application of agricultural pesticides or cross-resistance to other insecticides (WHO, 1985).

After detection of resistance to organochlorine insecticides in anopheline mosquitoes, organophosphorus insecticides, notably malathion, have been replacements in many areas where resistance was reported. Appearance of resistance to malathion in An. albimanus was first found in El Salvador (Breeland *et al*,. 1970), and then in 1968 in An. culicifacies in two states of Western India. By 1975 it had become common (Rajagopal, 1977). Subsequently malathion resistance in An. stephensi was first reported in Iran from the coast (Manouchehri *et al.*, 1976) and interior (Eshghi, 1978), then in Pakistan (Rathor & Toqir, 1980) and in Iraq in Basreh province (Manouchehri *et al.*, 1980).

The genetics and mechanisms of malathion resistance in An.stephensi from different areas have been studied. Malathion resistance in a population of An.stephensi from southern Iran was found to be inherited as a single gene. Synergist tests suggested the involvement of a carboxylesterase enzyme in malathion resistance. Piperonyl butoxide (PB) had antagonistic effects at all dosages tested, indicating that mixed function oxidases (MFO) were involved in the activation of malathion to toxic malaoxon (Herath & Davidson, 1981). In An.stephensi from Pakistan, synergist studies suggested the involvement of a carboxylesterase enzyme in malathion resistance. PB, a mixed function oxidase inhibitor, had a slight antagonistic effect on malathion, suggesting that carboxylesterase was the only mechanism in malathion resistance (Hemingway, 1982). Larvae of An.stephensi from Pakistan which were resistant to malathion, showed cross-resistance to phenthoate, suggesting that a highly specific type of resistance mechanism (Malathion carboxylesterase, MCE) is responsible for that resistance (Scott & Georghiou, 1986). The location of the gene for malathion resistance in adults of An.stephensi from Pakistan was studied by Rowland (1985), the gene for malathion resistance was found to be located between the gene for dieldrin resistance and diamond palpus in linkage group 3.

With the development of malathion resistance in a number of species of anopheline mosquitoes, many attempts have been made to search and evaluate some alternative insecticides for vector control programmes. The residual effect of pirimiphos-methyl was evaluated at a dosage of $1g/m^2$ against A.stephensi on the surface of thatch, cement and mud for 2, 3 and 4 weeks after residual spraying. Mortalities fell from 100% to 60.3, 73, and 68.9% respectively. Bioassay tests indicated that this insecticide

has an effective vapour toxicity for up to three days after residual spraying (Das *et al,.* 1981). The cross-resistance spectrum of pirimiphos-methyl was studied in adults and larvae of several organophosphorus resistant strains. Susceptibility tests indicated that there was no cross-tolerance in <u>An. arabiensis</u> and <u>Culex quinquefasciatus,</u> and only a slight cross-resistance was observed in <u>An. albimanus,</u> suggesting that pirimiphos-methyl could be used for control of organophosphorous and carbamate resistant strains of anopheline and culicine mosquitoes (Hemingway *et al,.* 1984).

2.4.3 Genetics of Insecticide Resistance in other Anopheline Mosquitoes

The genetics and biochemistry of insecticide resistance have been studied more fully in <u>An. albimanus</u> than in other anopheline mosquitoes. The mode of inheritance of DDT and dieldrin resistance in the larvae and adults of this species was studied by Davidson (1963). A strain from El Salvador resistant to both DDT and dieldrin (double resistant) was purified for individual DDT and dieldrin resistance, and susceptibility, by means of mass selection and single family selection. The results indicated that resistance to DDT and dieldrin is inherited independently, and that dieldrin resistance is dependent on a single semidominant genetic factor which imparts cross-resistance to other cyclodiene compounds, while DDT resistance is inherited as a single recessive genetic factor and shows cross-resistance to DDT analogues. A similar mode of inheritance of resistance was found in the larvae and adults. A strain of <u>An. albimanus</u> from El Salvador was subjected to larval selection with propoxur. Three generations of selection resulted in an increase in resistance to propoxur more than 100-fold. The resistance also extends to

a number of other insecticides. The selected larvae showed 65.3, 6.8, 11.2, 10.9, 5.3 and 4.3-fold increases in resistance to carbaryl, malathion, parathion, methyl parathion, fenitrothion, and DDT respectively, when the selected strain was compared with the parental stock. Synergist tests suggested that mixed function oxidases are involved in detoxication of propoxur and carbaryl, whereas in parathion, methyl parathion, fenitrothion and fenthion resistance, mixed function oxidases were involved in oxidative desulphuration of these compounds. Tests with TPP, a carboxylesterase inhibitor, suggested the involvement of carboxylesterase in malathion resistance (Ariaratnam & Georghiou, 1971). Further biochemical studies were carried out on the resistant strain by Ayad & Georghiou (1975). Two homozygous resistant strains were obtained, when sub-strains from this species were subjected to selection: (a) A homozygous resistant strain (OP-R), as a result of larval selection with ethyl parathion; and (b), (Carb-R), as a result of propoxur selection in the adult stage. AchE activity of resistant strains was studied _in vivo_. Both strains showed a high degree of AchE insensitivity to inhibition by paraoxon and propoxur. An altered AchE type resistance mechanism was postulated for the observed resistance. These results were subsequently supported by Georghiou and Pasteur (1978) and Hemingway and Georghiou (1983). In another study by Herath and Davidson (1981), adults of a laboratory strain of An. albimanus originating from Panama (PALB) and a strain provided from El Salvador in 1974 (FERNS/RR) were compared for susceptibility to a number of different insecticides. The FERNS/RR strain showed resistance to DDT, a number of OP compounds and propoxur but susceptibility to permethrin and deltamethrin. The effect of synergists, triphenyl-phosphate, piperonyl butoxide, O,O-dimethyl O-phenyl

phosphorothioate (SV,) and S,S,S,-tributyl phosphorotrithioate (DEF), in combination with malathion, suggested the involvement of at least 2 different detoxication resistance mechanisms, carboxylesterases and mixed function oxidases in the adults of the multiple resistant strain.

The nature of malathion resistance in a strain of An. culicifacies from India was studied by Herath & Davidson (1981). Resistance was found to be dominant in its expression, and crossing experiments indicated the possible involvement of more than one genetic factor in malathion resistance. Synergist studies suggested the presence of at least two mechanisms, a specific carboxylesterase and a less specific, mixed function oxidase system.

Different sub-strains derived from a multiple resistant strain of An. atroparvus from Spain were subjected to selection at the adult stage with fenitrothion, propoxur, malathion and fenthion for 12, 6, 20 and 12 generations respectively. Lack of synergism with TPP, a carboxylesterase inhibitor, in the malathion selected line, and PB, a multi-function oxidase inhibitor, in the fenitrothion selected line, suggested the involvement of a non-metabolic resistance factor, possibly an altered site of action, in those strains which were resistant to OP compounds. In the propoxur selected line, the mixed function oxidase inhibitor had no synergistic effects on propoxur after 2 hrs exposure time, but an increase in mortality was recorded in conjunction with a 6hr exposure to propoxur. This suggested the involvement of a mixed-function oxidase inhibitor in the propoxur selected line (Hemingway and Davidson, 1983).

Adults of An. arabiensis from Sudan were found to be resistant to malathion. Malathion was synergized by triphenyl phosphate (TPP), but not by piperonyl butoxide (PB). This suggested that a carboxylesterase enzyme

may be the basis of malathion resistance in the adults of this strain (Hemingway, 1983).

2.4.4 Pyrethroids

2.4.4.1 Mode of Action

The symptoms of pyrethroid poisoning in invertebrates are hyperexcitation, tremors and convulsion followed by paralysis and death. DDT and pyrethroids share a number of similar properties and possess a clear negative temperature coefficient of action on the nervous system (Blum & Kearns, 1956). The specific location of the target site(s) within the nervous system and the precise mode of action of pyrethroids is still controversial.

The most compelling evidence that pyrethroids have a similar mode of action to DDT comes from genetics. Busvine (1951) documented cross-resistance to pyrethrum in an Italian strain of housefly that was resistant to knockdown by DDT. Both DDT and pyrethroid compounds act on the peripheral and central nervous systems, the other major groups of insecticides lack any action on peripheral axons.

The peripheral nervous system in insects consists of sensory neurons and their axons, motor axons and their terminals, and all neurosecretory axon and neurohaemal organs that lie outside the ventral nerve cord and paired ganglia. The central nervous system (CNS) is considered to be the ganglia, connectives, and commissures from the brain to the terminal abdominal ganglion. These definitions make the insect CNS analogous to the mammalian CNS, which consists of the brain and spinal cord.

Pyrethroids produce a variety of symptoms which have been grouped

into two main categories designated 1 and 2, plus a third category that is neither entirely 1 nor 2 (Clements & May 1977; Scott & Matsumura, 1983). Type 2 pyrethroids (largely 1-cyanophenoxybenzyl insecticides) are thought to act on the central nervous system (CNS). Type 1 (non 1-cyano substituted) compounds, including allethrin, bioresmethrin, and most other pyrethroids, are regarded as acting on the peripheral nervous system in mammals (Gammon et al., 1981; Staatz et al., 1982). However there are always exceptions to these categories (Gammon et al.,1981).

Lowenstein (1942) in his very early studies of pyrethrin extracts on the ventral nerve cord (VNC) of Blatta orientalis, reported greatly increased nerve activity followed by block of nerve conduction. In another study, by Burt & Goodchild (1971), the same procedure was repeated, on the VNC from adult Periplaneta americana., which was exposed to pyrethrin 1 for various periods of time. An observation that pyrethroids are more potent when administered close to the CNS of rats (Staatz et al., 1982) matches the observation of Burt & Goodchild (1977) in cockroaches, and represents the main evidence for an action on the CNS of animals. They also considered the knockdown and lethal actions of pyrethroids to be the result of action on the CNS. In contrast, Gammon (1978) found that pretreatment of cockroaches with a sub-lethal injection of tetrodotoxin, which because of a barrier to penetration is thought to act on the peripheral nervous system, offered a measure of protection against subsequent allethrin poisoning. He concluded that allethrin was acting entirely peripherally. From other work with rats, non-cyano-containing pyrethroids such as permethrin are thought to produce symptoms mainly through action on the peripheral nervous system (Verschoyle & Aldridge, 1980), whereas 1-cyano-3-phenoxybenzyl pyrethroids such as

deltamethrim produce symptoms thought to be due to action on the CNS, or at least to originate in higher nerve centres of the brain (Staatz *et al.*, 1982). The same distinct set of symptoms are known to occur in insects (Staatz *et al.*, 1982).

The optimum temperature and mode of action of pyrethroids have been inseparably connected since Vinson & Kearn (1952) concluded that DDT was more toxic at lower temperatures. Some 3-phenoxybenzyl pyrethroids lacking 1-cyano substitution, such as permethrin and phenothrin, have high negative temperature coefficients similar to DDT. However on Heliothis virescens, 1-cyano-substituted 3-phenoxybenzyl pyrethroids are weakly or moderately positively correlated with toxicity (tralomethrin, deltamethrin), or only slightly negatively correlated (flucythrinate and cypermethrin).

Considerations of the toxicity of pyrethroids at various temperatures should be a vital part of any pest control programme. In 1981, widespread failure of permethrin to control tobacco budworm on cotton in the Imperial Valley, California, occurred during a 7 to 10 day period when the temperature at night stayed abnormally high, about 40°C. The negative temperature coefficient was thought to explain the failure of control. When night time temperatures returned to normal, cooler values, permethrin treatments again were seen to control budworm (Miller & Salgado, 1985). A dose of DDT that completely knocked down houseflies in 63 minutes at room temperature (65°F) caused only 50% mortality when the flies were immediately removed to 100°F (Lindquist *et al.*, 1945).

2.4.4.2 Knock-down

The term "knockdown" means the rapid action of insecticide, from which insects may or may not subsequently recover. Non-recovery is given a separate name (kill). Some pyrethroids have excellent knockdown but vary in their effects on recovery, depending on their structures (Elliott & Janes, 1978; Ohsumi *et al.*, 1981). It has not been clarified whether recovery from knockdown is merely a matter of metabolism of the insecticide to a non-lethal level, or whether those poisoning processes leading to knockdown are the same as those that eventually lead to kill (Miller & Salgado, 1985). Some potent pyrethroids (such as bioethanomethrin) have little or no knockdown effect, which suggests that the two processes are indeed separate (Miller & Adams, 1977).

Pyrethroids that act very quickly are said to possess good knock-down. There are two opposing views as to the cause of knock-down, conveniently represented by the work of Burt & Goodchild (1974) and Clements & May (1977). Burt and Goodchild contended that all poisoning symptoms resulted from actions on the CNS of insects. Clements & May (1977), on the other hand, subscribed to the interpretation of Page *et al.*, (1949) that knock down is so fast that there is not enough time for penetration of a compound into the CNS, and they concluded that knock down is due to excessive sensory hyperactivity in the peripheral nervous system. The majority of evidence supports the theory that some actions on sensory nerves are largely responsible for early knock down systems (Miller & Salgado 1985). Knock down by topical treatment was slower in the German cockroach (<u>Blattella germanica</u>) than knock down by surface contact. Uptake and distribution of ^{14}C permethrin showed that rapid knock down by surface contact corresponded closely with the involvement

of radiolabelled compound within the legs (see Miller & Salgado, 1985)).

This supports the conclusion of Clements & May (1977) that knock down is

associated with the peripheral nervous system, and is compatible with the

finding that houseflies knocked down by deltamethrin are still capable of

flight but cannot maintain posture (Bloomquist, 1983).

2.4.4.3 Metabolism

Synergism provides very strong circumstantial evidence that

microsomal oxidase systems participate in the degradation of pyrethroids.

Methylendioxyphenyl compounds are probably specific inhibitors of these

enzymes. Pyrethroids are often destroyed by oxidation rather than by

hydrolysis (Hassal, 1982).

Most of the work on the metabolism of pyrethroids has been done on

rats and mice, because they are important models for toxicity assessment.

Although the insects are the targets for pyrethroids, the metabolism of

these compounds by insects has not received the same detailed level of

investigation as that by mammals.

In the metabolism of the natural pyrethroid insecticide, pyrethrin 1,

only one of the methyl groups in the isobutenyl side chain of the

chrysanthemic acid is oxidised to a hydroxymethyl group in an NADPH-

dependent reaction. Then it is readily converted to a carboxylic acid

group. In pyrethrin 2, the major metabolites are identical to those

formed from pyrethrin 1 (Yamamoto & Casida, 1966; Yamamoto, et al.,1971)

(see Fig 2.8).

Pyrethrin I

hydroxymethylation (p.55)
NADPH

Hydroxymethyl
derivative of
chrysanthemum
monocarboxylic
acid ester

an alcohol
dehydrogenase?

Pyrethrin II

O-demethylation (p.56)
NADPH

Chrysanthemum
dicarboxylic
acid ester

(From Hassall, 1982)
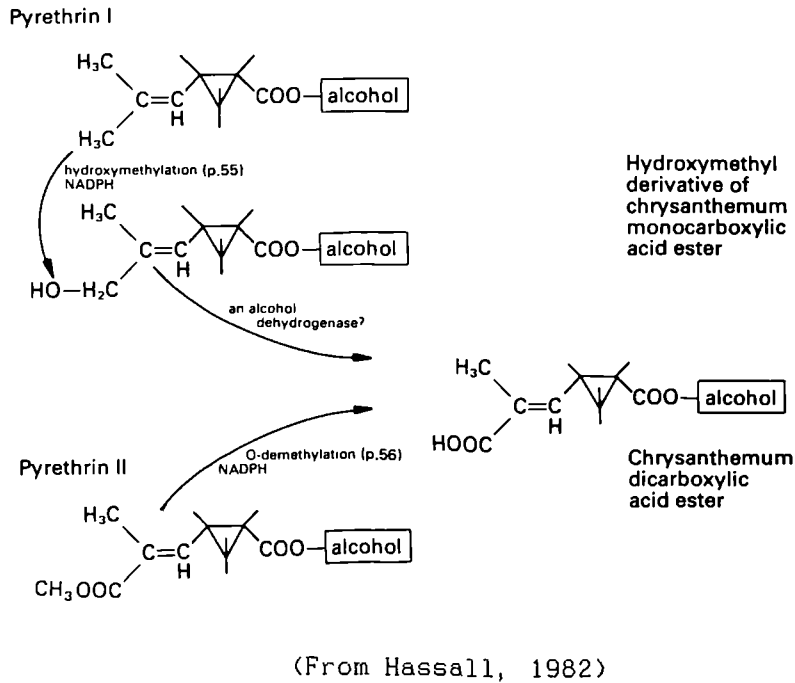
Fig 2.8 Probable oxidative metabolism of the acid moiety of

pyrethrins 1 and 2.
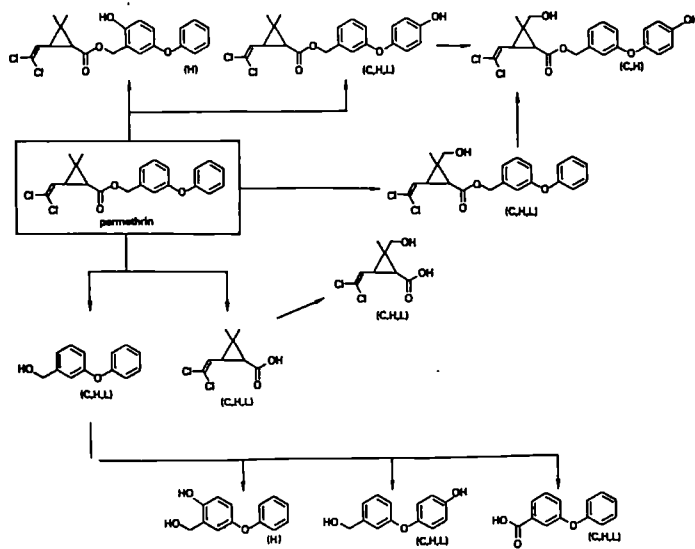
(Taken from Leahey, 1985)

Fig 2.9 Metabolism of permethrin by the cockroach (C), housefly (H) and cabbage looper (L); the insects in which a metabolite has been found are indicated below each structure

The metabolism of permethrin has been studied in a number of insect species, but the most detailed evaluation of the metabolic pathway has been achieved in a study with cockroach and housefly adults, and larvae of the cabbage looper moth (Shono *et al.*, 1978); the results are illustrated in Fig 2.9. In all three insects, ester cleavage and hydroxylation at the 4-position of the alcohol moiety and oxidation of 3-phenoxybenzylalcohol (generated by cleavage) to 3-phenoxybenzoic acid are the major metabolic processes in all three insects. Hydroxylation of the geminal dimethyl group of the acid moiety is also a major reaction in the cockroach, although it is of minor importance in the housefly and cabbage looper moth.

2.4.4.4 Genetics of Pyrethroid Resistance

About 10 species of anophelines have already developed resistance to pyrethroids, but often as a result of cross-resistance, selected by other insecticides, particularly DDT (WHO, 1986). The slow development of resistance to pyrethroids could be due to the limited use of these insecticides in the field (Brown, 1986).

Early research on resistance to pyrethroid insecticides repeatedly pointed out the close relationship between resistance to DDT and pyrethroids in different species, Among these, the housefly is one of the most studied species.

Busvine (1951), examined the relationship between DDT and pyrethroids in two strains of houseflies from Italy and Sardinia. The Italian strain showed cross-resistance to pyrethrin and DDT analogues. The Sardinian strain showed susceptibility to pyrethrin, and dehydrochlorination of DDT as the major resistance mechanism was detected only in the Sardinian

strain. In a later study, a strain of the housefly <u>Musca domestica</u> which had been selected for knockdown resistance to DDT proved to be cross-tolerant to pyrethrins. Busvine suggested the existence of an important DDT resistance mechanism, characterised by delayed knockdown to DDT and associated with resistance to pyrethroids (Busvine, 1951, 1953).

More detailed studies on the genetics and biochemistry of pyrethroid resistance have been done by Farnham (1971, 1973 and 1977), and Farnham and Sawicki (1976). In a careful study by Farnham (1973), four resistance factors were identified in the NPR strain of the housefly, <u>Musca domestica,</u> which was known to be resistant to natural pyrethrins:

(a) <u>pen</u> (chromosome 3): Reduced rate of penetration of insecticide through cuticle had been reported before in houseflies (Sawicki and Farnham, 1967, 1968; Hoyer & Plapp, 1968). This factor could be selected by almost any insecticide; it does not impart resistance, but can increase the effect of other mechanisms (Sawicki, 1970). <u>Pen</u> was associated with resistance to tributylin acetate, it showed no resistance to pyrethroids and DDT, but showed slight resistance to all synergised pyrethroids.

(b) <u>kdr-NPR</u> (chromosome 3): This factor, shown to be the major pyrethroid resistance mechanism in houseflies, provided resistance to pyrethroids, strong resistance to 5-benzyl-3-furymethyl, and cross-resistance to DDT. It was unaffected by the synergist sesamex. Two other factors, <u>kdr</u> and <u>kdr-o</u> have been described in houseflies showing cross-resistance to DDT and natural pyrethrins (Plapp & Hoyer, 1968). <u>Kdr-NPR</u> may be identical with one of these factors (Farnham, 1973).

(c) py-ses (chromosome 2): It gave slight resistance to pyrethrolone esters, natural pyrethrins and allethrin, susceptibility to synergised pyrethrins and no cross-resistance to DDT and tributylin acetate.

(d) py-ex (chromosome 2): This factor was not isolated in the homozygous state. However it gave slight resistance to natural pyrethrins and pyrethrolone esters.

Two knockdown resistance factors have previously been identified on chromosome 3 in DDT resistant houseflies by Milani (1954) and Hoyer & Plapp (1966). The characteristics of kdr-NPR with these two factors was compared by Farnham (1977). After purification of each factor, cross-over rates between these factors and the morphological mutants, brown body and green eye, indicated that kdr and kdr-o factors are probably identical with each other and with kdr-NPR.

It could be concluded that in the pyrethroid resistant houseflies described by Farnham (1973, 1977), increases in metabolic degradation are not of primary importance in resistance. The genes py-ses and py-ex confer less resistance than do pen and kdr. pen involves decreased absorption. kdr is not a detoxification gene; more likely, it involves a change in the target of pyrethroid-DDT action against the insect nerve.

Among the mosquitoes, larvae of Culex tarsalis, resistant to DDT, showed cross-resistance to pyrethrins, and to mixtures of pyrethrins and PB. Breeding experiments demonstrated that resistance to DDT and pyrethrins were genetically linked and possibly controlled by a similar mechanism, which is not metabolic. A kdr factor similar to that in the housefly was postulated for that resistance mechanism (Plapp & Hoyer

1968).

In a study by Rongsriyam & Busvine (1975), five species of mosquito, including Ae. aegypti and An. gambiae, highly resistant to DDT, were tested with different larvicides. Ae. aegypti and An. gambiae showed 2.9 - 5.8-fold cross-resistance to allethrin and bioallethrin. DMC and PB had a slight synergistic effect on DDT, but PB reduced the permethrin resistance. This suggested that the low level of pyrethroid resistance could be dependent on microsomal oxidation systems.

In a study by Parasittisuk & Busvine (1977), eight strains of Aedes aegypti from Central America and the Caribbean, and two species of anopheline mosquitoes, An. gambiae and An. quadrimaculatus, in which resistance to DDT ranged from 2-fold to 73-fold, were tested for resistance to pyrethroids. All strains showed a low level of cross-resistance to permethrin (1.4 to 3.4), except Ae. aegypti from East Coast, Demerara, with a 30-fold cross-resistance to permethrin. Synergist tests indicated that a considerable part of DDT resistance in this species was due to a dehydrochlorination mechanism. Test with PB suggested that microsomal oxidase systems were also involved in permethrin and DDT resistant larvae.

A strain of Ae. aegypti from Bangkok was subjected to selection with permethrin. The process started with mass selection, and then continued with single family selection. 30-fold resistance was produced and the strain was shown to be homozygous for permethrin resistance (Malcolm & Wood, 1982).

Further studies on the homozygous resistant strain indicated two major DDT resistance genes; R^{DDT}, which was located on chromosome 2, controls the dehydrochlorination resistance mechanism, and confers no

cross-resistance to permethrin; R^{DDT}, on chromosome 3, is allelic to R^{PY}, and confers resistance to DDT at a level 3-4 fold less than that of the former, and also cross-resistance to permethrin (Malcolm, 1983).

Two sibling strains of OP-multiresistant larvae of Culex pipiens quinquefasciatus were placed under selection pressure with d-trans permethrin and d-cis permethrin. 18 generations of selection with d-trans, produced about 4000- fold resistance. The d-cis strain showed far less response to selection pressure; 22 generations of selection produced a resistance ratio of 46-fold. Crosses between the d-trans resistant and susceptible strains revealed that the resistance is inherited as an intermediate character. The effect of synergists PB and DEF on the resistant strains suggested that non-metabolic mechanisms such as reduced sensitivity of the target site may be the primary source of resistance (Priester & Georghiou, 1978).

Further studies on this strain indicated that resistance to d-cis permethrin is inherited as a partially recessive character. Cross-resistance studies on two strains indicated that the larvae are resistant to a number of pyrethroid insecticides (Priester & Georghiou, 1979). Adults of d-cis and trans permethrin selected larvae were tested with trans and cis permethrin; the tests indicated that the resistance had been increased by larval selection (Priester et al., 1980)

The relationship between DDT and permethrin resistance was studied by Omer et al., (1980) in An. stephensi from Pakistan. Larvae of An. stephensi from Pakistan initially showed low levels of resistance to DDT, but susceptibility to pyrethroids. The larvae were subjected to selection with DDT for 4 generations. Selection increased resistance to DDT about 98-fold and cross-resistance to permethrin about 12-fold. A further 2

generations of selection with DDT in conjunction with synergists (DMC + PB) resulted in even higher levels of DDT resistance (187-fold) and cross-resistance to permethrin (23-fold). Synergist studies provided no evidence for enhanced metabolism due to dehydrochlorinase, or oxidases. This suggested that the resistance mechanism was of the kdr type (Omer et al, 1980).

Temperature is one of the most important factors which influence the toxicity of an insecticide to the target insect. A negative temperature coefficient for the activity of pyrethroid insecticides has been reported in various species (cited in Joon et al., 1987). However recent studies indicate that relationship between the temperature and pyrethroid activity may vary depending on the insect species and compound used (Spark et al, 1982, 1983; Song Hao, 1986).

Joon et al., (1987) examined the action of DDT and some pyrethroids on the susceptible and kdr-type houseflies, at different temperatures ranging from 15 to 35°C. The two classes of insecticides showed a negative temperature coefficient of toxicity for both strains and the penetration of (IRS)-trans-[^{14}C]-permethrin was positively correlated to temperature. It was suggested that increased nerve sensitivity may be the reason for a negative temperature coefficient of pyrethroid toxicity to the two strains of houseflies.

In another recent study, the toxicity of six pyrethroid insecticides, based on knock down, was determined at two post treatment temperatures (20 and 30°C) for a number of stored-product insects. Some species showed positive and some negative coefficients (Subramanyam & Cutkomp, 1987).

2.5 Synergists in insecticide resistance

The effectiveness or toxicity of organophosphorus insecticides is remarkably influenced by the action of other chemicals, including solvents for formulation, impurities in technical products, and other pesticides mixed in for simultaneous control of several other pests.

The joint action of two or more chemicals, in which the toxicity or biological effect is much greater than that expected from the simple summation of the effects caused by the individual components, is called synergism. Thus synergism gives a co-toxicity coefficient or synergistic ratio (SR), by measuring the value of LD50 of toxicant alone/LD50 of toxicant in mixture. A value significantly greater than one indicates synergistic action, while values significantly less than one indicate antagonistic action. The toxicity of the synergist itself is usually insignificant in comparison with the insecticide component.

Insecticide synergists usually act by blocking the enzymes affecting insecticide detoxication, and fundamental investigation of synergism and synergistic action has led to an improved appreciation of the mechanisms of detoxication in insects, of the basic biochemical process involved in insecticide resistance, and of the mode of action of the insecticide.

Metcalf (1967) has suggested that synergists may alter the biological activity of formulations by increasing the stability of insecticides, altering the rate of penetration through the cuticle and modifying the amount of insecticide picked up by the insect. However it is known that other metabolic mechanisms are more important.

Many compounds have been developed as synergists. They are involved in different reactions such as oxidation, hydroxylation, hydrolysis,

dealkylation, desulphuration, and inhibition of esterase activity.

In DDT resistant strains, there have been some non-metabolic mechanisms, such as slower penetration and decreased sensitivity of nervous tissue, but among the metabolic mechanisms, enzymatic conversion of DDT to DDE by dehydrochlorinase is the major detoxication mechanism in certain DDT resistant strains. The compounds 1,1-bis(p-chlorophenyl) ethanol (DMC), 1,1-bis(p-chlorophenyl)-2,2,2-trifluoroethanol (F-DMC), and N-din-p-chlorobenzenesulfonamide (WARF), have been used as effective DDT synergists, which inhibit the in vivo activity of dehydrochlorinase.

DDT-dehydroxylation is the second metabolic pathway of DDT-detoxication. This type of detoxication was first demonstrated in Drosophila and the cockroach (see Metcalf, 1966). The hydroxylatin of DDT has been rarely identified, but it has been shown that it can result from microsomal oxidation (Devonshire, 1973).

OP compounds undergo a variety of mixed function oxidase catalysed metabolic reactions; oxidative desulphuration results in activation, whereas dearylation and dealkylation also proceed oxidatively and lead to detoxication. Diethyl phenylphosphorothionate (SV1) effectively inhibited the microsomal oxidative dealkylation of paraoxon in an in vivo preparation from some resistant strains of houseflies (Oppenoorth et al., 1972). The best established mixed-function oxidase (MFO) inhibitors are methylenedioxyphenyl compounds (MDP), which were originally developed as the synergists of pyrethroids. The best known of these compounds is piperonyl butoxide. In the metabolism of OP insecticides, the MFO systems are concerned with both activation and degradation. Thus, the effect of microsomal enzyme inhibitors on the toxicity of OP compounds are complicated and are often difficult to interpret. In houseflies, MFO

inhibitors reduced both the desulphuration and dearylation of parathion (Nakatsugawa, *et al.*, 1969). The effect of sesamex on the toxicity of various OP compounds against the housefly NADIAM strain has been studied; some insecticides were synergised and others were antagonised by sesamex (Sun and Johnson 1960, 1969). Therefore the synergistic ratio of any particular OP compound depends on a metabolic balance between the critical pathways responsible for activation and degradation of the compound catalysed by the MFO system.

The mode of action of MFO inhibitors is not fully understood. Casida (1970) suggested that the MDP compounds and some other MFO inhibitors may act as alternative substrates for the MFO systems, saving the insecticide from the MFO dependent biotransformation.

Carboxylesterases are important in the inactivation of OP's such as malathion, phenthoate, malaoxon and acethion. This detoxication reaction involves the hydroxylation of a carboxylester linkage resulting in the production of the mono acid of malathion, a non-toxic ionic product (O'Brien 1960).

$$R\text{-}COO\text{-}C_2H_5 \xrightarrow{\quad +\ [H_2O]\quad} R\text{-}COOH + C_2H_5OH$$
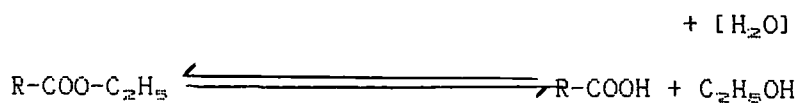
In many insect species carboxylesterase activity is slow or missing from susceptible individuals; however it is present in certain resistant insects.

In malathion resistant strains of houseflies and Culex tarsalis, some excellent OP synergists of malathion , such as triphenylphosphate (TPP) and tributyl phosphrotrithioate (DEF) were found by Plapp *et al.*, (1963).

Oppenoorth & Van Asperen (1961) demonstrated a synergistic action of N-propyl paraoxon with malathion in the resistant strains. The synergists toluylphosphate, triphenylphosphate, tributylphosphotrithioate, tributylphosphotrithioite and trimethylphosphotetrathioate, are effective synergists which caused an increase in the amount of malaoxon in resistant mosquito larvae (Plapp *et al.*, 1963).

Among the pyrethroid insecticides, sesamine is one of the active principles of sesame oil, which has long been known to synergise the action of pyrethrin (Cremlyn, 1978). Certain compounds such as sesamin and piperonyl butoxide, contain methylenedioxyphenyl groups which are probably the specific synergist for pyrethroid compounds. A mixture of one part of natural pyrethroids with two parts of PB was seven times more effective than an equal concentration of pyrethroid used alone (see Hassall, 1982). Pyrethroids are the only insecticides which are commercially formulated with synergist, because it enhances the effectiveness of the insecticide during its relatively limited life (Cremlyn, 1978). The mechanism of action of pyrethrum-synergists primarily involves inhibition of oxidases which detoxify the action of the compound. The methylenedioxyphenyl synergists are metabolised by the same oxidation process as are involved in the break-down of the pyrethroids, and consequently they serve as substitutes for pyrethroids in the enzyme system and inhibit their metabolism (Martin, 1973).

2.6 Aims of Research.

The aims of this research were as follows.

1. To compare the base-line susceptibility of DUB-S, a wild strain of An, stephensi, with IND-S, a laboratory stock.

2. To study the genetics and mechanisms of DDT resistance in larvae of the DUB-S strain.

3. To select larvae and adults of the IND-S and DUB-S strains for resistance to pirimiphos-methyl for a number of generations, to determine the genetics and mechanisms of resistance/tolerance and the cross- resistance spectra of the selected strains to a number of insecticides.

4. To select the larvae and adults of DUB-S strain with permethrin for permethrin resistance.

4.1 Study the genetics and mechanisms of permethrin resistance and the role of the kdr gene in resistant larvae and adults.

4.2 To determine the cross-resistance spectra of the permethrin selected line to other pyrethroids and also the responses to permethrin of the larvae of the adult-selected line and the adults of the larval-selected line.

Chapter 3: Materials Methods

3.1 **Mosquito strains:** The following strains of <u>An. stephensi</u> were used in this investigation:

(a) IND-S strain: A laboratory stock of Indian origin, which has been maintained in the Liverpool School of Tropical Medicine (LSTM) for at least 10 years; it is resistant to both DDT and dieldrin at the adult stage and susceptible to all insecticides at the larval stage.

(b) IND-LPM: a sub-strain from the IND-S strain, selected with pirimiphos-methyl (PM) at the larval stage.

(c) IND-APM: a sub-strain from the IND-S strain, selected with pirimiphos-methyl (PM) at the adult stage.

(d) DUB-S strain: A wild strain based on larvae, collected from Dubai (U.A.E.), and colonised at LSTM in May 1986.

(e) DUB-LPM strain: A sub-strain from DUB-S, selected with pirimiphos-methyl (PM) at the larval stage.

(f) DUB-APM strain: A sub-strain derived from DUB-S, selected with pirimiphos-methyl at the adult stage.

(g) DUB-LPR strain: A sub-strain from DUB-S, selected with permethrin (PR) at the larval stage.

✓(h) DUB-APR strain: A sub-strain from DUB-S, selected with permethrin (PR) at the adult stage.

(i) IRN strain: A laboratory stock of Iranian origin, colonised in 1959, resistant to DDT and dieldrin at the adult stage and susceptible to all insecticides at the larval stage.

3.2 **Insecticides:** The following insecticides were used:

(a) **Chlorinated hydrocarbons:**

- DDT: pp DDT, technical grade (98%) and the DDT solution

  for the larval tests were supplied by WHO.

  -Dieldrin, (Octalox): (1R, 4S, 5S, 8R)-1, 2, 3, 4, 10, 10-

  hexachloro- , 4, 4a, 5, 6, 7, 8 a-octahydro- 6, 7- epoxy-

  , 4, 4a, 5, 6, 7, 8, 8a-octahydro-6, 7-epoxy-1, 4: 5, 8-dimethanonaphthalene.

☀ (b) Pyrethroids:

  -Permethrin (PR): 3-phenoxybenzyl-(1RS)-*cis, trans*-3-

  (2, 2-. dichlorovinyl)-2, 2-dimethylcyclopropanecarboxylate

  technical grade (90 %).

  lambdacyhalothrin (ICON): 1-cyano-3-phenoxybenzyl 3-(2-chloro-

  3, 3, 3-trifluoroprop-1-enyl), technical grade (70. 2%),

  supplied by ICI.

(c) Organophosphates:

  -Pirimiphos-methyl (Actelic): 0-2-diethyl-amino-0-6-

  methylpyrimidin-4-yl 0, 0-dimethyl-phosphorothioate.

  -Chloropyrifos (Dursban): 0, 0-diethyl 0-(3, 5, 6-trichloro-2-

  pyridyl)- phosphorothioate, technical grade (94 %).

  - Malathion: S-1, 2-bis(ethoxycarbonyl)ethyl 0, 0-dimethyl

  phosphorodithioate.

  -Fenthion (Baytex): 0, 0-dimethyl 0-4-methylthio-m-tolyl

  phosphorothioate, technical grade (98. 9 %) .

  -Temephos (Abate): 0, 0, 0, 0-tetramethyl 0, 0-thiodi-*p*-

  phenylene bis(phosphorothioate).

(d) Carbamates:

  -Propoxur, (Baygon): 2-isopropoxyphenyl methylcarbamate.

(e) Synergists:

  -Piperonyl butoxide (PB): 5-(2-(2-butoxyethoxy) ethoxymethyl)-6-

propyl- 1,3-benzodioxole (90%).

-Triphenyl phosphate (TPP): (99 %)

-Chlorofenethol (DMC) : 1,1-bis(4-chlorophenyl)ethanol.

-Silicone Fluids: (DC 566), Dow Corning Ltd.

(g) Other chemicals:

-DTNB : 5,5-dithiobis(2-nitrobenzoic acid).

-Acetylcholinic iodide.

3.3 Impregnated papers

3.3.1 Pyrethroids

In order to determine the susceptibility level of adults, WHO standard impregnated papers such as malathion, propoxur, DDT and dieldrin were supplied by WHO.

Permethrin and lambdacyhalothrin impregnated papers were prepared in our laboratory according to the method of Chadwick et al., (1977), with the modification that Whatman No 1 filter paper was used rather than normal duplicating paper. The papers were cut into rectangles 15x12 cm. Acetone solutions of insecticides were diluted with an equal volume of silicone fluid (DC 566 Dow Corning) and 1.5 ml of this mixture was applied evenly by pipette onto paper at 10 and 1.2 $\mu g/cm^2$ for permethrin and lambdacyhalothrin respectively. The control papers were prepared by applying 1.5 ml of an equal volume of a mixture of acetone and silicone oil onto filter paper. These papers were stored for 4 to 5 hrs in the dark to allow the oil to spread, before being stored in a sealed container. Papers were used once within one week of impregnation.

3.3.2 Pirimiphos-methyl

Pirimiphos-methyl impregnated papers were prepared by spreading 2 ml of a 22% solution of insecticide in acetone, by means of a pipette, onto Whatman No 1 filter paper (12X15 cm) at 2.44 $\mu g/cm^2$. Control papers were prepared by applying 2 ml acetone onto the filter paper. The treated papers were allowed to dry for 15 minutes, then stored in the refrigerator in a sealed container. The pirimiphos-methyl impregnated papers were used once within 24 hours of impregnation.

3.3.3 Synergists

The synergist impregnated papers were prepared by applying 2 ml of a known concentration of synergist in acetone, by means of a pipette, onto Whatman No 1 filter paper (12 x 15 cm) to give a sub-lethal concentration, i.e. 3.6 $\mu g/cm^2$, following the method described by Hemingway (1984) and Prasittisuk & Busvine (1977).

Piperonyl butoxide (PB), a mixed function oxidase inhibitor, chlorofenethol (DMC), a dehydrochlorinase inhibitor, and triphenyl phosphate (TPP), a carboxylesterase inhibitor, were used in these studies.

3.4 Rearing Methods

Mosquito rearing and maintenance was carried out in an insectary at 27-28°C and 75-80% relative humidity, with a 12 hour photoperiod. Rearing was carried out in tap water at a temperature of 23-25°C; larvae were fed with yeast tablets for stages 1 and 2, then continued with Bemax (wheat germ flakes) for stages 3 and 4. The amount of food given dependent on the stage and number of larvae in the breeding tray.

Great care was taken to maintain uniformity of rearing conditions, and this is reflected in the comparability of replicate insecticide tests.

Adults were maintained in 30×30×30 cm cages. The females were allowed to feed on an anaesthetised guinea-pig three times a week. A pad of cotton wool soaked in 10% sucrose was placed on the top of each cage to supply sustenance for males and carbohydrate supplement for females.

The DUB-S strain was collected from larval breeding place in Dubai (U. A. E). Some 170 larvae at stages 3-4 were received and subjected to colonisation in the insectary. These larvae were first transferred into fresh water and fed with Bemax. To reduce the risk of contamination with parasites from the field, the water was changed each day for the first three days. During successive days, 167 pupae (87 females and 80 males) were collected and placed in a cage for emergence. The emerged adults were left in the cage for 4-5 days, in order to increase the mating chance.

An attempt was made to feed these females on laboratory standard animals such as Guinea-pigs, hamsters and mice. They were reluctant to feed, so had to be fed on human blood from the hand. The other difficulty encountered was with egg-laying. The females began to lay eggs after 3-4 blood meals, rather than after the second feed as with other laboratory stocks. This may have been related to the small pupae produced during the first generation, both phenomena being related to inadequacies of larval nutrition. In subsequent generations the feeding and egg-laying behaviour approached that of other laboratory stocks.

3.5 Insecticide testing methods:

3.5.1 Larvae

Larvae were tested with insecticides at the early fourth instar, in a room under controlled temperature (27- 28° C), according to the method described by WHO (1970). To obtain the required concentration of insecticide for larval tests, a known volume of technical grade insecticide was diluted in absolute ethanol; 1 ml of this solution in 249 ml of water gave the required concentration. The control larvae were treated with 1 ml ethanol. A mortality count was made after a period of 24 hr exposure to the insecticide. In permethrin tests, because of the low solubility of permethrin in water (<0.1mg tech/l), the insecticide was thoroughly stirred using a glass-rod prior to adding the larvae to the testing container. At high concentrations (greater than 10 ppm) the mixture turned cloudy, but it remained as a stable emulsion during the testing period.

In pyrethroid tests, because of the appearance of delayed mortality in a number of larvae following the exposure to insecticide, normally at high concentrations, the larvae were transferred to fresh water, then scored after a 24 hr recovery period.

In order to score the results, the total larvae (i.e. live and dead larvae) were transferred into a tray containing about 1 litre of fresh water, then the live larvae were readily collected and counted.

Uniformity of size and physiological age of the larvae were also important factors in the larval tests, i.e, smaller larvae and late developed larvae being more susceptible. A similar effect was found at the adult stage.

3.5.2 Adults

Tests on adult were carried out in a insectary under controlled condition, using the method recommended by WHO (1970). To reduce variability in the replicates, 2-3 day old sugar fed adult females and males were used. At each exposure time or concentration, 100 mosquitoes representing four individual replicates of 25 larvae or adults were tested.

Due to the knock-down effect of pyrethroid insecticides on the adults, particularly during the exposure time (i.e. early knock-down which occurred during the exposure time, and late knock-down during the holding period), some mosquitoes recovered later, during a 24 hr holding period. This was probably because they picked up an insufficient dose of insecticide to cause mortality. As a result, in selection studies, resistance gene(s) are more difficult to select, and in the WHO standard susceptibility test the probit regression line takes the form of a curve rather than a straight line (see Hemingway, 1981; Malcolm, 1983). To eliminate these problems, a modification was made to the WHO standard test in that exposure and holding tubes were placed in a horizontal position rather than the normal vertical position during both exposure and holding periods (for more details see chapter 6).

3.6 Knock-down testing method

The knock-down behaviour and recovery from knock-down, of adults of different strains exposed to permethrin and lambdacyhalothrin were determined, with the testing tubes held in the vertical position. Unfed adult females were exposed to 10 and 1.2 $\mu g/cm^2$ permethrin and

lambdacyhalothrin respectively and the knock-down count was made during the exposure time; recovery from knock-down was assessed after a period of 24 hr. The test was carried out on 4 replicates of 20-25 adult females for each strain (for more detail see section 6.3).

3.7 Synergist testing method

In an effort to investigate the mechanisms of DDT and pyrethroid resistance, and those in pirimiphos-methyl selected strains, the larvae were pretreated with a sub-lethal dose of synergists 4 hrs before treatment with insecticide, according to the method described by Ariaratnam & Georghiou, 1971). The synergists piperonyl butoxide (PB), chlorofenethol (DMC) and triphenyl phosphate (TPP) were used at sub-lethal concentrations, i.e, 10, 5 and 5 ppm respectively in these investigations.

Similarly in the adults, the adult females were pretreated with synergist at 3.6 $\mu g/cm^2$ 45 minutes before treatment with insecticides The effects of synergists were assessed by means of a synergist ratio (SR) which expressed the ratio of the LC50 of insecticide alone to the LC50 of the insecticide in the presence of the synergist. A value greater than one indicates synergism, while a value below one indicates antagonism. Similarly, a resistance ratio (RR) was calculated as the ratio of the LC50 of each strain compared with the susceptible strain.

3.8 Irritability testing method.

The irritability levels of permethrin selected and unselected strains were measured according the method described by WHO (1964).

20 unfed 2-3 day old adult females of each strain were individually

exposed to permethrin at 10 μg/cm² in an exposure chamber and the number of take-offs were counted during a 15 minute exposure time (for more details see section 6.9, irritability studies).

The differences between the number of take-offs for two strains were calculated according to the method described by Brown (1964). The mean and standard error of number of take-offs for each strain were calculated. The standard error of the difference between the means was computed as the square root of the sum of the squares of each of the two standard errors of the means. Therefore Student t was the difference between the means divided by the standard error of the difference, with $n_1 + n_2 - 2$ degree of freedom.

3.9 Selection methods:

Pirimiphos-methyl selection was carried out on adults and larvae of sub-strains derived from the IND-S and DUB-S strains respectively, and permethrin selection on adults and larvae of the two sub-strains derived from the DUB-S strain.

3.9.1 Adult selection: The sexes having been separated at the pupal stage the males and females were exposed separately and the survivors released into a cage and allowed to mate. Selection with pirimiphos-methyl and permethrin was carried out using concentrations sufficient to produce about 80 -90% mortality; further details are given in the related results section.

3.9.2 Larval selection: The DUB-LPR, IND-LPM and DUB-LPM strains were subjected to selection with permethrin and pirimiphos-methyl. Selection

was performed by exposing early fourth instar larvae for 24 hours, in lots of 200-250 larvae in 1000 ml of tap water in a tray, with one ml of insecticide solution added to give the appropriate concentration. Live larvae were then transferred into fresh water, and maintained to produce the next generation.

Initially permethrin susceptibility tests on the DUB-LPR strain showed that this population was highly heterogeneous for permethrin resistance. Therefore to avoid rapid elimination of background genetic material leading to reduced genetic variation, selection began at a moderate level (60-65% mortality) for the first two generations.

Pirimiphos-methyl selection on adults and larvae was performed at the level to give 80-90% mortality. Great care was taken at each generation of selection to ensure a large enough number of surviving mosquitoes to maintain genetic variability in the following generation. However, at least 150 adult females mosquitoes and sufficient males were supplied in the cage and allowed to breed for the next generation.

3.10 Crossing method: To obtain the hybrid F1 generation, the susceptible and resistant strains were reciprocally crossed by mass mating 150 virgin adults of each sex, the sexes being separated at the pupal stage. The single gene hypothesis was tested by reciprocal crosses of the F1 to the resistant and susceptible strains (back-crosses). To obtain the F2 generation, the F1 populations were allowed to inbreed. All these generations were tested over a wide range of insecticide concentrations. Nine to fifteen concentrations for permethrin resistant larvae and 11 to 12 concentrations for DDT resistant larvae, were used in back crosses and F2 generations with a

minimum of 100 larvae per concentration.

In another method to determine whether the inheritance of resistance was monofactorial or polyfactorial, the progeny of back-crosses were exposed to a discriminating dose of insecticide, at a level sufficient to kill heterozygotes and leave homozygous resistant larvae. Survivors were again back-crossed to the F1 hybrid for a number of successive back-crosses (i.e. back-cross with selection). If resistance is inherited monofactorially, mortality is not expected to increase over several repeated back-crosses. In the case of polyfactorial inheritance, the level of resistance should fall through successive back-crosses, giving increasing mortality (Crow, 1957). The possible involvement of one or two recessive genetic factors in the DDT resistant larvae could be shown as follows.

One gene model (recessive resistance).

```
P       *SS    X    *RR

F1             SR

F2        F1 X F1

          SR x SR

      SS     SR     RS    RR   Ratio 3: 1


BC        SR X RR

   SR     SR     RR    RR    Ratio 1: 1


BC        SR X SS

   SS     SS     RS    RS    Ratio 1: 1
```

Two gene model (recessive resistance).

P S_1S_2 X R_1R_2
        S_2S_2 R_2R_2

F1 S_1R_1
         S_2R_2

        S_1R_1 X S_1R_1
        S_1R_2 S_2R_2

F2	S_1S_2	R_1R_2	S_1S_2	S_1S_2	S_1R_2	S_1R_2
	S_1S_2	R_1R_2	R_1R_2	R_1R_2	S_1R_2	R_1R_2
	1/16	1/16	4/16	2/16	4/16	4/16
	all	live	½ die	½ die	all	½ die
	die				die	

BC S_1R_1 X R_1R_1
        S_2R_2 R_2R_2

    S_1S_2 S_1R_2 R_1S_2 R_1R_2
    R_1R_2 R_1R_2 R_1R_2 R_1R_2 Ratio 1:1

The progeny of back-crosses and F2 generations were exposed to a discriminating dose to kill susceptible and heterozygote resistant larvae
*SS = susceptible strain   *RR = resistant strain.

3.11 Statistical methods: Dosage mortality regression lines for insecticide tests and crosses were determined by the probit analysis method of Finney (1971), using the SPSSX statistical package on an IBM 3083 computer. Goodness of fit of the points to a straight line were tested by χ^2 analysis. The expected F2 segregation, on the basis of single factor Mendelian inheritance, was calculated by the formula:

X(F2)= a_1(SS)0.25 + a_2(SR)0.5 + a_3(RR)0.25. Similarly, the expected segregation of the back-crosses of F1 to both susceptible and resistant strains was calculated by the formulae X(BC)= a_2(SR)0.5 + a_1(SS)0.5 and X(BC)= a_2(SR)0.5 + a_3(RR)0.5, respectively, where X is the expected response to a given dose and a_1, a_2 and a_3 are the observed responses.

SS, SR and RR refer to susceptible IND-S, F1 hybrids, and resistants respectively, reading from their respective regression lines. The agreement of the observed response to the expected was calculated by the χ^2 method (Georghiou & Garber, 1965).

The degree of dominance was calculated by Falconer's formula as modified by Stone (1968):

$$D = \frac{2X_2 - X_1 - X_3}{X_1 - X_3}$$

where X_1, X_2 and X_3 are the log LC50's of the resistant homozygotes (RR), the heterozygotes (SR) and susceptible homozygotes (SS) respectively, and D is the degree of dominance. D = 1 indicates complete dominance; $0 < D < 1$ indicates incomplete dominance; D = -1 is completely recessive, and $-1 < D < 0$ indicates incomplete recessivity. Significant deviation between observed and estimated D values, and significant difference between of D1-D2 of progeny from reciprocal crosses F1 were tested by the t test.

$$t = \frac{D_1 - D_2}{\sqrt{V(D_1) - V(D_2)}}$$

3-12 Acetylcholinesterase assay.

Acetylcholinesterase (AchE) activity was assessed according to the method of Hemingway (1986). Individual larvae were homogenized in 1 ml of phosphate buffer (0.02 M, pH 7.5). 0.25 ml of the homogenate was used to assay normal uninhibited AChE activity, a further 0.25 ml was used for propoxur inhibition studies, and 0.25 ml for bendiocarb inhibition. Three replicate aliquots of homogenate from a single mosquito were placed in cuvettes and 10 µl of propoxur or bendiocarb solution was added to each replicate. After 1.5 min, 25 µl of the

acetylthiocholine iodide solution plus 20 μl of the DTNB solution were added to all replicates. The enzyme reaction was then allowed to run for 20 min. Then the volumes were adjusted to 1.5 ml with phosphate buffer in the cuvette and the absorbance read at 420 nm. The test was carried out for 16-20 individual mosquitoes for each strain

3.13 Electrophoretic analysis of esterase activity.

Esterase activity was studied using horizontal starch gel electrophoresis, using a modification of the method described by Townson (1969). The gels were prepared from a mixture of 22g electrostarch with 10ml Tris-DTA-maleate buffer pH 7.4 (TEM), made up to 250 ml with distilled water. This mixture was used for the preparation of two gels. Individual mosquitoes were homogenised in 10 μl of water which was absorbed onto cellulose acetate papers; these were inserted into the gel and Bromophenol blue was used as a marker. The gel was then run in TEM buffer for about 2 hrs at 200V, approximately 85 mA, until the marker had migrated about 8 cm. For detection of esterase patterns, Fast Red TR salt was used as the staining reagent and 1-naphthylacetate as a substrate, by adding 1ml of substrate (2% 1-naphthylacetate in acetone) onto a mixture of 50 mg stain and 60 ml phosphate buffer (pH 6.6, 0.066M). The gels were sliced horizontally and transferred to a sandwich box containing the substrate mixture, and incubated in a shaking water bath at 37°C for 10-15 minutes. The esterase patterns were then scored and photographed.

Chapter 4. Pirimiphos-methyl selection on the adults and larvae of two strains of <u>Anopheles stephensi</u>

4.1 Introduction

The organophosphorus (OP) compounds include a large and extremely important group of organic insecticides which demand attention. After the detection of resistance to organochlorine insecticides in anopheline mosquitoes, organophosphorus insecticides have replaced them in many areas where resistance was reported. In addition to the use of these compounds as residual insecticides, almost all the present-day larvicides used in mosquito control programmes are organophosphates. The most severe problem encountered has been the development of resistance in agricultural areas, because of the wide use of these insecticides in agricultural pest control programmes.

OP-resistance in anophelines has been reported in 31 species. (Brown, 1986). Among these, 26 species were malathion resistant, 20 fenitrothion resistant, 10 fenthion resistant, 6 chloropyrifos resistant and 5 temephos resistant in either the larvae or adults (WHO, 1987).

Acetylcholinesterase (AchE) is known to be a lethal target for organophosphorus compounds (OP). The most important resistance mechanisms involving detoxification of OP-compounds are mixed function oxidase systems; carboxylesterase as the major resistance mechanism in mosquitoes, and glutathion-dependent alkyl and aryl-transferase system, which have been demonstrated in houseflies (Plapp, 1976).

In this study, pirimiphos-methyl selection was carried out on the

adults and larvae of two strains of <u>An. stephensi</u> to investigate the development of tolerance in adults and larvae, the cross-tolerance spectrum of the selected strains to some traditional insecticides important in mosquito control programmes. and subsequently to study the mechanism of observed tolerance.

4.2 Pirimiphos-methyl selection on the adults

The adult males and females of the IND-S and DUB-S strains were separately submitted to selection with pirimiphos-methyl for 12 and 9 successive generations respectively. Under selection these strains become designated IND-APM and DUB-APM strains respectively. The results are shown in tables 4.1 to 4.4 and Figs. 4.1 to 4.6.

Selection on adult females of IND-APM strain with an original LT50 of 20.6 minutes, resulted in a gradual increase in LT50 to a maximum level of 65 minutes, i.e, a 3-fold increase in tolerance by the fifth generation (table 4.1 and Fig 4.1). Thereafter, the LT50 did not rise despite selection, and indeed showed an inexplicable dip in the F8 and F9 generations. A similar pattern was seen in the adult males (see table 4.2). Comparison between the slope of probit regression lines of selected females and males with parental stock at the F12 generation, indicated that the probit regression lines have become slightly steeper, with significant change in the slopes (d= 3.42 and 5.8 P<0.05 for selected males and females respectively)

9 generations of selection on the adult females and males of the DUB-APM strain, with initial LT50's of 23.7 and 20.6 minutes, resulted in a steady increase in the LT50 to a maximum value of 91 and 64.7 minutes respectively at the F9 generation (see tables 4.3 and 4.4 and

Fig 4.4).

Comparisons of the slope of probit regression lines of selected males and females, with parental stock, showed no significant change in their slopes (d = 1.47 and 1.4   P>0.05 respectively).

4.3 Pirimiphos-methyl selection on the larvae

The larvae of IND-LPM and DUB-LPM, two sub-strains derived from the IND-S and DUB-S strains, were subjected to pirimiphos-methyl selection for 12 and 9 generations respectively The results are shown in tables 4.5 and 4.6, and Figs 4.3 and 4.6.

Selection on larvae of the IND-LPM strain, with an initial LC50 of 0.0081 mg/l, resulted in a gradual increase in LC50 to a level of 0.024 mg/l at the F12 generation, an increase in tolerance of 3.0-fold (table 4.5, Fig 4.3).

Pirimiphos-methyl selection on larvae of the DUB-LPM strain, with an initial LC50 of 0.0144 mg/l, resulted in a steady increase in LC50 to reach a level of 0.053 mg/l at the F9 generation, an increase in tolerance of 3.7 fold compared with the DUB-S strain and some 6.5 fold that of the IND-S strain (table 4.6 and Fig 4.6). Comparison of the slopes of the probit regression lines of the IND-LPM with those of the parental stocks showed parallel lines, (d = 0.825 P > 0.05), but the slope of the DUB-LPM strain became steeper (d=3.78 P < 0.05).

4.4 Responses to pirimiphos-methyl selection on adults and larvae.

The responses to pirimiphos-methyl of the larvae of the adult-selected line, and the adults of the larval selected line were determined. The results, presented in tables 4.11 and 4.12, indicate

that adult and larval selection did not induce tolerance in larvae and adults respectively. This suggests the involvement of different genetic factors in adults and larvae of the selected strains.

4.5 Cross-resistance

The cross-tolerance spectrum of pirimiphos-methyl selected larvae and adults was determined for various OP insecticides, propoxur and for DDT. The results are shown in tables 4.13 to 4.16.

12 and 9 generations of successive selection with pirimiphos-methyl on the larvae of IND-LPM and DUB-LPM strains caused significant increases in tolerance, 1.4 and 2.1 fold to malathion, 2.3 and 1.8 fold to temephos, 1.9 and 4.4 fold to dursban, and 1.5 and 1.85 fold to fenthion respectively (tables 4.13 and 4.14). No cross-tolerance was observed when the IND-LPM strain was tested with DDT and propoxur (table 4.13).

Pirimiphos-methyl selection on adults of the IND-APM and DUB-APM strains, resulted in increases in tolerance of 1.2 and 2.13 fold to malathion, and 0.9 and 1.9-fold to propoxur respectively (See tables 4.15 and 4.16).

4.6 Synergist studies.

Piperonyl butoxide (PB) a mixed function oxidase inhibitor, and triphenyl phosphate (TPP), an inhibitor of carboxylesterase, were tested in the presence and absence of pirimiphos-methyl on the selected and unselected larvae and adults. The results are shown in the tables 4.17 and 4.18.

In both adults and larvae of the selected and unselected strains, PB had some antagonistic effect on pirimiphos-methyl with synergist ratios between 0.35 and 0.77.

TPP had no significant synergistic effect on the larvae of the IND-S, DUB-S and IND-LPM strains, but a slight synergistic effect (synergistic ratio = 1.4) was seen on larvae of the DUB-LPM strains. Similarly, in the adults, TPP had no significant synergistic effect on the IND-S, DUB-S and IND-APM strains, but a synergistic ratio of 1.5 was recorded for the DUB-APM strain (see table 4.18).

4.7 Further studies of tolerance

In order to determine whether pirimiphos-methyl tolerance behaves as a dominant character, adults and larvae of the selected strains were reciprocally crossed with unselected stocks. The F1 generations were tested with pirimiphos-methyl. The results are shown in tables 4.19 and 4.20, and Figs 4.9 and 4.10.

The F1 generations of reciprocal crosses between the selected IND-LPM, IND-APM and IND-S strains showed no significant difference in their responses to pirimiphos-methyl, their tolerance being intermediate between that of the parental strains.

Similar results were obtained when the DUB-APM strain was reciprocally crossed with the DUB-S strain (see Fig 4.9 and table 4.20). The reversion of tolerance was studied, when the selected strains were released from insecticide pressure. Among larvae of the IND-LPM strain, (LC50 =0.0242 mg/l at the F12 generation of selection), the LC50 gradually decreased over the succeeding 8 generations to around 0.018 mg/l (see table 4.21). In the absence of a high level of

resistance in the selected stocks, it was not considered worthwhile carrying out a series of back-crosses to look in detail at the mode of inheritance.

Among the adults of the IND-APM strain (LT50 = 58.2 minutes at the F12 generation of selection), the LT50 fell progressively to 25.5 minutes at the 8th generations, when the population was released from insecticide pressure (table 4.22).

Similar reversion was observed when the DUB-LPM and DUB-APM strains were released from insecticide selection pressure. Among the larvae of DUB-LPM strain (LC50 = 0.053 mg/l at the F9 generation of selection), the LC50 fell to 0.025 mg/l after 6 generations (table 4.23 and Fig 4.8). Similarly, in the adult females of the DUB-APM strain (LT50 = 91 minutes at the F9 generation of selection), The LT50 dropped to 34.3 minutes, after 6 generation, when the selected strain was released from insecticide pressure (table 4.24 and Fig 4.7).

4.8 Identification of the biochemical basis of tolerance

Resistance may be mediated by various mechanisms, the most important being metabolic break-down of the insecticide to less toxic compounds (see section 2.4.2).

The primary target of organophosphorus and carbamate compounds is acetylcholinesterase (AchE) which is present in the nervous system. The OP compounds containing a thiophosphoryl group (P=S) are usually very weak inhibitors of AchE in in vivo, unless bioactivated in the insect body by conversion of P=S bond to P=O.

Change in AchE sensitivity has been reported in mosquitoes and other insect species (WHO, 1985). The altered AchE gene in mosquitoes

was first detected in a multiple-resistant strain of <u>An. albimanus</u> from Central America (Ayad & Georghiou, 1975). This resistance mechanism was then reported in 4 anopheline and 3 culicine mosquitoes (WHO, 1985). In most cases, this mechanism has produced a broad spectrum of resistance to many organophosphorus and carbamate insecticides. Levels of resistance produced by this type of mechanism are generally higher for carbamates than for organophosphorus compounds (WHO, 1985; Hemingway, 1986). The altered AchE type mechanism in <u>An. nigerrimus</u> showed no evidence of synergism with either esterase or oxidase synergists, and no increase in the level of metabolism of malathion in resistant strains, compared with susceptible strains (Hemingway, 1986).

Esterases are also known to produce resistance to insecticides in various species and have been shown to be responsible for metabolic resistance in mosquitoes. The activity of esterases is often linked with resistance to OP compounds. The organophosphorous insecticides are inactivated and in certain cases hydrolysed by carboxylesterases.

In spite of obtaining only a moderate level of tolerance to pirimiphos-methyl in larvae and adults of the IND-S and DUB-S strains, an attempt was made to measure AchE and esterase activities in the selected strains as well as in unselected stocks.

4.9 Acetylcholinesterase assay

AchE activity was assessed according to the Hemingway method (1986) described earlier in section 3.12

The enzyme activity of individual unfed 2-3 day adult females of the selected strains were compared with unselected stocks. The tests were carried out on 16-20 mosquitoes of each strain. Acetylthiocholine

iodide was used as the substrate and bendiocarb and propoxur as the inhibitors. Absorbance was read at 420 nm. The results are shown in tables 4.25 and 4.26.

An apparent difference in colours by eye in the cuvette solution was observed when the tests were performed in the presence or absence of inhibitors. The former appeared pale, indicating that the enzyme activity was inhibited by inhibitor, while the latter was yellow, indicting   enzyme activity in the aliquot. A uniform colorimetric response was observed in this for all strains tested

Propoxur and bendiocarb showed no significant difference in their inhibitory effect for all strains tested, but significant differences were observed when the tests was carried out in the presence and absence of inhibitor (see table 4.26). There was no correlation in the AchE activity between selected and unselected strains, probably because of the absence of any specific resistance gene in the selected strains. On the other hand the IRN strain, a laboratory strain of An. stephensi, originating from IRAN, susceptible to OP compounds, showed higher enzyme activity in absence of inhibitor than the other strains. This suggests that the observed differences in enzyme activity might be due to differences in the genetic back-ground of the strains rather than the selection.

4.10 Electrophoretic analysis of non-specific esterase activity.

The non-specific esterase activity of pirimiphos-methyl selected and unselected strains was assayed using horizontal starch gel electrophoresis. Individual homogenized adults or larvae of each strain were used as the source of enzyme, and 1- naphthylacetate as a

substrate. The results are shown in Fig 4.11.

There was no obvious increase in esterase activity in the pirimiphos-methyl selected adults and larvae, compared with unselected strains, suggesting, the absence of a specific resistance gene (s) in the selected strains.
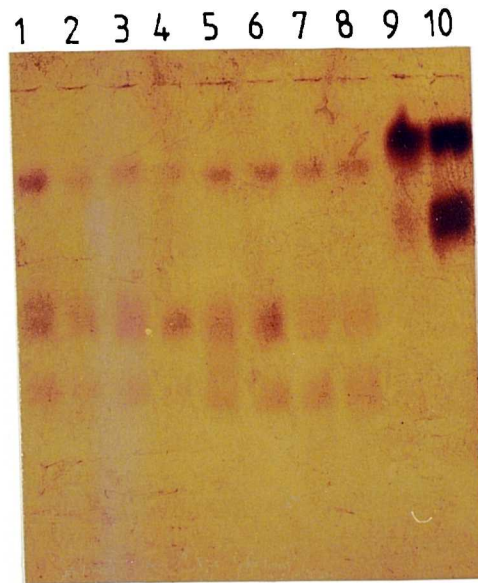
The tracks labelled above correspond to the following strain: 1 & 2, DUB-S; 3 & 4, IND-S; 5 & 6, DUB-APM; 7 & 8, IND-APM; 9 & 10, Culex quinquefasciatus, OP-resistant strain.

Fig 4.11 Non-specific esterase activity of adults of different strains of An.stephensi following starch gel electrophoresis.

4.11 Discussion

Pirimiphos-methyl selection on the larvae and adults of the IND and DUB strains was carried out for 12 and 9 successive generations respectively. Selection on the larvae produced 3.2 and 3.7 fold increases in LC50 in the IND-LPM and DUB-LPM strains respectively. Similarly in the adult females, 2.8 and 3.8 fold tolerance was recorded in the IND-APM and DUB-APM strains respectively. In the selected strains the probit regression lines showed an irregular movement in successive generations. It has often been seen that when a major gene for resistance is not present in a population, and selection results in tolerance, the probit regression lines will nearly always show irregular movement. This has also been seen in DDT, dieldrin and BHC selection in <u>Ae. aegypti</u> (Shidrawi, 1957), selection for DDT-tolerance in larvae and adults of <u>An. stephensi</u> (Davidson, 1958) and selection for malathion tolerance in adults of <u>Ae. aegypti</u> (Rees, 1983).

The larvae of the DUB-S strain initially showed more tolerance to pirimiphos-methyl than the IND-S strain, with a resistance ratio of 1.8. Susceptibility tests on the adults indicated that there was no such difference in the LT50 between the IND-S and DUB-S strains. It is often found that field strains are more insecticide-tolerant than the laboratory strains (Brown & Pal, 1971). In this case, the observed differences in the LC50's in the larvae could be due to the use of pirimiphos-methyl as a larvicide over a number of years in the malaria control programmes in the U.A.E. (Malaria Control in U.A.E, 1983)

Resistant strains developed by laboratory selection have nearly always reverted towards susceptibility on release from insecticide pressure. This is because the superior fitness of heterozygotes makes

it difficult to achieve a strain entirely homozygous for resistance factors (Crow, 1957). From published data on reversion of resistance in mosquitoes when the insecticide had been withdrawn, Curtis *et al.,* (1978) calculated the coefficient of selection against resistance genotypes which would be required to produced the observed rates of reversion. In An. culicifacies, the selection coefficient was found to be inversely related to the initial frequency of the susceptible gene. Wood & Bishop (1981) reviewed the factors influencing the reversion of resistance in the field and laboratory strains of different mosquitoes. In Ae. aegypti, a rapid reversion to susceptibility occurred when selection was relaxed in the early stages of selection. Further selection on this strain resulted in stabilization of resistance, even in the absence of insecticides. This indicates that the development of resistance was still in progress. Similarly in An. stephensi from Iran DDT resistance in the field reverted to susceptibility on withdrawal of the insecticide. Following further DDT application resistance was then stabilized (Mofidi, 1960; Zaim, 1988).

In the present study, pirimiphos-methyl tolerance reverted to susceptibility a few generations after withdrawing the insecticide. The IND-S strain has been used in this study as a laboratory stock. The effect of inbreeding on the genetic variance and gene frequency is apparent as "inbreeding depression". Inbreeding normally tends to reduce fitness, genetic variability and eventually leads to uniformity. Failure of selection for pirimiphos-methyl resistance in this strain could be mainly due to inbreeding depression and loss of genetic variability as the result of inbreeding of the strain in the laboratory.

The DUB-S strain was used in this study as a wild strain. In spite of the use of pirimiphos-methyl as an adulticide and larvicide in the U.A.E, pirimiphos-methyl selection on the adults and larvae resulted in only a slight increase in tolerance. A number of factors may explain the failure of selection for pirimiphos-methyl resistance in this strain such as: the effective population size is small (i.e., the gene pool is restricted); the genes concerned are subjected to insufficient selection pressure; the sub-population has been isolated and probably the gene(s) for resistance is rare. Therefore the observed reduction in susceptibility might be due to the interaction of multiple or ancillary genes, each of which may only have a slight effect on tolerance (i.e., there is an absence of major gene(s)).

The cross-tolerance spectra of the IND and DUB selected strains were tested with some OP insecticides. The selected adults and larvae showed 1.4 - 2.3 fold increases in tolerance to malathion, temephos, and fenthion. A greater level of tolerance was observed when the DUB-LPR strain was tested with chloropyrifos (resistance ratio = 4.4), which was found to be higher than that to pirimiphos-methyl itself. No cross-tolerance was observed when the selected strains were tested with DDT and propoxur.

The cross-resistance spectrum of the selected adults was tested with malathion and propoxur. Only the DUB-APM strain showed a moderate level of cross-tolerance, 2.1 and 1.9 fold to malathion and propoxur respectively.

Malathion resistance in a population of A. stephensi from southern Iran was found to be inherited as a single gene. Synergist tests suggested the involvement of carboxylesterase enzyme in malathion

resistance. Piperonyl butoxide (PB) had continuous antagonistic effects at all dosages tested, indicating that mixed function oxidases (MFO) were involved in the activation of malathion by conversion to toxic malaoxon (Herath & Davidson, 1981). In A. stephensi from Pakistan, synergist studies suggested the involvement of a carboxylesterase enzyme in malathion resistance. PB, a mixed function oxidases inhibitor, had a slight antagonistic effect on malathion, suggesting that carboxylesterase is probably the basis of malathion resistance in this strain (Hemingway, 1982). Larvae of A. stephensi from Pakistan which were resistant to malathion, showed cross-resistance to phenthoate, suggesting that a specific type of resistance mechanism (Malathion carboxylesterase, MCE) is responsible for that resistance (Scott & Georghiou, 1985).

In the present study, crossing experiments indicated that the observed tolerance in the adults and larvae of the two strains to pirimiphos-methyl is inherited as an autosomal intermediate character.

The activity of mixed function oxidases in the selected and unselected strains was assessed by using piperonyl butoxide (PB), a mixed function oxidase inhibitor. Pretreatment of the adults and larvae of pirimiphos-methyl selected and unselected strains with PB produced a continuous antagonism at all the dosages tested. This could be attributed to the inhibition of those oxidases which are involved in the oxidative conversion of P=S to P=O during the activation of pirimiphos-methyl to a more toxic compound. There was no evidence to suggest any MFO involvement in pirimiphos-methyl detoxification.

The esterase activity of selected and unselected strains was assessed using TPP, a carboxylesterase inhibitor. Only the DUB-LPM and

DUB-APM strains showed a slight synergistic effect. This suggests the possibility of low esterase activity in these strains.

When the non-specific esterase activity of pirimiphos-methyl selected and unselected strains was compared, there was no obvious increase in esterase patterns of selected and unselected strains.

The AchE activity of the unselected stocks and selected strains was compared. Propoxur and bendiocarb inhibited the activity of AchE in both selected and unselected strains. However, higher AchE activity was recorded for the IRN strain in the absence of inhibitor, compared with unselected and selected strains. There was clearly no correlation between enzyme activity in selected and unselected strains. This indicated that the observed differences in enzyme activity might be due to differences in the genetic background of strains rather than to pirimiphos-methyl selection.

The response to pirimiphos-methyl of the larvae of the adult selected line and the adults of the larval selected line, were determined. The results indicate that different genetic factors are responsible for the observed tolerance in adults and larvae.

Table 4.1 Probit regression line parameters of successive generations of adult females of An. stephensi (IND-APM strain) under pirimiphos-methyl selection.

Genera	Slope ± S.E	Y intercept ± S.E	X^2 (df)	P	LT50 (min)	95% C.L	LT90 (min)	95% C.L
IND-S	4.080 ± 0.114	-0.362 ± 0.158	6.105 (4)	0.191	20.62	20.02 21.23	42.508	40.76 44.50
F1	3.879 ± 0.363	-0.557 ± 0.526	4.797 (4)	0.309	27.07	24.61 29.78	57.925	50.00 70.79
F2	3.043 ± 0.339	1.0393 ± 0.441	4.119 (4)	0.390	20.03	17.74 22.73	52.825	42.69 72.36
F3	5.652 ± 0.393	-3.449 ± 0.6143	7.690 (3)	0.053	31.26	26.15 35.93	52.692	44.89 59.07
F4	5.366 ± 0.395	-3.697 ± 0.684	2.073 (3)	0.557	41.78	39.44 44.20	72.404	66.53 80.57
F5	7.740 ± 0.544	-9.029 ± 1.0014	7.903 (4)	0.095	64.95	59.58 70.20	95.094	86.06 110.87
F6	9.618 ± 0.738	-11.821 ± 1.310	6.340 (3)	0.096	56.10	50.61 61.24	76.241	68.83 90.86
F7	7.115 ± 0.538	-7.456 ± 0.956	5.184 (3)	0.159	56.33	53.78 58.87	85.28	80.08 92.33
F8	7.018 ± 0.504	-5.634 ± 0.767	2.247 (3)	0.532	32.74	31.14 34.43	49.855	46.47 54.42
F9	5.713 ± 0.419	-2.468 ± 0.557	4.657 (3)	0.199	20.29	19.14 21.44	34.008	31.32 37.70
F10	7.025 ± 0.477	-7.004 ± 0.83	0.730 (4)	0.948	51.14	48.79 53.50	77.841	73.29 83.77
F11	8.572 ± 0.673	-10.045 ± 1.188	2.799 (3)	0.424	56.88	54.87 58.92	80.256	76.00 86.10
F12	7.785 ± 0.629	-8.741 ± 1.115	1.686 (3)	0.640	58.24	56.05 60.48	85.081	80.01 92.21

Table 4.2 Probit regression line parameters of successive generations of
adult males of An. stephensi (IND-APM strain) under pirimiphos-
methyl selection.

Gener	Slope ± S.E	Y intercept ± S.E	X² (df)	P	LT50 (min)	95% C.L	LT90 (min)	95% C.L
IND-S	4.068 ± 0.1574	-0.2723 ± 0.2157	4.889 (4)	0.299	19.77	18.95 20.59	40.83	38.54 43.55
F1	3.955 ± 0.317	-0.382 ± 0.451	4.364 (5)	0.498	22.95	21.09 24.85	48.41	43.16 56.10
F2	3.399 ± 0.402	0.568 ± 0.538	5.204 (3)	0.157	20.13	17.82 22.64	47.97	39.72 63.65
F3	5.051 ± 0.394	-1.795 ± 0.578	3.650 (3)	0.302	22.14	20.24 23.93	39.71	36.58 43.80
F4	4.933 ± 0.367	-2.1896 ± 0.543	3.965 (3)	0.265	28.68	26.96 30.48	52.16	47.54 58.69
F5	5.843 ± 0.452	-4.759 ± 0.781	3.655 (3)	0.301	47.09	44.41 49.67	78.10	72.71 85.46
F6	5.761 ± 0.455	-4.438 ± 0.778	2.681 (3)	0.443	43.50	40.80 45.98	72.55	67.60 79.26
F7	5.561 ± 0.453	-3.992 ± 0.770	1.197 (3)	0.754	41.41	38.66 43.95	70.40	65.48 77.11
F8	5.964 ± 0.427	-3.780 ± 0.634	3.841 (3)	0.279	29.66	28.07 31.34	48.65	44.96 53.68
F9	5.879 ± 0.432	-2.765 ± 0.578	4.160 (3)	0.245	20.93	19.78 22.14	34.58	31.89 38.26
F10	6.086 ± 0.390	-4.983 ± 0.655	2.790 (4)	0.594	43.70	41.46 45.95	70.96	66.40 76.85
F11	5.618 ± 0.442	-4.367 ± 0.764	2.984 (3)	0.394	46.48	43.73 49.11	78.60	73.00 86.29
F12	5.686 ± 0.446	-4.435 ± 0.769	0.088 (3)	0.993	45.66	42.93 48.23	76.70	71.34 84.03

Table 4.3 Probit regression line parameters of successive generations of adult females of <u>An. stephensi</u> (DUB-APM strain) under pirimiphos-methyl selection.

Gener	Slope ± S.E	Y intercept ± S.E	X² (df)	P	LT50 (min)	95% C,L	LT90 (min)	95% C,L
DUB-S	6,100 ± 0,410	-3,392 ± 0,567	1,506 (3)	0,681	23,75	22,51 25,07	38,52	35,68 42,33
F1	6,781 ± 0,502	-4,853 ± 0,739	3,048 (3)	0,384	28,38	27,02 29,78	43,86	40,96 47,77
F2	7,128 ± 0,503	-6,064 ± 0,881	2,325 (3)	0,508	35,68	34,17 37,20	53,99	50,66 58,60
F3	8,805 ± 0,587	-8,42 ± 0,98	7,385 (3)	0,061	45,59	40,79 50,69	65,67	57,73 82,99
F4	7,393 ± 0,553	-8,253 ± 0,994	2,424 (3)	0,489	62,02	59,44 64,63	92,44	86,77 100,19
F5	6,651 ± 0,518	-6,759 ± 0,93	0,385 (3)	0,496	61,08	58,44 64,09	91,34	85,36 99,57
F6	6,957 ± 0,501	-7,07 ± 0,878	1,282 (3)	0,733	54,30	51,85 56,82	82,99	77,74 90,06
F7	8,263 ± 0,583	-9,556 ± 1,033	2,335 (3)	0,502	57,90	55,56 60,33	82,75	78,12 88,87
F8	7,609 ± 0,570	-9,456 ± 1,085	2,545 (3)	0,467	79,44	76,28 82,71	117,08	110,05 126,67
F9	7,065 ± 0,510	-8,839 ± 1,002	4,586 (3)	0,205	90,97	87,07 95,06	138,14	129,29 150,16

Table 4.4 Probit regression line parameters of successive generations of adult males of <u>An. stephensi</u> (DUB-APM strain) under pirimiphos-methyl selection.

Gener	Slope ± S.E	Y intercept ± S.E	X^2 (df)	P	LT50 (min)	95% C.L	LT90 (min)	95% C.L
DUB-S	5,726 ± 0.387	-2,524 ± 0.504	4,726 (3)	0,193	20,61	19,50 21,83	34,51	31,70 38,35
F1	5,230 ± 0.382	-1,841 ± 0.507	1,272 (3)	0,736	20,33	19,08 21,64	35,74	32,65 40,04
F2	6,924 ± 0.513	-5,262 ± 0.772	0,661 (3)	0,882	30,34	28,93 31,77	46,47	43,52 50,46
F3	8,531 ± 0.612	-8,677 ± 0.985	0,692 (3)	0,875	40,11	38,55 41,72	56,69	53,61 60,76
F4	7,782 ± 0.612	-8,581 ± 1.079	2,253 (3)	0,522	55,61	53,44 57,77	81,25	76,64 87,60
F5	6,900 ± 0.508	-6,875 ± 0.876	0,876 (3)	0,831	52,60	50,25 55,06	80,67	75,29 88,02
F6	6,227 ± 0.456	-5,424 ± 0.767	1,256 (3)	0,740	47,20	44,81 49,70	75,81	70,24 83,44
F7	7,002 ± 0.515	-6,633 ± 0.856	2,012 (3)	0,570	45,85	43,70 48,15	69,89	65,06 76,48
F8	7,316 ± 0.546	-8,266 ± 0.989	5,888 (3)	0,117	65,07	58,89 72,13	97,40	85,19 123,51
F9	6,582 ± 0.475	-6,921 ± 0.873	10,140 (3)	0,017	64,74	54,49 75,37	101,38	85,18 143,57

Table 4.5 Probit regression line parameters of successive generations of larvae of <u>An. stephensi</u> (IND-LPM strain) under pirimiphos- methyl selection.

Gener	Slope ± S.E	Y intercept ± S.E	X² (df)	P	LC50 mg/l	95% C.L	LT90 mg/l	95% C.L
IND-S	5,156 ± 0.274	15,796 ± 0.559	4,710 (3)	0,194	0,0081	0,0077 0.0084	0,014	0,044 0.015
F1	4,932 ± 0.411	14,547 ± 0.798	1,316 (3)	0,725	0,012	0,011 0.012	0,021	0,019 0.024
F2	5,315 ± 0.576	15,217 ± 1.096	2,594 (3)	0,458	0,119	0,011 0.013	0,021	0,019 0.025
F3	5,371 ± 0.351	15,555 ± 0.689	1,992 (3)	0,574	0,011	0,010 0.011	0,018	0,017 0.021
F4	5,299 ± 0.325	14,494 ± 0.575	0,788 (3)	0,852	0,016	0,015 0.017	0,028	0,026 0.031
F5	7,265 ± 0.535	19,206 ± 1.037	2,049 (4)	0,727	0,011	0,011 0.012	0,017	0,016 0.018
F6	4,284 ± 0.320	12,787 ± 0.584	1,490 (4)	0,828	0,015	0,014 0.016	0,030	0,027 0.035
F7	5,584 ± 0.387	14,851 ± 0.677	4,797 (4)	0,543	0,015	0,014 0.073	0,028	0,024 0.036
F8	5,106 ± 0.085	13,861 ± 0.597	3,089 (4)	0,543	0,017	0,016 0.018	0,029	0,027 0.032
F9	5,374 ± 0.374	13,939 ± 0.626	3,696 (4)	0,449	0,022	0,021 0.023	0,038	0,035 0.042
F10	5,079 ± 0.365	13,23 ± 0.606	1,011 (4)	0,908	0,023	0,022 0.025	0,042	0,038 0.047
F11	6,772 ± 0.491	15,726 ± 0.790	3,658 (4)	0,454	0,026	0,025 0.027	0,040	0,038 0.044
F12	5,620 ± 0.451	14,079 ± 0.735	0,543 (3)	0,909	0,024	0,023 0.026	0,041	0,038 0.046

Table 4.6 Probit regression line parameters of successive generations of larvae of <u>An. stephensi</u> (DUB-LPM strain) under pirimiphos- methyl selection.

Gener	Slope ± S.E	Y intercept ± S.E	X^2 (df)	P	LC50 mg/l	95% C.L	LC90 mg/l	95% C.L
DUB-S	4,661 ± 0.344	13,590 ± 0.684	2,142 (3)	0,543	0,0144	0,0134 0.0154	0,0270	0,0243 0.0321
F1	6,081 ± 0.468	15,889 ± 0.837	2,422 (3)	0,490	0,0162	0,0154 0.0170	0,0263	0,0244 0.0290
F2	6,294 ± 0.475	9,84 ± 0.373	3,732 (3)	0,292	0,0170	0,0162 0.0179	0,0272	0,0252 0.0300
F3	7,135 ± 0.546	16,993 ± 0.895	4,095 (3)	0,251	0,0229	0,022 0.024	0,0343	0,0322 0.0372
F4	7,555 ± 0.617	15,686 ± 0.855	3,080 (3)	0,379	0,0385	0,0368 0.040o	0,0570	0,0537 0.0603
F5	7,809 ± 0.628	15,884 ± 0.867	0,863 (3)	0,834	0,0404	0,0388 0.0419	0,0589	0,0556 0.0636
F6	5,09 ± 0.465	12,387 ± 0.651	8,374 (3)	0,039	0,0235	0,0280 0.0411	0,0632	0,0521 0.1008
F7	6,826 ± 0.549	14,620 ± 0.76	5,657 (3)	0,130	0,0390	0,0346 0.0430	0,0601	0,0529 0.0755
F8	8,078 ± 0.581	15,556 ± 0.759	2,403 (3)	0,493	0,0494	0,0474 0.0513	0,0711	0,0672 0.0764
F9	7,076 ± 0.539	14,025 ± 0.695	6,486 (3)	0,090	0,0530	0,0476 0.0594	0,0805	0,0700 0.1056

Table 4.7 Summary of pirimiphos-methyl selection on larvae of the

DUB-LPM strain.

Generation	Insecticide conc, mg/l	No, individuals exposed	No, survived	Selection pressure % mortalities
P	0,025	2814	454	83,87
F1	0,025	2501	410	83,61
F2	0,025	2890	522	81,94
F3	0,028	2456	438	82,17
F4	0,05	1500	275	81,67
F5	0,055	2406	370	84,62
F6	0,053	3514	560	84,06
F7	0,053	3233	670	79,28
F8	0,065	3183	410	87,12
F9	0,07	4266	598	85,98

Table **4.8** Summary of pirimiphos-methyl selection on adult females and males of the DUB-APM strain.

		♂				♀		
Gene,	Exposure time (min)	No, individuals exposed	No, survived	Selection pressure % mortalities	Exposure time (min)	No, individuals exposed	No, survived	Selection pressure % mortalities
P	34	1940	395	79,64	40	1954	409	79,07
F1	35	1274	198	84,46	40	1243	223	82,06
F2	40	1043	199	80,92	45	1037	204	80,33
F3	50	841	139	83,47	55	830	155	81,33
F4	70	1084	229	78,87	80	1091	218	80,02
F5	70	1148	172	84,98	85	1148	172	85,02
F6	65	1429	264	81,53	80	11469	205	86,05
F7	65	1873	260	86,12	80	1893	298	84,26
F8	90	1589	255	83,95	100	1628	237	85,44
F9	100	1594	179	88,77	130	1552	204	86,86

Table **4.9** Summary of pirimiphos-methyl selection on larvae of
the IND-LPM strain.

Generation	Insecticide mg/l	No, individuals exposed	No, survived	Selection pressure % mortalities
P	0,012	1870	340	81,82
F1	0,015	1522	269	82,33
F2	0,018	3169	525	83,43
F3	0,018	5050	703	86,10
F4	0,028	2187	270	87,65
F5	0,018	2974	354	89,0
F6	0,028	2835	348	87,73
F7	0,028	5156	720	86,04
F8	0,028	6702	1010	84,93
F9	0,03	4718	805	82,94
F10	0,035	7070	1268	82,07
F11	0,035	3855	755	80,42

Table 4.10 Summary of pirimiphos-methyl selection on adult females and

males of the IND-APM strain.

Gene.	♂				♀			
	Exposure time (min)	No, individuals exposed	No, survived	Selection pressure % mortalities	Exposure time (min)	No, individuals exposed	No, survived	Selection pressure % mortalities
P	35	768	179	76,69	40	633	164	74,09
F1	40	583	163	79,42	45	686	120	76,24
F2	35	965	179	81,45	40	748	131	82,49
F3	40	1260	122	90,32	50	1631	188	88,47
F4	70	1590	161	89,87	65	1610	141	91,24
F5	60	1916	249	87,0	80	1757	229	86,97
F6	60	3327	447	85,66	70	2095	295	85,92
F7	35	2223	296	86,68	70	2368	289	87,80
F8	30	1411	142	89,94	40	1444	198	86,29
F9	60	1496	152	89,84	35	1394	168	87,95
F10	65	1035	855	82,61	70	1049	850	81,03
F11	70	1356	255	81,19	75	1401	254	81,87

Table 4.11 The effect of pirimiphos-methyl selection of adults on the tolerance of larvae of <u>An. stephensi</u> (IND-APM and DUB-APM).

Strains		LC50 95% C.L	LC90 95% C.L	Slope (b)	X^2 (df)	P	Resistance ratio (RR)
IND	IND-S	0.0077	0.0135	5.156 ±	4.710	0.194	
		0.0081	0.0143	0.274	(3)		
		0.0084	0.0153				
	IND-APM	0.0104	0.0176	5.377 ±	2.034	0.730	1.36
		0.0110	0.0190	0.365	(4)		
		0.0116	0.0210				
DUB	DUB-S	0.0134	0.0243	6.661 ±	2.142	0.543	
		0.0144	0.0270	0.034	(3)		
		0.0164	0.0311				
	DUB-APM	0.0130	0.0216	5.481 ±	1.116	0.773	0.97
		0.0140	0.0235	0.438	(3)		
		0.0145	0.0260				

Resistance ratio = Ratio of LC50 of the selected strain to LC50 of the parental stock.

*The figures in the first line for each species represent the Lower C.L, in the middle lines LC50/LC90, and in the third lines Uper C.L.

Table 4.12 Effect of pirimiphos-methyl selection of larvae on the tolerance of adult males and females of <u>An. stephensi</u> (IND-LPM and DUB-LPM).

Strains		LC50 95% C,L	LC90 95% C,L	Slope (b)	X^2 (df)	P	Resistance ratio (RR) *
IND	IND-S♀	20,16 20,62 21,23	44,49 42,51 50,76	4,079± 0,114	6,105 (4)	0,191	-
	IND-S♂	18,95 19,77 20,59	38,54 40,83 43,55	4,068± 0,157	4,889 (4)	0,299	-
	IND-LPM♀	22,53 23,97 25,44	38,50 41,84 46,40	5,297± 0,380	3,505 (4)	0,477	1,16
	IND-LPM♂	19,08 20,52 21,98	36,52 40,09 44,95	4,407± 0,309	4,234 (4)	0,375	1,04
DUB	DUB-S♀	22,51 23,75 25,07	35,68 38,52 42,33	6,1003± 0,4104	1,506 (3)	0,681	
	DUB-S♂	18,95 19,77 20,59	38,54 40,83 43,55	4,068± 0,1574	4,889 (4)	0,299	-
	DUB-LPM♀	22,86 24,13 25,42	36,80 39,76 43,85	5,907± 0,445	1,317 (3)	0,725	1,02
	DUB-LPM♂	20,90 23,37 25,95	31,16 35,53 44,23	7,042± 0,525	5,338 (3)	0,149	1,18

Resistance ratio = Ratio of LC50 of the selected strain to LC50 of the parental stock.

Table 4.13 The cross-tolerance spectrum of pirimiphos-methyl selected larvae of IND-LPM strain to different insecticides.

Insecticides tested	Strains	LC50 95% C.L	LC90 95% C.L	Slope ± S.E	X² (df)	P	Resistance ratios(RR) *
malathion	IND-S	0.091 0.097 0.103	0.162 0.177 0.198	4.929± 0.381	0.370 (3)	0.946	1.41
	IND-LPM	0.128 0.137 0.145	0.224 0.245 0.274	5.069± 0.394	0.910 (3)	0.823	
temephos	IND-S	0.0014 0.0018 0.0023	0.0027 0.0035 0.0055	3.469± 0.247	6.196 (3)	0.102	2.28
	IND-LPM	0.0038 0.0041 0.0045	0.0082 0.0093 0.0109	3.634± 0.277	0.221 (3)	0.974	
chloropy-rifos	IND-S	0.00082 0.00088 0.00094	0.00147 0.00162 0.00182	4.816± 0.363	2.778 (3)	0.427	1.92
	IND-LPM	0.00158 0.00169 0.00180	0.0027 0.0030 0.0034	5.077± 0.373	0.784 (3)	0.583	
fenthion	IND-S	0.0055 0.0058 0.0060	0.0079 0.0084 0.0090	7.889± 0.646	3.408 (3)	0.330	1.51
	IND-LPM	0.00846 0.00874 0.00903	0.0112 0.0118 0.0126	9.807± 0.722	1.969 (3)	0.579	
DDT	IND-S	0.026 0.029 0.031	0.0629 0.0718 0.0846	3.189± 0.215	2.170 (4)	0.705	0.93
	IND-LPM	0.024 0.027 0.029	0.0585 0.0667 0.0784	3.412± 0.317	0.797 (4)	0.586	
propoxur	IND-S	0.40 0.42 0.44	0.65 0.70 0.77	5.74± 0.383	2.619 (4)	0.623	0.98
	IND-LPM	0.43 0.41 0.45	0.76 0.69 0.84	5.169± 0.355	1.375 (4)	0.849	

Resistance ratio = Ratio of LC50 of the selected strain to LC50 of the parental stock.

Table 4. 14 The cross tolerance spectrum of pirimiphos-methyl selected

larvae of the DUB-LPM strain to different insecticides.

Insecticides tested	Strains	LC50 95% C,L	LC90 95% C,L	Slope ± S,E	X^2 (df)	P	Resistance ratios(RR)
malathion	DUB-S	0,123 0,131 0,138	0,207 0,225 0,251	5,404± 0,422	2,309 (3)	0,511	2,05
	DUB-LPM	0,253 0,268 0,285	0,438 0,478 0,436	5,101± 0,387	2,317 (3)	0,509	
temephos	DUB-S	0,0023 0,0026 0,0028	0,0058 0,0068 0,0082	3,052± 0,232	2,475 (3)	0,480	1,77
	DUB-LPM	0,0042 0,0046 0,0051	0,0098 0,0113 0,0135	3,308± 0,241	4,133 (3)	0,248	
chloropy-rifos	DUB-S	0,00138 0,00130 0,00147	0,0023 0,0026 0,0029	4,795± 0,371	4,005 (3)	0,261	4,38
	DUB-LPM	0,0055 0,0057 0,0060	0,0079 0,0084 0,0090	7,89± 0,646	3,408 (3)	0,333	
fenthion	DUB-S	0,0071 0,0073 0,0076	0,0092 0,0097 0,0103	10,539± 0,777	5,087 (3)	0,166	1,85
	DUB-LPM	0,0125 0,0135 0,0146	0,0163 0,0179 0,0212	10,539± 0,758	6,006 (3)	0,111	

Resistance ratio = Ratio of LC50 of selected strain to LC50 of the DUB-S strain,

Table 4.15 The cross tolerance spectrum of pirimiphos-methyl selected

adults of the IND-APM strain to different insecticides.

Insecticides tested	Strains	LT50 95% C.L	LT90 95% C.L	Slope ± S.E	X² (df)	P	Resistance ratios(RR)
malathion	IND-S	17.50 19.20 21.17	29.51 33.75 40.57	5.231± 0.521	4.707 (3)	0.195	1.21
	IND-APM	24.63 23.20 26.07	42.65 39.33 47.20	5.375± 0.397	4.621 (3)	0.202	
propoxur	IND-S	15.78 16.73 17.76	20.73 22.38 24.90	10.133± 1.108	2.739 (4)	0.602	0.89
	IND-APM	17.25 14.91 19.48	26.78 23.14 34.98	6.705± 0.498	7.602 (3)	0.055	

Table 4. 16 The cross tolerance spectrum of pirimiphos-methyl selected

adults of the DUB-APM strain to different insecticides.

Insecticides tested	Strains	LT50 95% C.L	LT90 95% C.L	Slope ± S.E	X^2 (df)	P	Resistance ratios(RR)
malathion	DUB-S	25,70 27,28 28.77	42,08 45,37 49.98	5,797± 0,478	4,082 (3)	0,253	2,13
	DUB-APM	53,15 58,14 63.75	73,22 82,24 100.41	8,509± 0,624	5,773 (3)	0,123	
propoxur	DUB-S	16,73 20,33 24.63	28,38 35,45 55.93	5,308± 0,391	9,589 (3)	0,022	1,92
	DUB-APM	36,84 39,11 41.51	62,95 68,89 77.20	5,212± 0,383	4,056 (3)	0,255	

Resistance ratio = Ratio of LT50 of the selected strain to LT50 of the DUB-S strain.

Table 4.17 Effect of synergists PB and TPP on larvae of different strains of An.stephensi.

Insecticide/ synergist	Strains	LC50 95% C.L	LC90 95% C.L	Slope ± S.E	X^2 (df)	P	Resistance ratio (RR)	Synergist ratio (SR)%
*PM	IND-S	0.0077 0.0081 0.0084	0.0135 0.0143 0.0153	5.156± 0.274	4.710 (3)	0.194	-	-
PM+PB	IND-S	0.0216 0.0229 0.0244	0.0364 0.0400 0.0448	5.321± 0.386	0.754 (3)	0.860	-	0.35
PM+TPP		0.0079 0.0089 0.0110	0.0139 0.0153 0.0168	5.177± 0.401	2.419 (3)	0.490	-	0.91
PM	IND-LPM	0.0193 0.0203 0.0214	0.0293 0.0316 0.0346	6.179± 0.485	2.347 (3)	0.503	2.51	-
PM+PB	IND-LPM	0.0417 0.0435 0.0452	0.0606 0.0644 0.0697	7.498± 0.578	4.129 (3)	0.248	-	0.47
PM+TPP		0.0197 0.0210 0.0220	0.0330 0.0359 0.0400	5.454± 0.451	1.373 (3)	0.712	-	0.97
PM	DUB-S	0.0134 0.0144 0.0164	0.0243 0.027 0.0311	4.661± 0.344	2.142 (3)	0.543	-	-
PM+PB	DUB-S	0.0290 0.0306 0.0324	0.0466 0.0504 0.0557	5.923± 0.430	2.825 (3)	0.419	-	0.47
PM+TPP		0.0108 0.0115 0.0123	0.0192 0.0211 0.0238	4.895± 0.369	1.917 (3)	0.590	-	1.25
PM	DUB-LPM	0.00476 0.0530 0.0594	0.0700 0.0C05 0.1055	7.076± 0.539	6.486 (3)	0.090	3.68	-
PM+PB	DUB-LPM	0.112 0.105 0.096	0.148 0.159 0.168	5.885± 0.439	3.403 (3)	0.334	-	0.50
PM+TPP		0.0314 0.0385 0.0463	0.048 0.057 0.066	6.458± 0.489	4.015 (3)	0.260	-	1.4

* Synergist ratio = Ratio of LC50 of insecticide alone to LC50 of insecticide in the presence of synergist.

* Pirimiphos-methyl

Table 4.18   Effect of synergists PB and TPP on adult females of

different strains of <u>An.stephensi</u>

Insecticide/ synergist	Strains	LT50 95% C.L	LT90 95% C.L	Slope ± S.E	X^2 (df)	P	Resistance ratio (RR) [1]	Synergist ratio (SR) [1]
PM		20.02 20.62 21.23	40.76 42.51 44.49	4.08± 0.114	6.105 (4)	0.191	-	-
PM+PB	IND-S	34.46 37.10 39.97	66.88 74.60 85.34	4.223± 0.280	2.959 (4)	0.565	-	0.56
PM+TPP		18.55 20.01 21.49	36.80 40.55 45.67	4.179± 0.292	4.797 (4)	0.309	-	1.03
PM		56.73 58.98 61.36	79.80 84.70 91.32	8.154± 0.594	0.832 (3)	0.842	2.86	-
PM+PB	IND-APM	73.25 76.58 80.07	111.91 119.61 129.82	6.617± 0.435	2.659 (4)	0.616	-	0.77
PM+TPP	—	43.50 46.35 48.99	74.68 81.26 91.05	5.256± 0.4805	0.690 (3)	0.875	-	1.27
PM		22.51 23.75 25.07	35.68 38.52 42.33	6.1003± 0.4104	1.506 (3)	0.681	-	-
PM+PB	DUB-S	34.66 36.18 37.71	51.20 54.52 59.09	7.196± 0.554	4.686 (3)	0.196	-	0.66
PM+TPP		18.69 19.77 20.90	30.47 33.08 36.67	5.732± 0.414	2.176 (3)	0.537	-	1.20
PM		87.07 90.97 95.06	129.29 138.14 150.16	7.065± 0.510	4.586 (3)	0.205	3.83	-
PM+PB	DUB-APM	142.88 148.78 154.80	204.92 217.40 234.25	7.780± 0.576	3.660 (3)	0.301	-	0.61
		57.56 60.08 62.65	85.26 90.93 98.73	7.121± 0.540	4.770 (3)	0.189	-	1.51

[1] Resistance ratio = Ratio of LT50 of the selected strain to LC50 of the parental stock.
[1] Synergist ratio = Ratio of LT50 of insecticide alone to LC50 of insecticide in presence of synergist for each strain.

Table 4.19   Pirimiphos-methyl tests on larvae of the F1 progeny from

crosses between pirimiphos-methyl selected larvae (IND-LPM)

and parental stock

Strains	LC50 95% C.L	LC90 95% C.L	Slope± S.E	X^2 (df)	P	Resistance ratio (RR)
IND-S	0,0077 0,0081 0,0084	0,0135 0,0143 0,0153	5,156± 0,274	4,710 (3)	0,194	-
IND-LPM	0,0230 0,0242 0,0255	0,0375 0,0410 0,0460	5,62± 0,451	0,543 (3)	0,909	2,99
F1 *R x *S ♂ ♀	0,0121 0,0128 0,0135	0,0192 0,0207 0,0228	6,123± 0,448	1,308 (3)	0,727	1,58
F1 *R x *S ♀ ♂	0,0129 0,0136 0,0144	0,0214 0,0233 0,0259	5,505± 0,414	3,646 (3)	0,302	1,68

Resistance ratio= Ratio of LC50 of the F1 progeny/IND-LPM strain to LC50 of the IND-S strain,
*R = IND-LPM, *S = IND-S

Table 4.20   Pirimiphos-methyl tests on adults of the F1 progeny from crosses between pirimiphos-methyl selected adults and parental stocks

Strains	LT50 95% C,L	LT90 95% C,L	Slope± S,E	X^2 (df)	P	Resistance ratio (RR)
IND-S	20,02 20,62 21,23	40,76 42,51 44,49	4,080± 0,114	6,105 (4)	0,191	-
IND-APM	56,05 58,24 60,89	80,01 85,08 92,21	7,785± 0,629	1,686 (3)	0,640	2,82
F1 R✻ x S✻ ♂ ♀	29,97 31,79 33,68	50,23 54,66 60,77	5,445± 0,396	2,184 (3)	0,535	1,54
R x S ♀ ♂	25,94 30,64 35,84	45,25 55,13 79,23	5,023± 0,371	6,950 (3)	0,074	1,49
DUB-S	22,51 23,75 25,07	35,68 38,52 42,33	6,1003± 0,4104	1,506 (3)	0,074	-
DUB-APM	87,07 90,97 95,06	129,29 138,14 150,16	7,065± 0,510	4,586 (3)	0,205	3,83
F1 D✻ x I✻ ♂ ♀	33,64 38,50 43,96	55,75 66,08 87,89	5,462± 0,396	5,515 (3)	0,138	1,62
D x I ♀ ♂	36,09 38,45 40,94	64,27 70,77 80,02	4,837± 0,363	1,525 (3)	0,677	1,61

✻R= IND-APM,   ✻S= IND-S   ✻D= DUB-APM   ✻I= DUB-S

Table 4.21   Reversion of tolerance in larvae of the IND-LPM strain in

successive generations after release from insecticide pressure.

Gen, after released	LC50 95% C,L	LC90 95% C,L	Slope± S,E	X^2 (df)	P	Resistance ratios(RR)*
F2	0,020 0,022 0,023	0,036 0,040 0,046	4,733± 0,362	4,034 (3)	0,258	2,72
F4	0,016 0,018 0,021	0,025 0,030 0,041	6,164± 0,460	7,014 (3)	0,071	2,22
F6	0,016 0,017 0,018	0,026 0,029 0,032	5,574± 0,433	0,735 (3)	0,865	2,1
F8	0,016 0,018 0,019	0,028 0,031 0,035	5,192± 0,39	0,897 (3)	0,826	2,22

* Resistance ratio; Ratios of LC50 of each generation to LC50 of the IND-S strain,

Table 4.22 Reversion of tolerance in adults of the IND-APM strain in successive generations after release from insecticide pressure.

Gen, after released	LT50 95% C,L	LT90 95% C,L	Slope± S,E	X^2 (df)	P	Resistance ratios(RR)*
F2	45,19 47,41 49,76	71,25 77,26 86,00	6,043± 0,492	3,468 (3)	0,325	2,30
F4	38,05 40,43 43,01	66,36 73,24 83,13	4,996± 0,376	2,593 (3)	0,459	1,96
F6	37,84 40,38 43,16	69,59 77,70 89,74	4,508± 0,356	0,489 (3)	0,921	1,96
F8	24,03 25,51 27,02	40,83 44,45 49,52	5,312± 0,403	1,878 (3)	0,598	1,24

* Resistance ratio; Ratio of LT50 of each strain to LT50 of the IND-S strain

Table 4.23 Reversion of tolerance in adults of the DUB-LPM strain in successive generations after release from insecticide pressure.

Gen, after released	LC50 95% C,L	LC90 95% C,L	Slope± S,E	X^2 (df)	P	Resistance ratios(RR)*
F2	0,044 0,041 0,047	0,069 0,063 0,077	6,693± 0,671	4,950 (4)	0,92	2,85
F4	0,037 0,034 0,039	0,059 0,054 0,066	6,246± 0,611	3,327 (4)	0,505	2,36
F6	0,027 0,025 0,029	0,039 0,036 0,044	7,624± 0,742	3,737 (3)	0,291	1,74

Resistance ratio = Ratio of LC50 of each generation to LC50 of the DUB-S strain

Table 4.24 Reversion of tolerance in adults of the DUB-APM strain

in successive generations after release

from insecticide pressure.

Gen, after released	LT50 95% C,L	LT90 95% C,L	Slope± S,E	χ^2 (df)	P	Resistance ratios(RR)*
F2	75,86 71,72 88,25	112,47 103,77 125,40	7,493± 0,710	5,869 (4)	2,09	3,02
F4	63,16 59,63 66,73	92,49 85,88 102,02	7,737± 0,725	3,518 (4)	0,475	2,51
F6	38,17 34,34 41,69	68,23 61,72 77,79	5,08± 0,517	3,184 (4)	0,527	1,45

Resistance ratio = Ratio of LT50 of each generation to LT50 of the DUB-S strain,

Table 4.25 Activity of AchE in the presence or absence of inhibitors (propoxur and bendiocarb) in adults of different strains of An. stephensi.

Strains	OD ± SD		
	Absence of inhibitor	Presence of propoxur	Presence of bendiocarb
DUB-S	0,252 ± 0,031 n=20	0,106 ± 0,033 n=20	0,108 ± 0,037 n=20
IRN-S	0,265 ± 0,057 n=20	0,089 ± 0,027 n=20	0,084 ± 0,024 n=20
DUB-APM	0,251 ± 0,035 n=18	0,078 ± 0,0236 n=18	0,084 ± 0,037 n=18
IND-S	0,175 ± 0,042 n=20	0,095 ± 0,029 n=20	0,081 ± 0,025 n=20
IND-APM	0,211 ± 0,021 n=16	0,101 ± 0,032 n=16	0,092 ± 0,032 n=16

Table 4.26 Comparison of AchE activity of different

strains of <u>An. stephensi</u>

Strains	IRN-S	DUB-APM	IND-S	IND-APM
	t=0.89	t=0.09	*t=6.58	*t=4.71
DUB-S	t=1.79	*t=3.26	t=1.12	t=0.68
	*t=2.42	t=2	*t=2.7	t=-1.33
		t=0.92	*t=5.69	*t=3.91
IRN-S		t=1.59	t=0.67	t=1.2
		t=1	t=0.39	t=0.83
			t=0.68	*t=4.08
DUB-APM			t=2.24	*t=2.58
			0=.3	t=0.67
				*t=3.27
IND-S				t=0.60
				t=1.12

The figures in the first line for each strain reperesent the t
values for AchE activity in the absence of inhibitors, and the
figures in the 2nd and 3rd lines represent the AchE activity in the
presence of the inhibitors propoxur and bendiocarb, respectively,
* P< 0.05

Fig 4.1 Probit regression lines of successive generations

of adult females of the IND-APM strain under

pirimiphos-methyl selection.
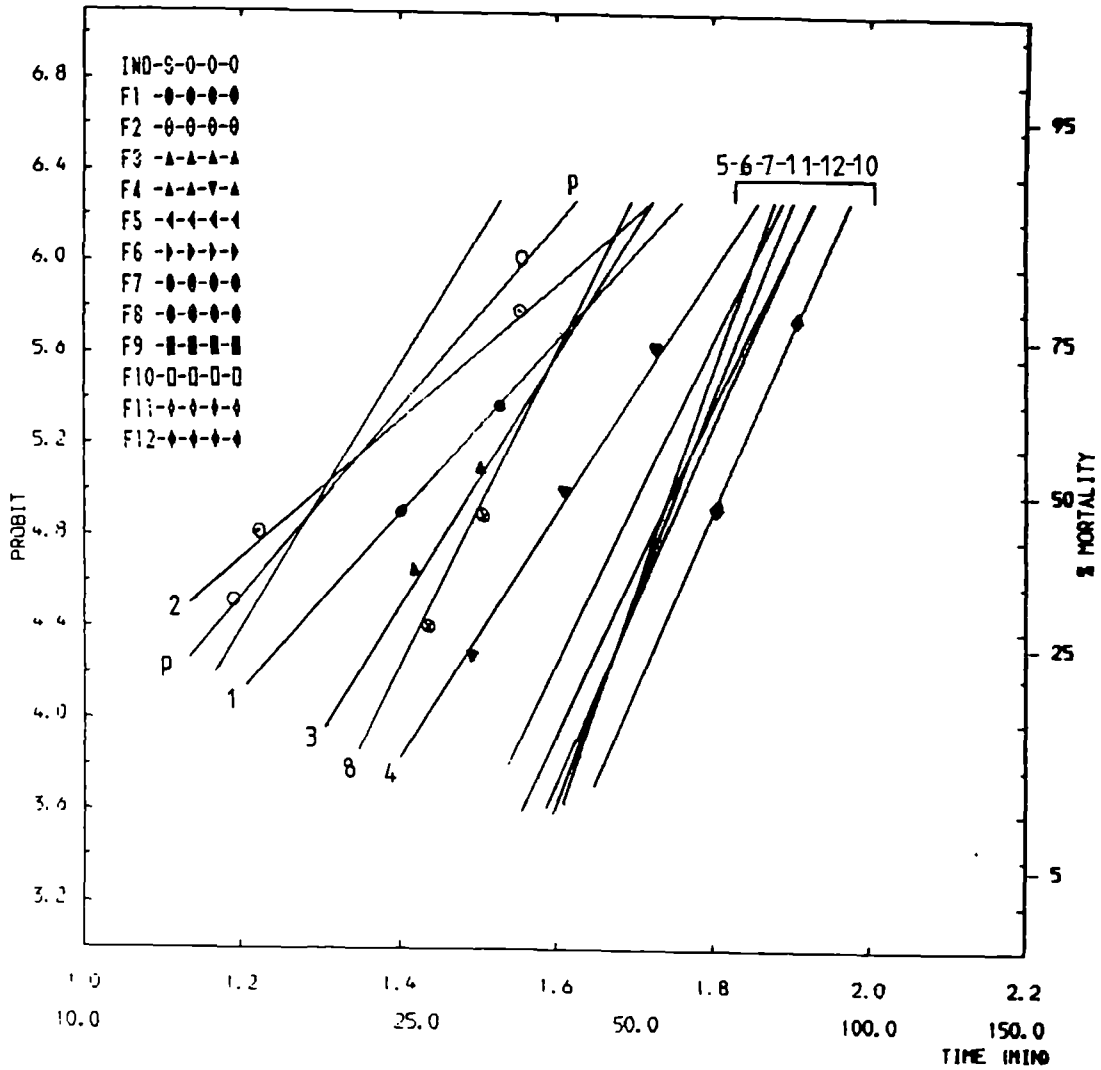
Fig 4.2 Probit regression lines of successive generations

of adult males of the IND-APM strain under

pirimiphos-methyl selection.
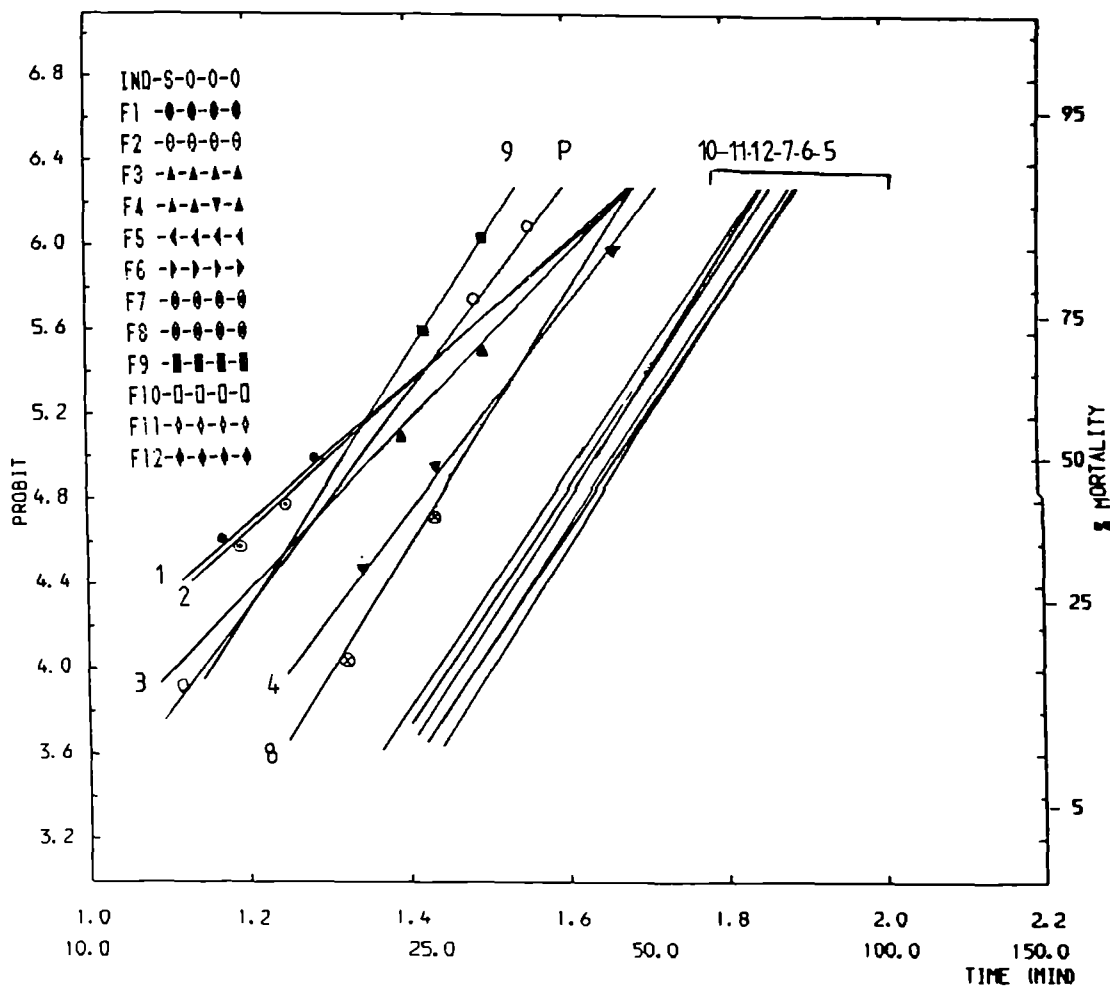
Fig 4.3 Probit regression lines of successive generations

of larvae of the IND-LPM strain under

pirimiphos-methyl selection.
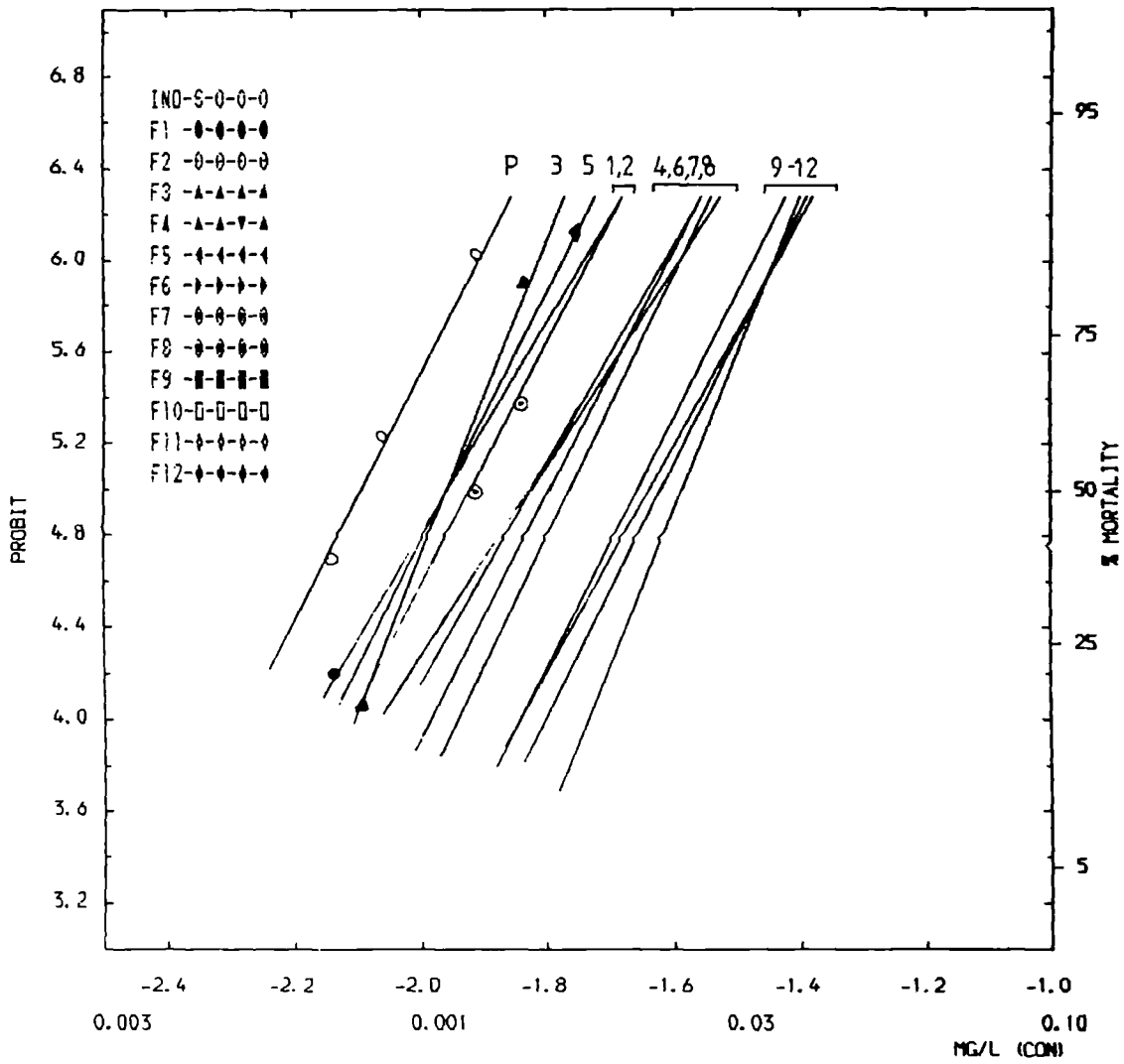
Fig 4.4 Probit regression lines of successive generations

of adult females of the DUB-APM strain under

pirimiphos-methyl selection.
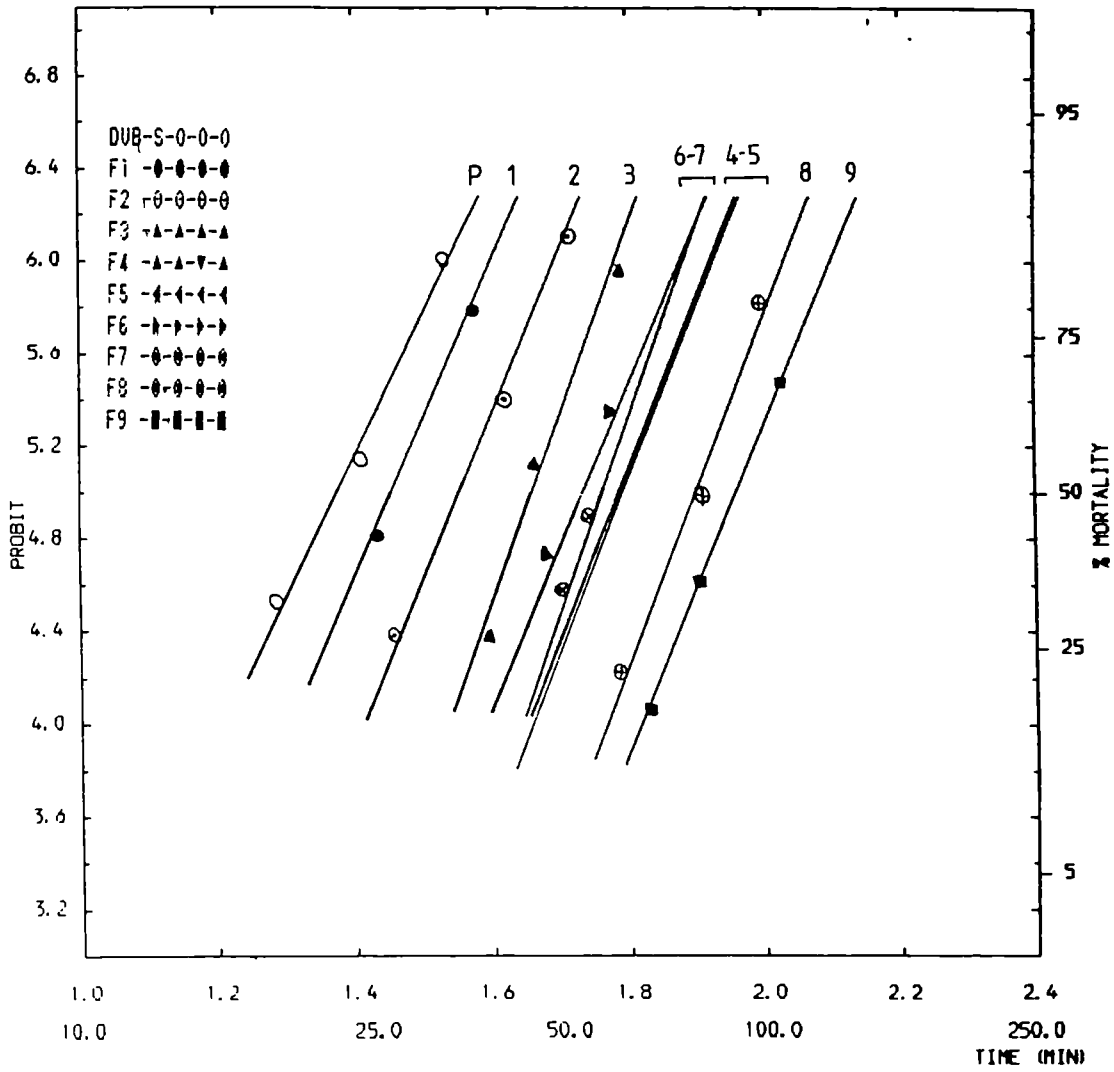
Fig 4.5 Probit regression lines of successive generations

of adult males of the DUB-APM strain under

pirimiphos-methyl selection.
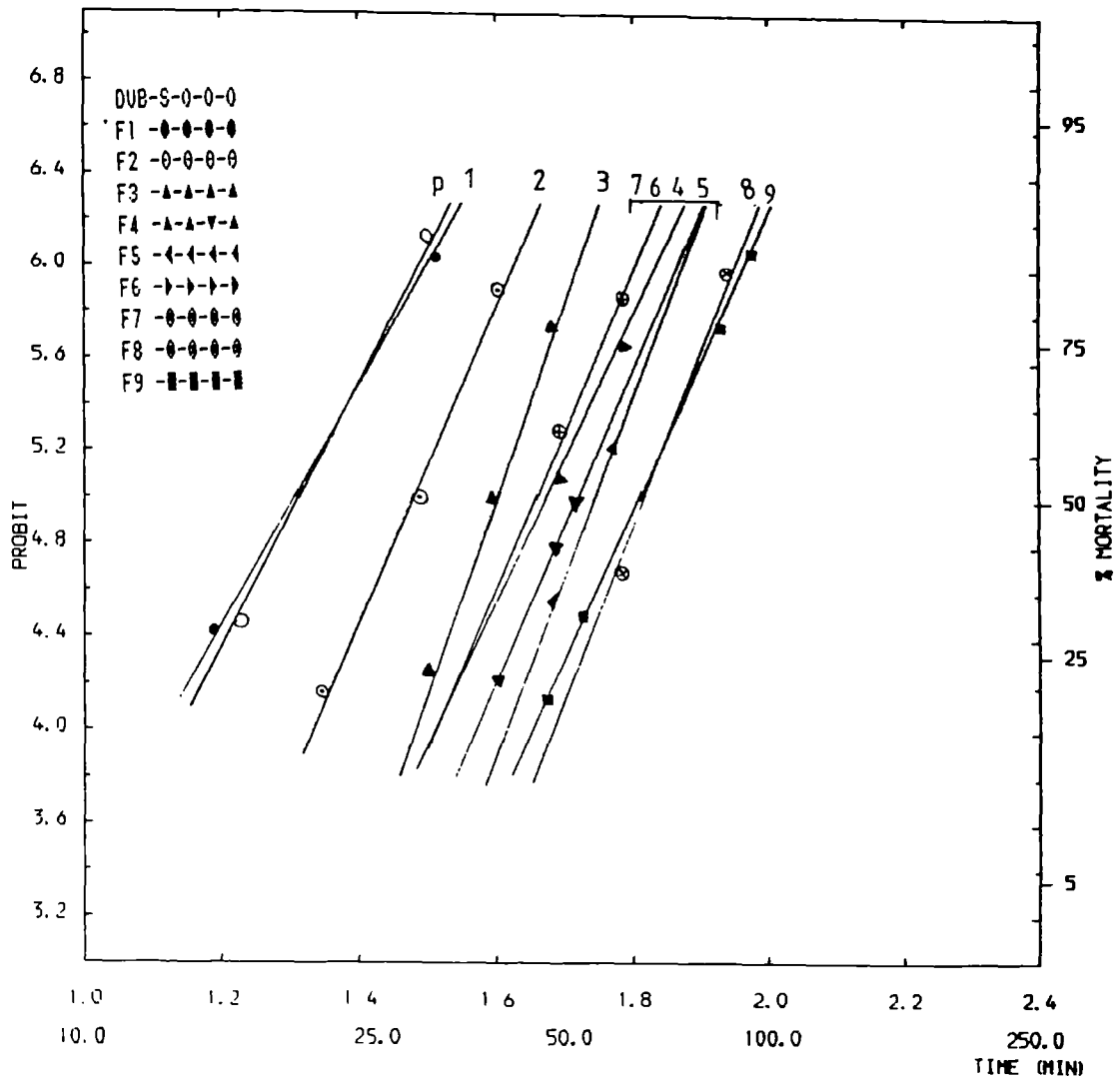
Fig 4.6 Probit regression lines of successive generations

of larvae of the DUB-LPM strain under

pirimiphos-methyl selection.
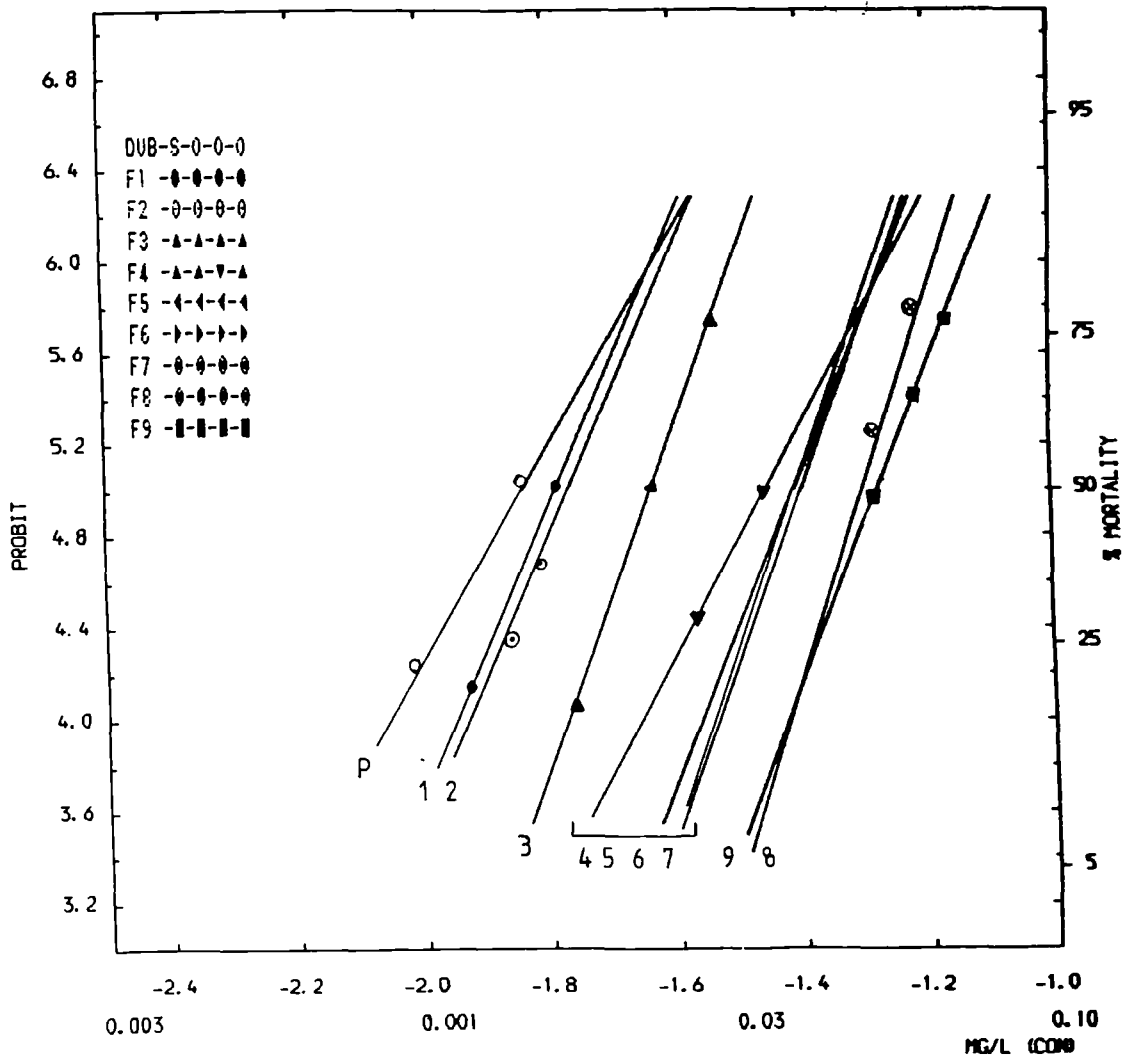
4.7 Reversion of tolerance in adults of the DUB-APM strain

in successive generations after release from

insecticide pressure.
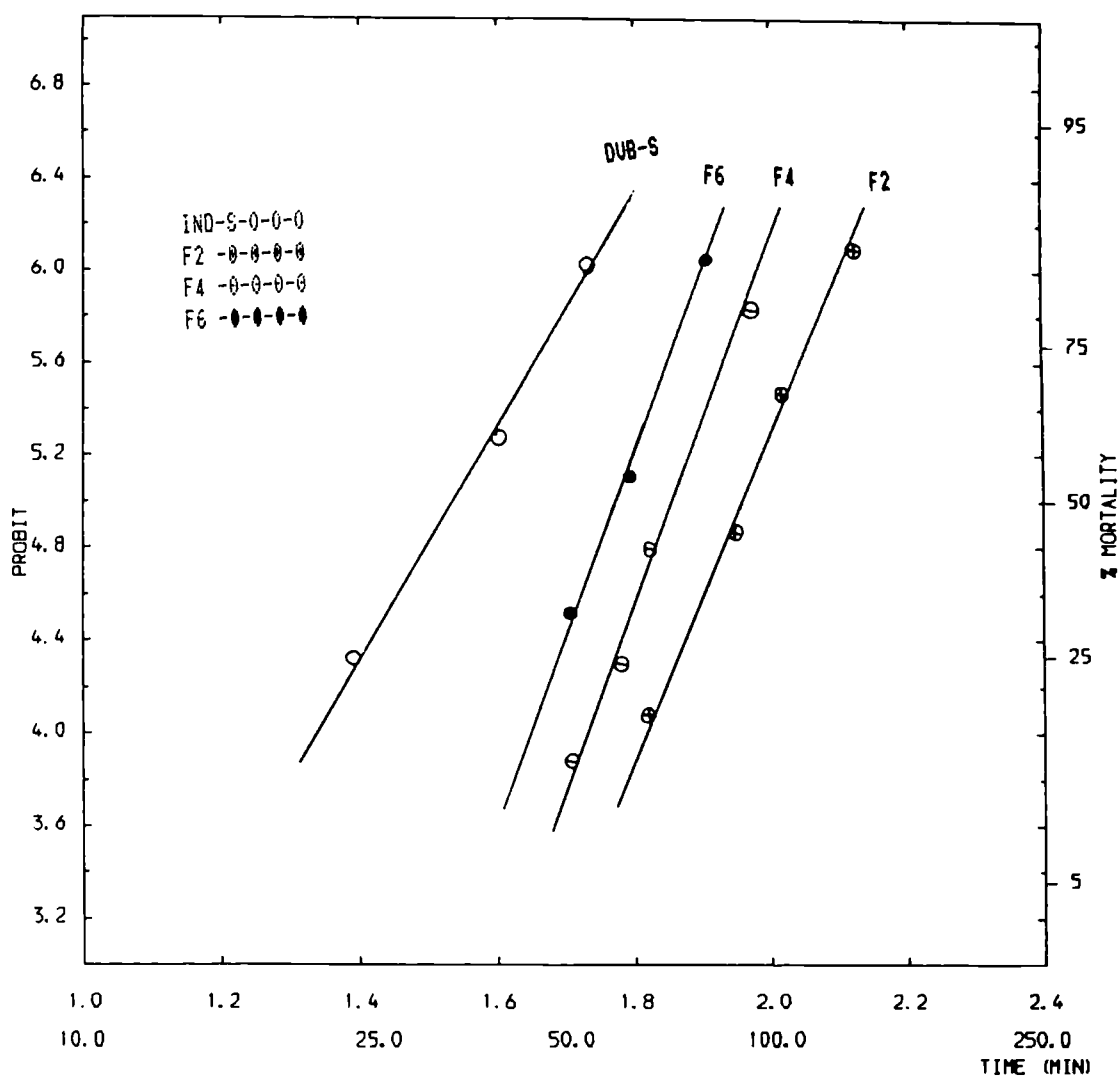
4.8 Reversion of tolerance in larvae of the DUB-LPM strain

in successive generations after release from

insecticide pressure.
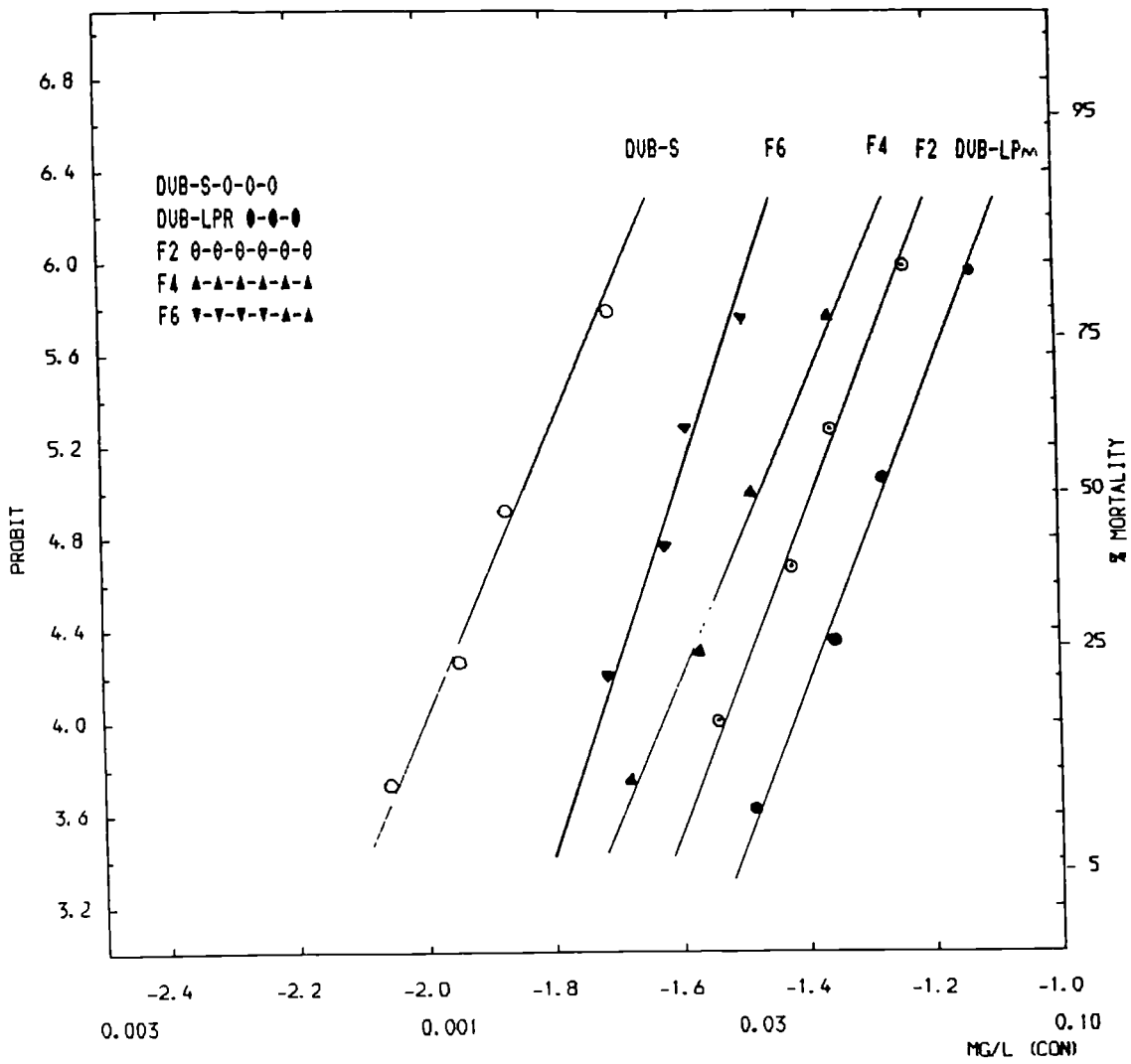
Fig 4.9 Probit regrtession lines of F1 progeny from crosses

between adults of DUB-APM and parental stock.
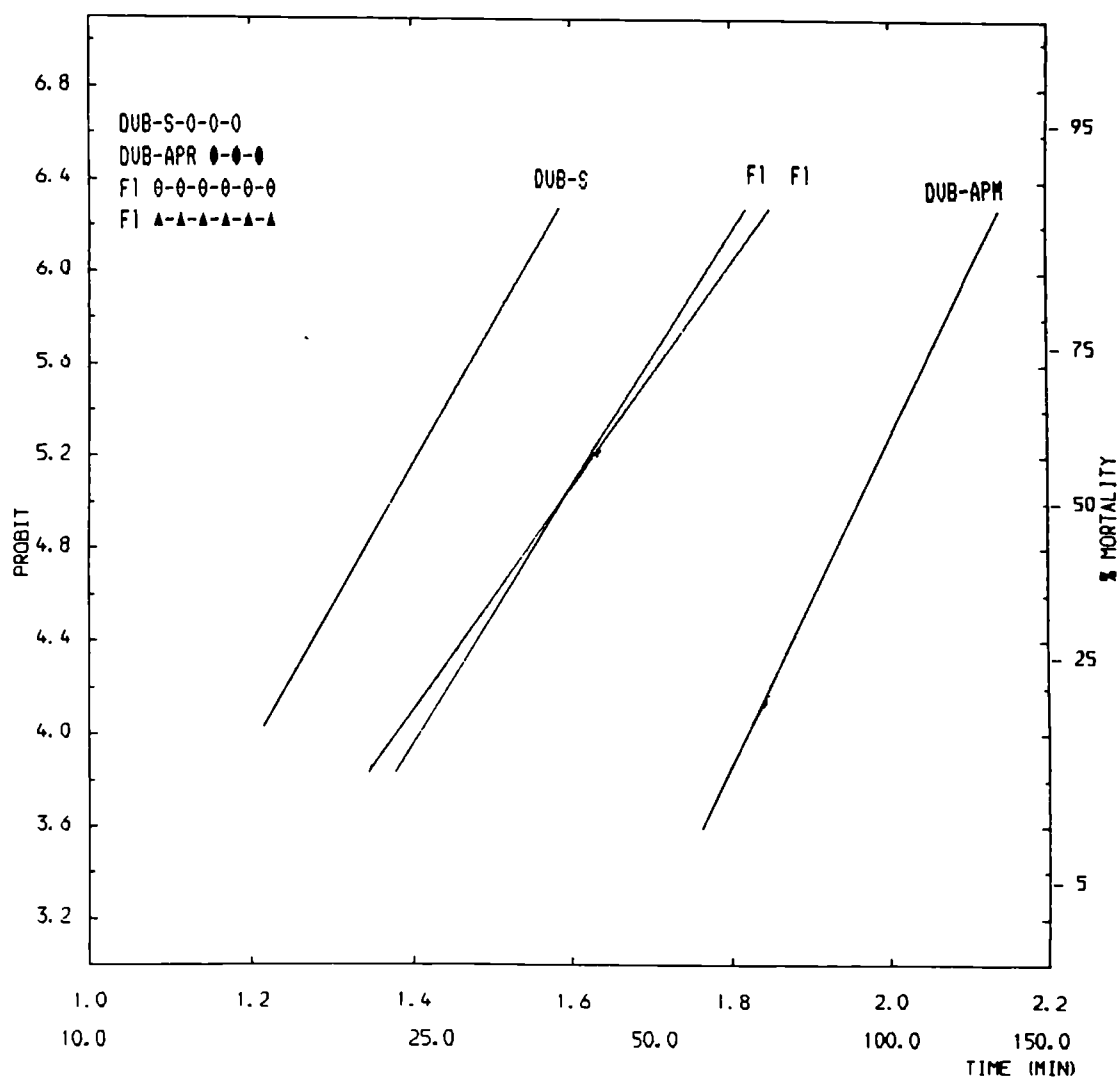
Fig 4.10 Probit regrtession lines of F1 progeny from crosses

between adults of IND-APM and parental stock.
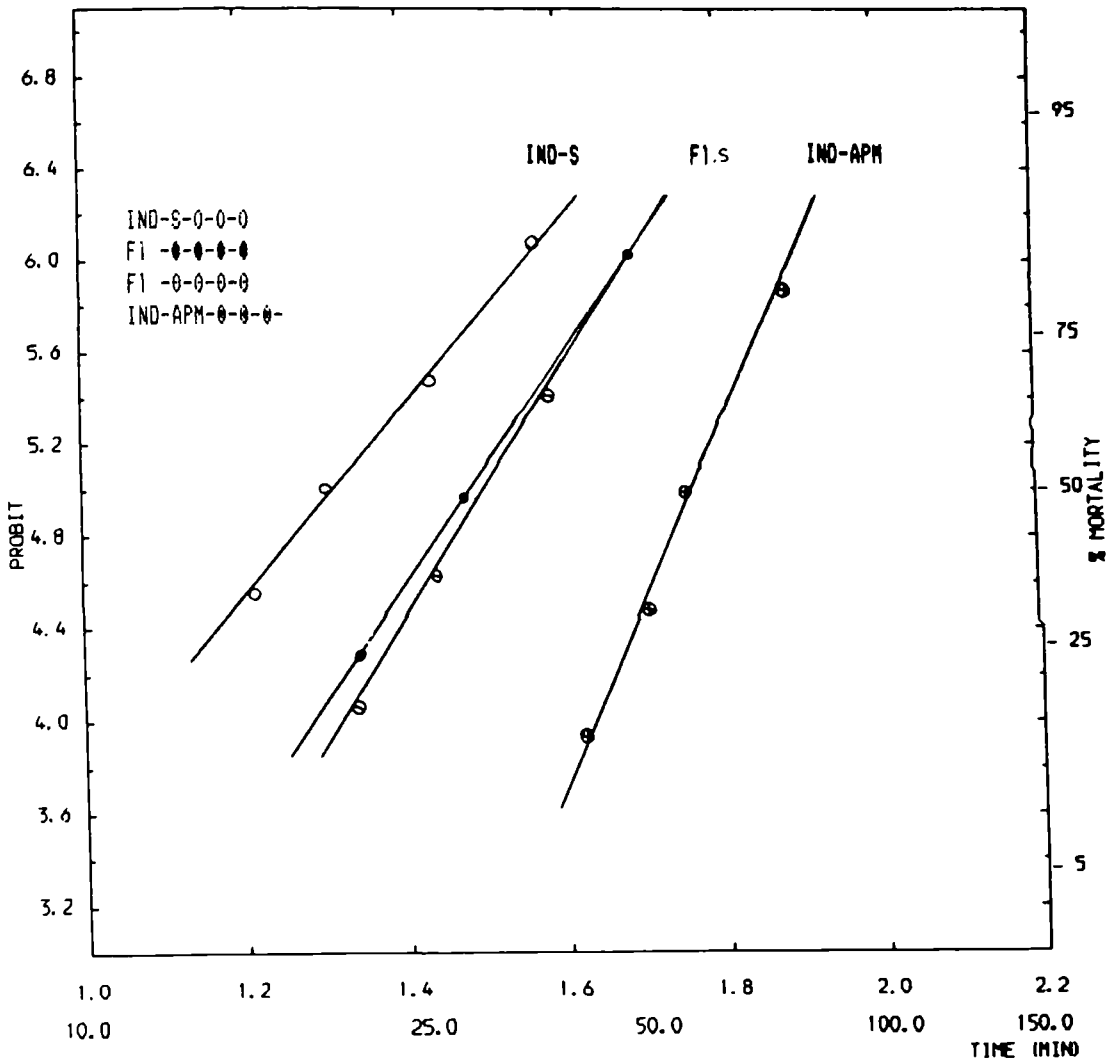
Chapter 5: Base-line susceptibility and genetics of DDT resistance in

larvae of An. stephensi

5.1 Introduction

A total of 58 species of anopheline mosquitoes have been recorded

as resistant to one or more pesticides, and of these, 56 species showed

resistance to DDT (Brown, 1986).

At least 11 anophelines have been recorded as most important

malaria vectors in different areas (WHO, 1985). Despite the development

of DDT resistance in such a large number of anopheline mosquitoes, the

genetics of DDT resistance has been studied in only eight species (see

Hemingway, 1981).

In this study an attempt has been made to determine the base-line

susceptibility of adults and larvae of the IND-S and DUB-S strains to

different insecticides, and the mode of inheritance and mechanisms of

DDT resistance in the larvae of An. stephensi from Dubai (U.A.E).

5.2 Susceptibility of the IND-S and DUB-S strains to different insecticides

After initial colonisation of the DUB-S strain, a wild strain of

An. stephensi collected from Dubai, it was maintained by mass rearing,

with appropriate action to improve feeding behaviour and egg-laying

(see 3.2 rearing of mosquitoes) and thus minimise loss of genetic

variation. Following two generations to increase numbers in the

breeding stock, experimental work was commenced in the 3rd generation.

Tests were carried out to determine the susceptibility of adults

and larvae of the DUB-S strain, and the IND-S, a laboratory stock of

Indian origin, to some traditional insecticides important in mosquito control programmes, such as chlorinated hydrocarbons, organophosphorus compounds, carbamate (propoxur) and pyrethroids such as permethrin, lambdacyhalothrin and deltamethrin. The results are presented in tables 5.1, 5.2 and 5.3

5.2.1 Larval susceptibility

Larvae of the IND-S strain were susceptible to the four main groups of insecticides tested, thus the strain was used throughout this study as a susceptible strain. The results are shown in table 5.1.

The susceptibility of larvae of the DUB-S strain to different insecticides was also determined. The result are shown in table 5.2.

The DUB-S strain showed extremely high resistance to DDT (LC50 = 3.41 mg/l) with a resistance ratio of 117.6 that of the IND-S strain. High levels of resistance to DDT have been recorded for larvae of An. stephensi from different areas. In a study by Rongsriyam & Busvine (1975), an LC50 of 4.6 mg/l was recorded for larvae of An. stephensi from Iraq. This strain was colonised in 1966 and then subjected to further selection with DDT. In another strain from India, an LC50 of 2.98 was recorded for the larvae (Verma & Rahman, 1984).

When larvae of the DUB-S strain were tested with a number of organophosphorus compounds, they showed moderate levels of tolerance to pirimiphos-methyl, malathion, chloropyrifos, fenthion and temephos, with resistance ratios of 1.8, 1.5, 1.5, 1.3 and 1.6 (table 5.2) respectively.

Permethrin tests on the DUB-S strain showed that this population is highly heterogeneous for permethrin resistance with a resistance ratio

of 7.5 (slope 0.84± 0.085), and to lesser extent for lambdacyhalothrin and deltamethrin with resistance ratios of 2 and 1.4 respectively.

5.2.2 Adult susceptibility

Susceptibility tests on adult females of the IND-S and DUB-S strains, indicated that both these strains are resistant to DDT and dieldrin; 0% and 7% mortalities were recorded when the DUB-S strain was tested with 4% DDT and dieldrin respectively for 1 hour, followed by a 24 hr holding period. Similarly in the IND-S strain, 39.5 and 39.6% mortalities were recorded when it was exposed to 4% DDT and dieldrin respectively for 1 hr. This indicates that the DUB-S strain is more highly resistant to DDT and dieldrin than the IND-S strain.

A slight decrease in susceptibility was observed when the DUB-S strain was tested with pirimiphos-methyl, propoxur and lambdacyhalothrin, with resistance ratios of 1.15, 1.2, and 1.3 respectively, and slightly higher tolerance for malathion and permethrin, with resistance ratios of 1.4 and 1.4 respectively (table 5.3).

5.3 Effect of synergists on resistance.

Piperonyl butoxide (PB), a mixed function oxidase inhibitor, and chlorofenethol (DMC), a dehydrochlorinase inhibitor in the presence and absence of DDT, were tested on the DDT susceptible (IND-S) and DDT resistant (DUB-S) strains. The results are presented in table 5.7.

PB had no significant synergistic effect on DDT in the IND-S and DUB-S strains (synergistic ratio = 1.16 and 1.24 respectively). A synergistic ratio of 4.7 was recorded when the DUB-S strain was tested

with PB in presence or absence of permethrin, indicating that mixed function oxidases are involved in permethrin tolerance in the larvae of this strain.

DMC had no significant significant synergistic effect on DDT in DDT susceptible and resistant larvae, with synergist ratios of 1.1 and 1.1 respectively, and this suggests that a dehydrochlorinase mechanism in not involved in DDT resistance in the DUB-S strain.

5-4 Inheritance of resistance

In order to determine the mode of inheritance of DDT resistance in larvae of the DUB-S strain, the two strains were reciprocally crossed by mass mating about 150 virgin adults of each sex, the sexes having been separated at the pupal stage. F1 progeny of the two reciprocal crosses were tested with DDT (see table 5.4 and Figs 5.1 and 5.2). The F1's showed no significant difference in their responses to DDT (t= 1.36, P>0.05), suggesting that resistance is autosomally inherited. The F1 results also indicate that resistance is recessive, the degree of dominance being D = -0.61 and -0.62 for the two reciprocal crosses. The single gene hypothesis was tested by reciprocal crosses of the F1 to the resistant strain (back-crosses), and the F2 generations. If DDT resistance is due to a major autosomal gene which is completely recessive, 1:1 ratios in each of the back-crosses and a 3:1 ratio in the F2 generations would be expected.

The expected mortalities for back-cross progeny and F2 generations, based on a single gene hypothesis were calculated. The agreement of the observed response to the expected in back-crosses and F2 generations was calculated by X^2 method. X^2 tests on the expected and observed

mortalities from the dose-response curve and dose-expected curve, corresponding to SR genotype in back-crosses and SS and SR genotypes in F2 generations (see table 5.5 and Fig 5.1), showed no significant deviation from those expected if a major gene is involved. However, comparisons of expected and observed mortalities from part of the dose-response curves, corresponding to RR genotype in back-crosses and F2 generations, showed significantly higher mortality in the response curve than the expected (P<0.05). Goodness of fit of the points to a straight line for back-crosses and F2 generations were then tested by X^2 analysis (Finney, 1971). X^2 tests on the back-cross progeny showed no significant deviation from a straight line (P > 0.05), but in the F2 generation the deviation was significant (P < 0.05).

In another method to determine whether DDT resistance was inherited in a monofactorial or polyfactorial manner, the offspring of crosses between the F1 and resistant strain (back-cross) were exposed to a discriminating dose to eliminate the heterozygous resistant larvae. This process was repeated through 5 further generations. The results are shown in table 5.6. There was a slight but progressive increase in mortality from 52.2% in the first generation of back-cross to 56.2% in the fifth generation. χ^2 tests indicated that the results for the first 3 repeated back-crosses did not differ significantly from those expected on the single gene hypothesis (p > 0.05). However at the fourth and fifth generations, mortalities began to increase (p <0.05), and this suggests the possible involvement of more than one genetic factor in DDT resistance in the larvae of <u>An. stephensi.</u>

5.5 Discussion.

An.stephensi is one of the main malaria vector in the Persian Gulf, the Indian subcontinent and the Middle-East areas. The first evidence of resistance to DDT in this species was reported in 1955 from Saudi Arabia (see Brown and Pal, 1971). By 1963 DDT resistance was developed in Iran, Iraq, Pakistan, and Afghanistan (Davidson & Mason, 1963). In spite of the development of DDT resistance in anopheline mosquitoes in different areas, DDT is still widely used as a residual insecticide in malaria control programmes (Brown, 1986).

An.stephensi and An.culicifacies have been recorded as the main malaria vectors in most parts of the U.A.E (Ministry of Health, U.A.E. 1981-1983).

The insecticides currently used in malaria control programmes in the U.A.E are mainly DDT and pyrethroids (neopybuthrin and deltamethrin) as residual insecticides, temephos in the form of granules and emulsion, and pirimiphos-methyl as an adulticide and larvicide (Ministry of Health, U.A.E, 1981-1983; Farid, 1981).

The larvicides are applied in the form of a space spray using Ultra Low Volume (ULV). DDT is applied as a residual insecticide using Hudson X-pert sprayers in small villages, hamlets and labour camps. Over large areas, DDT, pyrethroids and pirimiphos-methyl are applied by means of swing fog machines.

Due to the long history of insecticide application and the variety of insecticides used as larvicides or adulticides in mosquito control as well as in agricultural pest control programmes in the U.A.E, the DUB-S strain showed a quite different pattern of susceptibility when compared with the IND-S strain, varying from susceptible through

tolerance to resistance to some the insecticides tested. The DUB-S strain showed resistance to both DDT and dieldrin at the adult stage, and high resistance to DDT at the larval stage. The IND-S strain was resistant to DDT and dieldrin at the adult stage but susceptible to DDT at the larval stage.

Temephos has been widely used in the whole area as a potent larvicide and pirimiphos-methyl as an adulticide and a larvicide since 1983 (Ministry of Health, U. A. E, 1983). Regarding the use of these two insecticides in mosquito control programmes, the larvae showed about 1.8-fold increases in tolerance to pirimiphos-methyl and temephos respectively.

Pyrethroids (neopybuthrin and deltamethrin) have been used for indoor spraying using a swing fog machine. The adults showed 1.4 and 1.3-fold increases in tolerance to permethrin and lambdacyhalothrin respectively. Surprisingly the larvae were highly heterogeneous for permethrin resistance and tolerance to lambdacyhalothrin and deltamethrin, with resistance ratios of 7.4, 2 and 1.4 respectively. DDT resistance with cross-resistance to permethrin has been reported in several strains of mosquitoes (Hoyer & Plapp, 1968; Omer., et al 1980). Because of the lack of base-line susceptibility information and regular tests on the susceptibility of adults and larvae of anopheline mosquitoes to different insecticides in the area, it is difficult to make a precise prediction about the observed tolerance in the larvae. However, it is unlikely to be induced as the result of cross-tolerance conferred by DDT resistance, because synergist studies have indicated that different genetic factors are responsible for DDT and permethrin resistance (absence of a kdr genetic factor). The observed

tolerance in the larvae is probably due to the contamination of larval breeding places with pyrethroids by swing fog machine.

Mechanisms of DDT resistance have been studied in different strains of An. stephensi. A sub-strain derived from Delhi laboratory colony was selected with DDT. DDT selection resulted only in vigour tolerance. The adults converted more than one-third of the absorbed DDT into DDE in 24 hours (Perry, 1960). In a DDT resistant strain from Iraq, less DDT was converted to DDE than in the unselected Delhi strain, and the presence of DDT dehydrochlorinase was not demonstrated. PB, a mixed function oxidase inhibitor, and WARF, a dehydrochlorinase inhibitor, also did not make the resistant strain much more susceptible (see Brown & Pal, 1971).

In An. stephensi from Pakistan, DDT selection induced permethrin resistance in the larvae, but the synergists PB and DMC had no effect on resistance to DDT and pyrethroids. Reduced sensitivity of the active site was postulated as the major factor responsible for the observed resistance (Omer et al., 1980).

The inheritance of DDT resistance in An. stephensi from different areas has been studied by Davidson & Jackson (1961a, 1961b). Resistance was found to be inherited monofactorially in the adults and larvae, expression of the factor for resistance being dependent on the genetic background.

In the present study, synergists PB, a mixed function oxidase inhibitor, and DMC, a dehydrochlorinase inhibitor, had no synergistic effect on DDT in larvae of the DUB-S strain. This suggests that neither mixed function oxidase nor dehydrochlorinase are involved in DDT resistance in the larvae. PB had some synergistic effects on permethrin

in permethrin tolerant larvae, and this indicates that mixed function oxidases are probably involved in detoxication of permethrin in the DUB-S strain. These results suggest that different genetic factors are responsible for DDT and permethrin resistance in larvae of the DUB-S strain.

The crossing experiments and the progressive increases in mortality in back-cross generations indicate the involvement of more than one genetic factor in DDT resistance in larvae of the DUB-S strain.

Table 5.1 Susceptibility of larvae of IND-S strain to

to different insecticides.

Class of insecticides	Insecticide tested	LC50 95% C.L	LC90 95% C.L	Slope ± (b)	X^2 (df)	P
organochl- orine	DDT	0,026 0,029 0,031	0,063 0,072 0,085	3,19 ± 0,22	2,17 (4)	0,705
organopho- sphate	pirimiphos- methyl	0,008 0,008 0,008	0,0135 0,0143 0,0153	5,17 ± 0,27	4,71 (3)	0,194
	malathion	0,083 0,089 0,095	0,159 0,179 0,210	4,18 ± 0,34	4,30 (3)	0,231
	temephos	0,0012 0,0016 0,0021	0,0031 0,0043 0,0073	2,99 ± 0,22	5,76 (3)	0,124
	chloropy- rifos	0,00085 0,00091 0,00097	0,00148 0,00162 0,00181	5,07 ± 0,38	3,85 (3)	0,278
	fenthion	0,0050 0,0056 0,0060	0,0076 0,0077 0,0093	8,90 ± 0,73	5,92 (3)	0,115
carbamate	propoxur	0,397 0,418 0,440	0,647 0,699 0,769	5,74 ± 0,38	2,62 (4)	0,623
pyrethroids	permethrin	0,0403 0,0431 0,0461	0,075 0,083 0,095	4,51 ± 0,35	1,76 (3)	0,624
	lambdacy- halothrin	0,035 0,040 0,043	0,100 0,117 0,143	2,69 ± 0,20	0,25 (4)	0,993
	deltamethrin	0,44 0,56 0,69	1,90 2,70 4,60	1,86 ± 0,23	2,17 (3)	0,537

Table 5.2 Susceptibility of the larvae of the DUB-S strain
to different insecticides.

Insecticide tested	LC50 95% C.L	LC90 95% C.L	Slope ± (b)(df)	X²	P ratio RR‡	Resistance
DDT	3,10 3,41 3.74	6,23 7,11 8.43	4,01 ± 0,32	0,73 (2)	0,70	117,6
fenthion	0,0071 0,0073 0.0076	0,0924 0,0970 0.0103	10,54 ± 0,78	5,09 (3)	0,17	1,30
pirimiphos-methyl	0,0134 0,0144 0.0164	0,024 0,027 0.031	4,66 ± 0,34	2,14 (3)	0,54	1,75
malathion	0,123 0,131 0.138	0,207 0,225 0.251	5,40 ± 0,42	2,31 (3)	0,51	1,47
temephos	0,0023 0,0026 0.0028	0,0058 0,0068 0.0082	3,052 ± 0,232	2,475 (3)	0,480	1,63
chloropyrifos	0,0013 0,0014 0.0015	0,0023 0,0025 0.0029	4,79 ± 0,37	4,01 (3)	0,26	1,54
permethrin	0,20 0,32 0.47	6,59 10,76 21.17	0,84 ± 0,09	0,57 (3)	0,90	7,44
lambdacy-halothrin	0,07 0,08 0.09	0,24 0,31 0.44	2,20 ± 0,19	3,10 (4)	0,54	2,0
deltamethrin	0,58 0,80 1.05	4,16 6,51 13.15	1,40 ± 0,18	1,4 (3)	0,71	1,43

‡ RR = Ratio of LC50 of the DUB-S to LC50 of the IND-S strain.

Table 5.3   Susceptibility of adult females of the IND-S and

the DUB-S strains to different insecticides.

Strains tested	insecticide	LT50 95% C.L	LT90 95% C.L	Slope (b)	x^2 (df)	P	RR‡
IND-S	pirimiphos-methyl	20.02 20.62 21.23	40.76 42.51 44.49	4.08 ± 0.114	6.105 (4)	0.191	-
	malathion	17.50 19.20 21.17	29.51 33.75 40.47	5.231 ± 0.521	4.707 (3)	0.195	-
	propoxur	15.78 16.73 17.76	20.73 22.38 24.90	10.133 ± 1.108	2.739 (4)	0.602	-
	permethrin ‡	28.36 30.11 31.85	44.26 47.95 53.28	6.342 ± 0.563	2.725 (3)	0.436	-
	lambdacy-halothrin‡	17.11 18.49 19.88	33.13 36.69 41.74	4.306 ± 0.329	1.340 (3)	0.720	-
DUB-S	pirimiphos-methyl	22.51 23.75 25.07	35.68 38.52 42.33	6.1003 ± 0.4104	1.506 (3)	0.681	1.15
	malathion	25.70 27.27 28.77	42.08 45.37 49.98	4.324 ± 0.312	3.061 (3)	0.371	1.42
	propoxur	16.73 20.33 24.63	28.38 35.45 55.93	5.308 ± 0.391	9.589 (3)	0.022	1.22
	permethrin	33.34 42.98 51.16	67.97 84.53 135.40	4.363 ± 0.379	7.757 (3)	0.051	1.43
	lambdacy-halothrin	21.65 23.24 24.40	39.47 43.62 49.45	4.688 ± 0.336	3.460 (3)	0.326	1.26

‡ RR = Ratio of LT50 of the DUB-S to LT50 of the IND-S strain

Permethrin and lambdacyhalothrin impregnated papers were prepared in our

laboratory at 10 and 1.2μg/cm² respectively.

Table 5.4   DDT   test   on larvae of the F1 progeny from crosses between adults of the DUB-S and the IND-S strains

Strains/ crosses	LC50 95% C.L	LC90 95% C.L	Slope (b)	$\chi 2$	P	Resistance ratio*
IND-S	0,026 0,029 0,031	0,063 0,072 0,085	3,189± 0,215	2,170 (4)	0,75	
DUB-S	3,10 3,405 3,74	6,226 7,108 8,425	4,009 0,324	0,728 (2)	0,695	117,4
*F1 (A) (IND-S)♂ x (DUB-S)♀	0,066 0,073 0,079	0,148 0,168 0,197	3,534 0,305	0,546 (3)	0,909	2,52
*F1 (B) (IND-S)♀ x (DUB-S)♂	0,063 0,071 0,077	0,152 0,174 0,207	3,257 0,295	2,285 (3)	0,515	2,45
F2 A x A	0,061 0,095 0,126	0,414 0,561 0,912	1,661± 0,13	24,89 (9)	0,003	
F2 B x B	0,062 0,099 0,133	0,428 0,596 1,036	1,642 0,128	28,9 (9)	0,001	
BC A♂ x *R♀	0,133 0,155 0,178	0,893 1,092 1,404	1,513 0,102	11,00 (10)	0,358	
BC *R♂ x A♀	0,132 0,155 0,178	0,930 1,144 1,484	1,475 0,101	7,003 (10)	0,725	

* F1 = A   *F2 = B   *R = DUB-S

Table 5.5 DDT test on larvae of the IND-S, DUB-S strains, F1 progeny

from crosses between IND-S and DUB-S strain, F2 generations,

and back-crosses progeny .

Insecticide (mg/l)	IND-S (SS)‡	F1		F2				BC				DUB-S (RR)
		A‡	B‡	AxA	BXB	EXP 3:1	X²	AdxRq	RdxAq	EXP	X² 1:1	
0,02	27 (100)											
0,03	51 (100)											
0,046	74 (100)	23 (100)	30 (100)									
0,07	88 (100)	49 (100)	54 (100)	28 (100)	26 (100)	36	3,2	20 (100)	23 (100)	24	0,88	
0,1		70 (100)	72 (100)	44 (100)	42 (100)	51,8	2,4	40 (100)	44 (100)	35	1,09	
0,11	97 (100)											
0,13				70 (100)	69 (100)	60,8	3,6	49 (100)	49 (100)	40	3,0	
0,16		87 (100)	89 (100)									
0,17				76 (100)	76 (100)	67,5	3,3	51 (100)	53 (100)	45	1,45	
0,22		96 (100)	94 (100)	79 (100)	79 (100)	71,3	2,9	61 (100)	55 (100)	47,5	2,26	
0,3				81 (100)	80 (100)	75	1,9	70 (100)	67 (100)	50	11,56	
0,38				83 (100)	83 (100)	75	3,4	75 (100)	73 (100)	50	21,16	
0,5				88 (100)	87 (100)	75	9,0	81 (100)	79 (100)	50	33,64	
0,63				90 (100)	90 (100)	75	12	84 (100)	82 (100)	50	40,96	
1				94 (100)	92 (100)	75	19,3	89 (100)	90 (100)	50	64,0	
1,8				96 (100)	96 (100)	78,3	18,4	92 (100)	94 (100)	56,5	57,22	
3								95 (100)	95 (100)	70	29,76	
1,25												5 (98)
2,5												28 (100)
5												76 (103)
10												93 (95)

‡ SS = IND-S, RR = DUB-S, A = (Sd x Rq), B = (Rd x Sq), F2 = (A x A), F2 = B x B
The numbers in brackets represent the total tested.

Table 5.6 Results of 5 repeated back-crosses (*F1 x DUB-S) with

selection to distinguish between monofactorial

and polyfactorial inheritance.

Back-crosses	Number tested	No, of dying (proportion)	x^2
1st	341	178 (0.522)	1,42 NS
2nd	493	258 (0.523)	2,18 NS
3rd	548	291 (0.531)	3,67 NS
4th	449	245 (0.546)	5,57 *
5th	493	277 (0.562)	10,17 *

* $P < 0.05$ = significant deviation from 1;1 ratio
larvae were exposed to a concentration of 0,25 mg/l DDT for 24 hrs and the number of dying recorded,
*F1 = IND-S♂ x DUB-S♀

Table 5.7   Effect of synergist, PB and DMC, on larvae of

IND-S and DUB-S strains

Insecticide only +PB	Strains	LC50 95%C.L	LC90 95%C.L	Slope± S.E	X2 (df)	P	Resistance ratio RR‡	Synergist ratio SR‡
DDT		0,026 0,029 0.031	0,063 0,072 0.085	3,189± 0,215	2,170 (4)	0,705		
	IND-S						-	1,16
DDT+PB		0,027 0,025 0.027	0,0526 0,060 0.070	3,348± 0,231	2,601 (4)	0,627		
DDT		3,10 3,41 3.74	6,23 7,11 8.43	4,01± 0,324	0,728 (2)	0,695		
	DUB-S						119,47	1,24
DDT+PB		0,667 2,754 6.582	4,11 7,45 17.79	2,97± 0,257	10,527 (2)	0,005		
PR‡		0,040 0,043 0.046	0,075 0,089 0.095	4,505± 0,350	1,761 (3)	0,624		
	IND-S						-	0,9
PR+PB		0,046 0,048 0.051	0,074 0,079 0.088	5,809± 0,398	5,598 (4)	0,231		
PR‡		0,200 0,321 0.468	6,59 10,76 21.17	0,840± 0,085	0,574 (3)	0,902		
	DUB-S						7,45	4,72
PR+PB		0,062 0,068 0.074	0,130 0,147 0.173	3,803± 0,308	4,861 (3)	0,182		
DDT+DMC	IND-S	0,024 0,026 0.029	0,058 0,066 0.78	3,208± 0,218	4,729 (4)	0,316		1,12
DDT+DMC	DUB-S	2,794 3,067 3.364	5,52 6,27 7.39	4,124± 0,333	2,989 (2)	0,224		1,11

‡RR = Ratio of LC50 of the selected strain to LC50 of the IND-S strain
‡SR = LC50 of insecticide alone to LC50 of insecticide in the presence of synergist
‡PR = permethrin,

Fig 5.1 DDT test on larvae of the IND-S, DUB-S, the F1 progeny from crosses between IND-S and DUB-S, and F2 generation
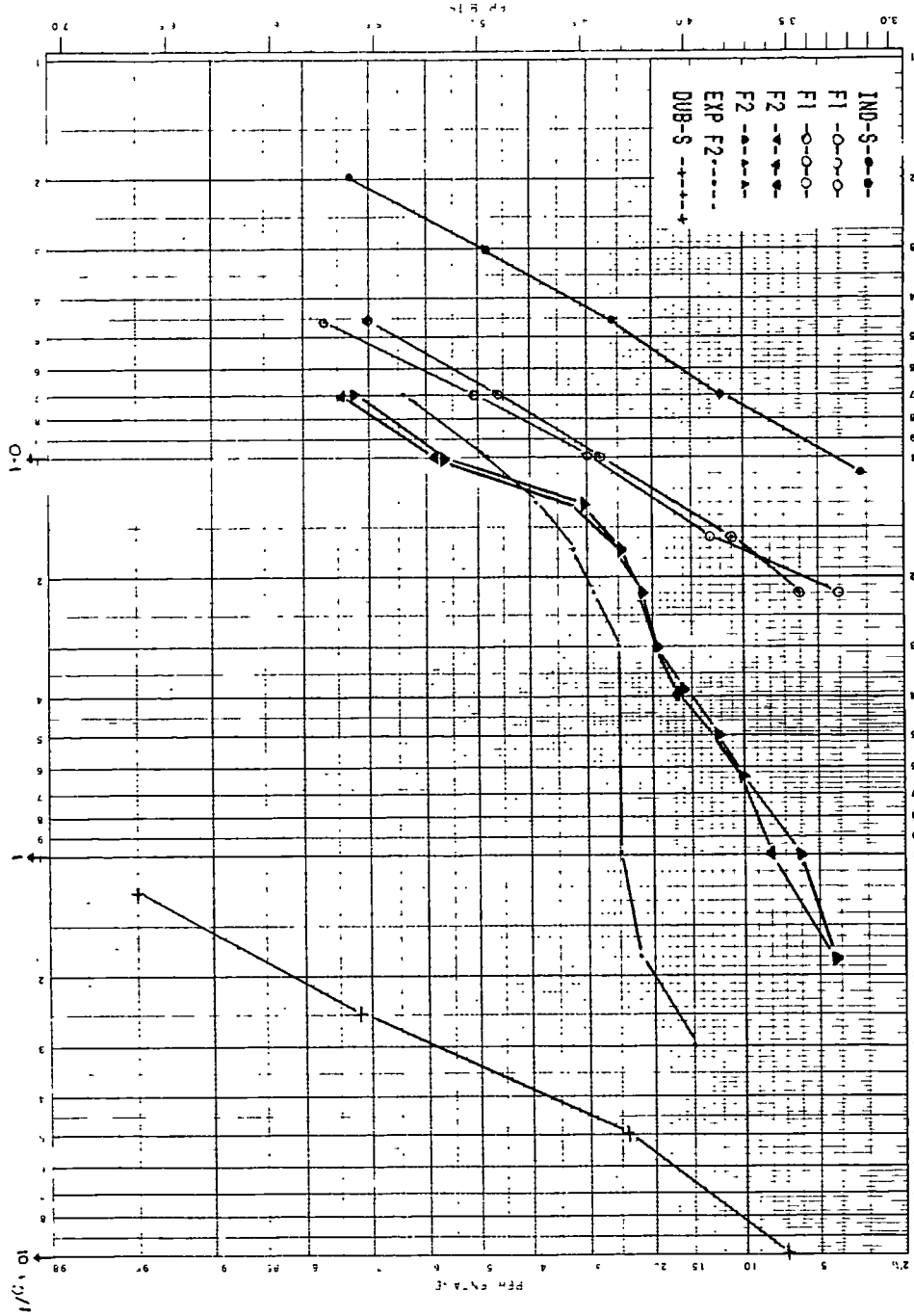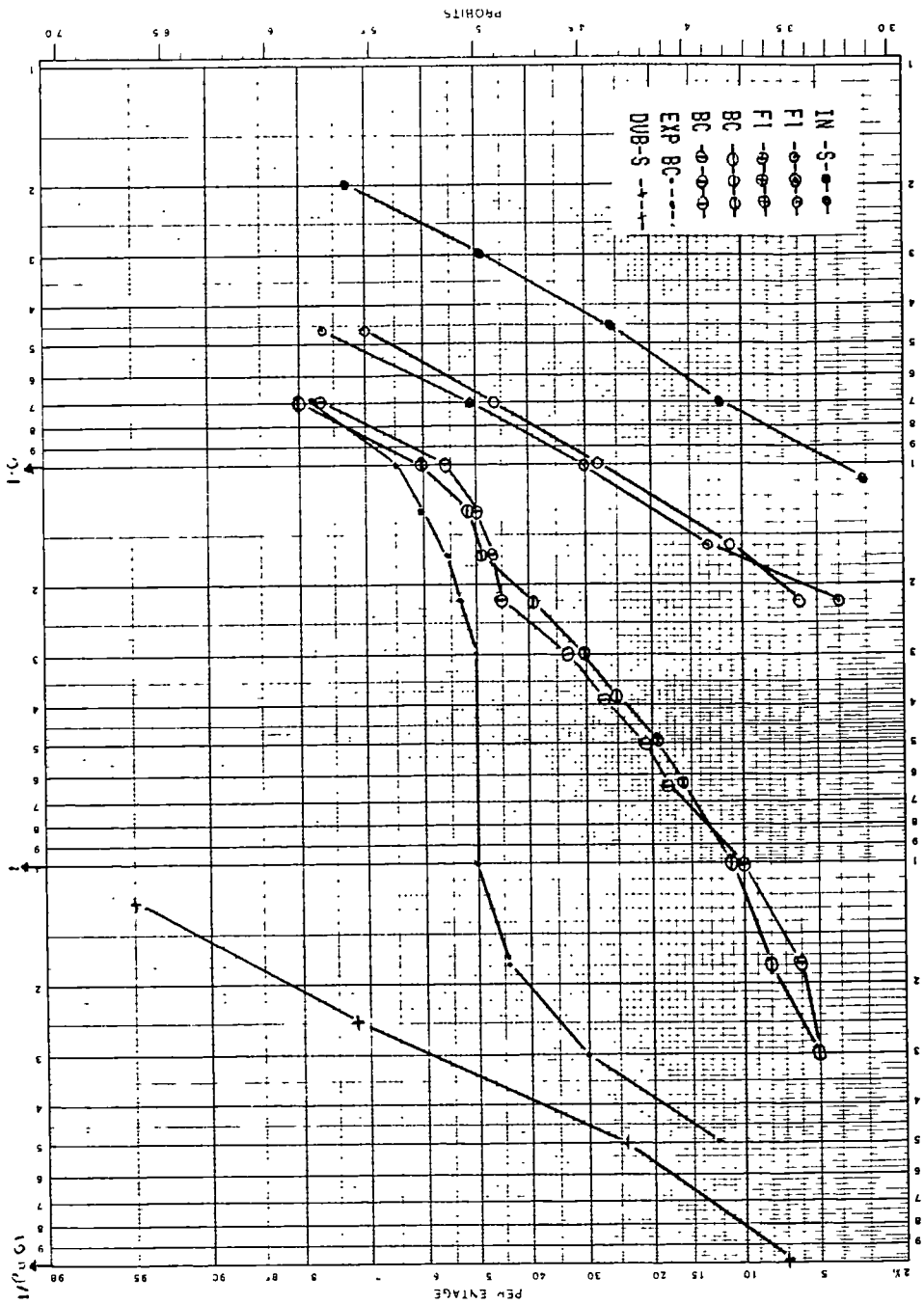
Fig 5.2   DDT test on larvae of back-cross progeny from
          crosses between F1 progenyand DUB-S strain

Chapter 6.   Genetics and mechanisms of permethrin resistance

in adults of <u>An. stephensi</u>

6.1 Introduction

It has been shown that resistance to DDT caused by action of the <u>kdr</u> gene imparts cross-resistance to pyrethroid insecticides (Busvine, 1953; Farnham, 1973). Subsequently it was demonstrated that pyrethroid selection confers or enhances resistance to DDT by the same gene (Farnham & Sawicki, 1976; Omer *et al*, 1980; WHO, 1985).

Rapid knock-down activity of some pyrethroid insecticides and recovery from knock-down have been reported in some mosquito species (Georghiou, 1962; Hitchen & Wood, 1974; Malcolm, 1988), probably because of picking up an insufficient amount of insecticide to cause mortality.

In this study, a strain of <u>An. stephensi</u> from Dubai, highly resistant to DDT at the adult and larval stages, was subjected to selection with permethrin at the adult stage. An attempt was then made to study the mode of inheritance of resistance, knock-down behaviour, and the role of <u>kdr</u> type genes in adults of the selected strain.

6.2 Determining the susceptibility of adults of theIND-S and DUB-S strains to permethrin and lambdacyhalothrin

Initially the susceptibility of adult males and females of the IND-S and DUB-S strains was measured using WHO permethrin impregnated paper (0.25%), and the standard WHO method (testing tubes in standing

position). The results are presented in table 6.1 and Figs 6.1 and 6.2. Due to the rapid action of permethrin and recovery of a number of knocked-down mosquitoes during the holding period, the observed mortality line for the IND-S, a permethrin susceptible strain was curved, mainly at the higher exposure times. Thus in selection studies, as a result of recovery behaviour, the resistance gene(s) probably would not be selected (see Hemingway, 1980; Malcolm, 1981).

In an attempt to eliminate the early knocked-down adults during the exposure times, the WHO adult susceptibility tests were carried out with some modifications. Testing and holding tubes were held in the horizontal rather than vertical position, so that the knocked-down mosquitoes were obliged to stay on insecticide impregnated paper, picking up a further quantity of insecticide which reduced the chance of recovery. The results of permethrin tests on the IND-S and DUB-S strains with 0.25% WHO permethrin impregnated paper for both positions of the testing tubes, are shown in table 6.1 and Figs 6.1 and 6.2.

The IND-S and DUB-S strains showed LT50's of 88.7 and 97.8 for vertical test compared with 45.3 and 79.7 minutes for horizontal tests.

Due to gradual loss of residual effect of WHO impregnated papers after normal use in 2-3 tests on adults, it was decided to carry out tests with fresh permethrin and lambdacyhalothrin impregnated papers prepared in our laboratory. Whatman No.1 filter papers were treated with a mixture of insecticide in acetone and silicon oil (for more detail see chapter 2: Material and Methods) to give concentrations of 10 $\mu g/cm^2$ for permethrin and 1.2 $\mu g/cm^2$ for lambdacyhalothrin respectively, providing about 90-100% mortality in IND-S, a permethrin susceptible strain after one hour exposure time followed by a 24 hour

holding period. The adult susceptibility tests were then carried out with these treated papers rather than the WHO impregnated papers.

The susceptibility level of the adult females of the IND-S and DUB-S strains to permethrin and lambdacyhalothrin was then determined using these laboratory papers, with the holding tubes held in both the vertical and horizontal test positions. The results are shown in table 6.1.

Adult females of the IND-S strain showed LT50's of 37.3 and 19.7 minutes to permethrin and lambdacyhalothrin respectively in vertical tests, compared with 30.6 and 18.5 in horizontal tests. Greater differences were observed at the LT90 level. Higher LT90's were recorded in the vertical tests rather than the horizontal tests (for more detail see table 6.1).

In horizontal tests the DUB-S strain showed LT50's of 43 and 23.3 minutes to permethrin and lambdacyhalothrin respectively, compared with 30.6 and 18.5 minutes for IND-S strain.

The observed mortality line for the DUB-S strain showed an inflection at about 75% mortality with permethrin (see Figs 6.4 and 6.2), but not with lambdacyhalothrin, probably indicating that this population is heterogeneous for permethrin resistance (containing about 25% heterozygotes).

6.3 Knock-down behaviour

The knock-down behaviour and rate of recovery of adult females from the IND-S and DUB-S strains to permethrin and lambdacyhalothrin were measured when the testing tubes were held in the vertical position. Knock-down was scored during the exposure time, and recovery from knock-down after a period of 24 hours. The results are shown in

table 6.2. Adult females of the DUB-S strain showed KT50's of 52.3 and 31.5 minutes with permethrin and lambdacyhalothrin respectively, compared with 43.3 and 24.7 minutes for the IND-S strain.

Knock-down behaviour in the DUB-S strain began at 30 and 15 minutes with permethrin and lambdacyhalothrin respectively, compared with 25 and 10 minutes for IND-S strain. The DUB-S strain showed 7% and 14% recovery from knock-down with permethrin and lambdacyhalothrin respectively, compared with 10% and 10% for the IND-S strain in one hr exposure time.

Apart from the observed knock-down during the exposure time (so called early knock-down), a further knock-down normally occurs during the first 5 hours of the holding period (late knock-down). Observations show that most of the late knock-down mosquitoes recover during the 24 hour holding period.

6.4 Permethrin selection on the adults

In selection studies, it was seen that under high selection, 80-90% mortality, a number of survivors lost some of their legs during the struggle for recovery from knock-down. However these survivors had a shorter life expectancy than the unselected stocks. The greatest difficulty was encountered with adult females; these showed some feeding difficulty and among the fed females some additional mortality occurred during oviposition on water in the plastic cups normally supplied for egg laying in the breeding cages. The surviving adult females produced fewer eggs than the normal laboratory stock.

To reduce delayed mortality in the breeding cage and improve the feeding behaviour, the adults were allowed to feed on sugar for 4-5

days, then were blood-fed on the hand for one or two feeds, with subsequent feeds from a guinea-pig. To minimise losses during oviposition, a petri- dish containing a layer of soaked cotton wool with a Whatman filter paper on the top was provided in the cage, rather than free water. Fewer eggs were collected, but further mortality was thereby reduced.

The adult males and females of the DUB-APR strain (a sub strain derived from DUB-S stock) were separately submitted to selection with permethrin for 8 generations at a selection pressure of 78-88% mortality. The results are shown in tables 6.3 and 6.4, and Fig 6.4.

When, as a result of egg-laying difficulty and post selection mortality in the breeding cage, the number of survivors was so reduced as to threaten the continued maintenance of the strain, the population was released from insecticide pressure. This occurred in the F3 and F7 generations.

The parental stock (DUB-S) showed an initial LT50 of 43 minutes. Eight generations of selection on the adults resulted in a steady increase in the LT50. At the F8 generation, the LT50 reached 299 minutes i.e. a 7-fold increase in resistance compared with the parental DUB-S stock and 10-fold that of the IND-S stock.

Knock-down activity of the selected strain was compared with the DUB-S and IND-S strains. Permethrin selection on the DUB-APR strain with an initial KT50 of 52.3 minutes, resulted in an increasingly delayed knock-down . At the F8 generation, the KT50 reached 336.8 minutes i.e. a 6.5-fold increase in resistance to knock-down , compared with the DUB-S stock and 7.8-fold that of the IND-S stock (table 6.2).

✓ Comparison between probit regression lines for the DUB-APR strain

(b= 8.98 ±0.69) and parental stock (b = 4.36 ± 0.38) shows that the slope has become steeper (d = 2.96 and P< 0.05).

6.5 Response of larvae to adult selection

The response to permethrin of the larvae from the adult-selected line was determined, using methods described earlier. The results are presented in table 6.5 and Fig 6.5. At the F7 generation of adult selection, the larvae of DUB-APR strain were tested with permethrin. The LC50 reached 1.59 mg/l, i.e. a tolerance 3.6 times greater than the DUB-S stock and 36.8 times that of the IND-S stock. Comparison between the probit regression lines for larvae of the DUB-APR strain and the parental stock (DUB-S) showed no significant difference in their slopes (d= 1.347 P>0.05).

6.6 Cross-resistance

The degree of cross-resistance to lambdacyhalothrin of the adult selected line (DUB-APR) was determined. The results are shown in table 6.6.

The parental stock showed an initial LT50 of 23.3 minutes with lambdacyhalothrin. At the F8 generation of permethrin selection, a test with lambdacyhalothrin on the adult females showed a LT50 of 81.4 minutes, i.e. a cross-tolerance of 3.5-fold, compared with that of the DUB-S stock, and 3.8 fold that of the IND-S stock (see table 6.6).

Similarly the knock-down responses of the selected strain to lambdacyhalothrin was compared with DUB-S and IND-S strains. At the F8 generation of permethrin selection, the KT50 for lambdacyhalothrin reached 101.2 minutes, i.e. delay to knock-down 3.2 times greater than

the DUB-S strain and 4.1 times that of the IND-S strain (see table 6.2).

6.7 Synergist studies

Piperonyl butoxide (PB), a mixed function oxidase inhibitor, and chlorofenethol (DMC), a dehydrochlorinase inhibitor, were tested in the presence and absence of permethrin and DDT on different strains of An. stephensi. The results are presented in tables 6.7 and 6.8.

PB and DMC had no significant synergistic effect with DDT on the DUB-S strain (see table 6.8). This result suggests that neither mixed function oxidases nor dehydrochlorinases are involved in DDT resistance in the adults.

Permethrin in the presence and absence of PB on the adults of IND-S and DUB-S strain showed synergistic ratios of 1.13 and 1.08 respectively, indicating that PB had no significant synergistic effect in these strains. A synergistic ratio of 1.94 compared with resistance ratio of 10 was recorded for the DUB-APR strain (see table 6.7) , which suggests that mixed function oxidases might contribute to permethrin resistance in the adults of this strain.

6.8 Inheritance of resistance

In order to determine the mode of inheritance of permethrin resistance in adults of the DUB-APR strain, the IND-S (permethrin susceptible) and DUB-APR (permethrin resistant) strains were reciprocally crossed by mass mating about 150 virgin adults of each sex, the sexes having been separated at the pupal stage. F1 progeny from the two reciprocal crosses were tested with permethrin for

resistance to kill and resistance to knock-down (see table 6.9 and Fig 6.5). The F1's showed no significant difference in their responses to permethrin either in resistance to kill or delay to knock-down (t = 0.013 P> 0.05). This result suggests that permethrin resistance and delay to knock-down is autosomally inherited and this applies equally to the gene(s) conferring resistance to kill or resistance to knock-down. The F1 results also indicated that resistance is partially recessive, the degree of dominance being D_1 and D_2 = -0.40 and -0.39 for the two reciprocal crosses. The single gene and polygene hypotheses were tested with reciprocal crosses of the F1's to the resistant strain (back-crosses) and the F2 generations. If permethrin resistance is due to a major autosomal gene which is completely recessive, 1:1 ratios in each of the back-crosses and 3:1 ratios in the F2 generations would be expected. If a single major gene is not involved and resistance is inherited as a polygenic character, no distinctive segregation would be expected in back-crosses and F2 generations.

The observed mortalities for back-cross progeny and F2 generations showed no distinctive segregation at 50% and 75%. Goodness of fit of the points to a straight line for back-crosses and F2 generations were then tested by χ^2 analysis (Finney, 1971) and showed no significant deviation from a straight line, which suggests that permethrin resistance is inherited as a polyfactorial character (see table 6.9 and Fig 6.6).

In order to carry out further investigations on resistance to kill (LT50), and resistance to knock-down (KT50), knock-down behaviour, and recovery from knock-down in adult females, a permethrin test was performed on the F1 progeny of crosses between the IND-S and DUB-APR

strains. The results are shown in table 6.11.

At 45 minutes (the lower exposure time), no differences between resistance to kill and delay to KD were observed (no recovery from knock-down). At 55 and 70 minutes, 6.7% and 6% recovery from the knock-down were observed respectively. At higher exposure times of 90 and 120 minutes, there was no recovery from knock-down but an increase in mortality compared with resistance to knock-down (8%) was recorded (see table 6.11). This suggests that recovery from knock-down is greater at the shorter exposure time rather than at longer exposure time. No recovery from knock-down was recorded when the DUB-APR strain was tested with permethrin.

6.9 Irritability studies

It has been observed that anopheline and culicine mosquitoes resting on residual deposits of DDT are excited and fly away from insecticide a short time before being knocked-down. Ae. aegypti resistant to DDT have been shown to be less DDT-irritable than the susceptible strain, probably because of the involvement of detoxication mechanisms and the conversion of DDT to less toxic compounds (see Brown, 1964; Brown and Pal, 1971).

Behaviouristic resistance, i.e, an increased ability to escape from houses sprayed with DDT, has been detected in several strains of anopheline mosquitoes in the absence of physiological resistance (see Brown & Pal, 1971).

The irritability level of adult females of the IND-S, DUB-S (stock strains) and the DUB-APR and DUB-LPR strains to permethrin was measured according to the method described by WHO (1964). The results are shown

in tables 6.12,   6.13 and Fig 6.6

20 individual unfed 2-3 day adult females of each strain   were exposed to permethrin at 10 µg/cm² in an exposure chamber, and the number of take-offs were counted during the following 15 minutes of exposure time.

During the exposure time, the mean number of take-offs per female was   49.7 and 37.3 for the IND-S and DUB-S strains respectively. Analysis of the   mean and variance of the number of take-offs for two stock strains showed no significant difference in their irritability to permethrin (t= 1.79 P> 0.05). Mean take-offs of 6 and 18.6 for adult females of the DUB-APR and DUB-LPR strains were recorded respectively. Comparison of the mean and variance of the number of take-offs for the two strains indicates that the DUB-APR is significantly less irritable to permethrin than the DUB-LPR strain (t= 7.43, P<.05). The two selected strains were significantly less irritable to permethrin than the parental stock (t= 11.37 and 6.11,   P<0.05) (see table 6.13).

6.10 Discussion

Pyrethroids possess many desirable properties, including high toxicity to insects, low toxicity to mammals, and high biodegradability (Plapp, 1976). DDT and pyrethroids share many characteristics in their actions as insecticides, such as a negative temperature coefficient of toxicity, rapid knock-down (kd) effect and action on the peripheral nervous system as well as central nervous system (Miller & Salgado, 1985).

Busvine (1951, 1953) pointed out the relationship between resistance to DDT and pyrethroids in houseflies. Since then this

relationship has been detected in numerous other arthropods including Pediculus humanus, ticks (Boophilus microplus), Heliothis armigera, acarines (Plutetta xylostella), the hornfly (Haematobia irritans) and mosquitoes (see Plapp, 1976: Miller and Salgado, 1985).

The housefly, Musca domestica, is one of the most important insect species with a great ability to develop resistance to insecticides. The relationship between DDT and pyrethroid resistance has been more widely studied in this species than any other pest species. Detailed genetic and biochemical studies on pyrethroid resistance in houseflies have been reported by Farnham (1971, 1973, 1977). Four genetic factors have been identified in this species; kdr factor is one of the important resistance factors, conferring resistance to pyrethroids, cross-resistance to DDT and greatly delayed knock down (Farnham 1973).

The gene(s) conferring DDT and pyrethroid knock-down (kdr) resistance has been shown to be not a metabolic factor, but more likely to involve target site insensitivity to pyrethroid-DDT action in insect nerves (Elliott & Potter, 1978).

95 species of mosquitoes (56 anopheline, 39 culicine) have been recorded as resistant to DDT in the field (Brown, 1986). Among the anophelines, surprisingly only two species, An. albimanus and An. sacharovi have been shown to be resistant to pyrethroids (see WHO, 1985; Malcolm, 1988). No additional information about the mode of action and relationship between DDT and pyrethroid resistance in these two species has been published. Low levels of pyrethroid tolerance have been reported in 6 anopheline species (see Malcolm, 1988). Of these only An. gambiae from Burkina Faso showed comparatively high tolerance (5.6-fold) to bioallethrin and the observed tolerance was reduced by

piperonyl butoxide (PB).

A <u>kdr</u>-like or an analogous mechanism in culicine mosquitoes in the larvae of <u>Culex tarsalis</u> was first found by Plapp & Hoyer (1968); it conferred resistance to DDT, pyrethrins and pyrethrin plus piperonyl butoxide. Breeding experiments demonstrated that resistance to DDT and pyrethrins was genetically linked and resistance to DDT and pyrethrin possibly controlled by a similar mechanism which was not metabolic.

In <u>C. quinquefasciatus</u> DDT resistance developed by permethrin selection (Priester & Georghiou, 1978; 1979). A <u>kdr</u>-like mechanism of"site insensitivity" was postulated for the observed resistance in the larvae.

Different strains of <u>Ae. aegypti</u> from different areas, resistant to DDT at the adult stage were examined for resistance to pyrethroids, but only one species from East Coast Demerara showed 30 fold cross-resistance to permethrin. Metabolic breakdown mechanisms were postulated for the observed resistance (Prasittisuk & Busvine, 1977).

Osborne & Hart (1979) reported that sensory nerves in a <u>kdr</u> strain of housefly were 1000 times less sensitive to permethrin than sensory nerves from a susceptible strain. Omer et al. (1980) reported that neuromuscular preparations from a strain of <u>An. stephensi</u> from Pakistan, possessing <u>kdr</u>-type resistance mechanism, were 20-fold more resistant to repetitive firing induced by (IR)-cis-permethrin.

It has been thought that because of similarities between the mode of action of DDT and pyrethroids and the development of DDT resistance in many mosquito species, pyrethroids may suffer a similar fate to DDT with the development of resistance. However, as mentioned above, of the 95 DDT resistant species of mosquito, only three have been recorded

as resistant to pyrethroids in the field and kdr type resistance has been detected in only three species, always as the result of selection studies. Therefore it is difficult to make a certain prediction about the development of pyrethroid resistance in mosquitoes.

Following the use of DDT and pyrethroid insecticides as larvicides and adulticides in the U.A.E (Ministry of Health, U.A.E, 1983.), both adults and larvae of the DUB-S strain showed high resistance to DDT and were heterogeneous for permethrin resistance.

In this study, 8 generations of permethrin selection on the adults resulted in a 10-fold increase in resistance and 7.8-fold increase in resistance to knock-down, compared with the IND-S strain, a permethrin susceptible strain. Cross-tolerance and delayed knock-down by lambdacyhalothrin were also developed in adults, with resistance ratios of 3.8 and 4, compared with the DUB-S and IND-S strains respectively. In a study by Omer et al (1980), larvae of An. stephensi were selected with DDT alone, and DDT plus synergists and permethrin; the adults showed 9.4, 11 and 10 fold cross-resistance to permethrin respectively. No further study on the genetics and mechanisms of permethrin resistance in the adults of this strain has been published. In the present study, adult selection produced cross-tolerance in larvae, 36.6-fold compared with the IND-S strain and 3.6-fold that of the DUB-S strain.

In a study by Malcolm and Wood (1982) a strain of Ae. aegypti from Bangkok was subjected to selection with permethrin. The process started with mass selection, then continued with single family selection. A 30-fold increase in resistance was produced. Synergist studies indicated no evidence of a kdr type resistance mechanism in the adults.

Changes in fecundity, oviposition rate, post-emergence mortality and disturbances in oogenesis, in houseflies and mosquitoes, have been reported frequently, usually as the result of selection studies with organochlorine and organophosphate insecticides (see Brown and Pal, 1971). At a sub-lethal concentration of pyrethrin, an anti-feeding response or repellency has been observed in mites (see Leahey, 1985). Following anti-vectorial treatment with deltamethrin, reduction in density, parasite and parous rate has been reported in anophelines (see Acad, *et al*, 1986).

In this study, during adult permethrin selection, a clear reduction in the rate of oviposition was observed. Thus to avoid loss of the strain, the population was released from insecticide pressure at F3 and F7 generations.

Reciprocal crosses between the selected strain and a susceptible strain revealed that resistance is partially recessive. Tests on back-cross progeny and F2 generations also indicated that permethrin resistance is inherited as an autosomal polyfactorial character. Knock-down response of the F1 generation, F2 and back crosses were determined. The results indicated that resistance to knock-down is genetically inherited and the genes for permethrin resistance and delay to knock-down are probably identical.

Synergist, PB, a mixed function oxidase and chlorofenethol (DMC), a dehydrochlorinase inhibitor, had no effects on DDT in the DDT resistant strains. A synergistic ratio of 1.9 with PB, compared with resistance ratio of 10, was recorded for permethrin in selected adults, indicating that PB has a minor effect on permethrin resistance, and the oxidative detoxication of permethrin by mixed

function oxidases is not the major resistance mechanism in the adults of _An. stephensi._ These results raise the possibility of involvement of reduced sensitivity at the target site as the primary mechanism for permethrin resistance.

In a similar study by Priester & Georghiou (1978, 1979), larvae of _C. pipiens fatigans_ were subjected to selection with d-cis and d-trans permethrin. A high level of permethrin resistance (RR >4000) was induced with d-trans permethrin and adult resistance was increased by larval selection. The result of crosses and back-crosses indicated that the resistance was inherited as a polyfactorial character. PB eliminated some resistance to pyrethroids but a major part of the resistance remained at high levels. Therefore reduced sensitivity of the active site was postulated as responsible for the major part of permethrin resistance.

Irritability tests on the IND-S and DUB-S the stock strains indicated no significant differences in their irritability, but the adults of the two selected strains (DUB-APR and DUB-LPR) showed significant difference in their irritability to permethrin, compared with stock strains and also with each other. Unfortunately no comparative studies on the irritability of adult mosquitoes to permethrin have been published, although irritability levels to DDT of different strain of mosquitoes, susceptible and resistant to DDT, have been examined. The resistant strains have been shown to be less irritable to DDT than the susceptible strains, particularly when the resistance mechanism is due to detoxication of DDT to a less toxic compound.

In this study the selected strains showed less irritability to

permethrin than the stock strains. This is likely to be caused by reduced sensitivity of the active site, i.e., a _kdr_ type mechanism, although mixed function oxidases may play a minor role effecting oxidative detoxication of permethrin in the adult of _An. stephensi_ selected with permethrin.

Table 6.1 Comparison of susceptibility of the IND-S and DUB-S strains
to permethrin and lambdacyhalothrin (testing tubes held in
vertical and horizontal positions).

Type of impregnated paper	strains	position of tubes	LT50 95% C.L	LT90 95% C.L	Slope ± S.E	χ^2	P
0,25% permethrin (WHO)	IND-S	vertical	74,07 88,69 118.72	146,85 215,59 672.92	3,32 ± 0,36	11,75 (4)	0,019
		horizontal	43,83 45,34 46.91	58,81 61,85 65.91	9,50 ± 0,69	2,76 (3)	0,431
	DUB-S	vertical	88,11 97,83 113.29	206,92 276,59 453.68	2,84 ± 0,39	1,891 (3)	0,595
		horizontal	– 79,69 –	– 147,69 –	4,51 ± 0,48	25,33 (3)	–
permethrin 10µg/cm² (lab paper)	IND-S	vertical	34,38 37,25 40.22	70,53 80,31 95.87	3,84 ± 0,35	5,60 (4)	0,231
		horizontal	29,02 30,60 32.10	45,18 48,16 52.19	6,51 ± 0,51	1,07 (3)	0,784
lamb* 1,2µg/cm² (lab paper)	IND-S	vertical	16,27 19,71 23.01	38,79 47,90 67.74	3,32 ± 0,24	17,53 (6)	0,008
		horizontal	17,11 18,49 19.88	33,13 36,69 41.74	4,31 ± 0,33	1,34 (3)	0,720

* lamb= lambdacyhalothrin

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Table 6.2 Comparison between mortality and knock-down on exposure

of adult females of different strains of <u>An. stephensi</u> to

permethrin and lambdacyhalothrin.

Insecticide tested	Strains	Mortality			knock-down (KT)		
		LT50 95%C.L (min)	LT90 95%C.L (min)	Slope ± S.E	KT50 95%C.L (min)	KT90 95%C.L (min)	Slope ± S.E
Permethrin	IND-S	28.36 30.11 31.85	44.26 47.95 53.28	6.94 ± 0.56	40.82 43.26 45.75	60.73 65.99 74.02	6.99 ± 0.40
	DUB-S	33.34 42.98 51.16	67.97 84.53 135.40	4.36 ± 0.38	49.95 52.28 54.54	91.33 98.64 108.79	4.65 ± 0.32
	DUB-APR	268.50 299.12 330.98	367.54 415.40 531.76	8.98 ± 0.69	326.02 336.75 347.32	494.19 517.82 547.43	6.86 ± 0.38
lambd*	IND-S	19.79 21.18 22.64	35.7 39.2 44.02	4.79 ± 0.34	22.91 24.72 26.39	37.67 40.66 44.77	5.93 ± 0.33
	DUB-S	21.70 23.27 24.91	40.07 44.26 50.15	4.59 ± 0.33	30.31 31.53 32.70	47.50 49.74 52.41	6.47 ± 0.36
	DUB-APR	74.51 81.36 88.12	126.70 141.39 164.84	5.34 ± 0.58	88.63 101.22 114.26	212.72 231.62 250.16	5.04 ± 0.41

*lambd = lambdacyhalothrin

Table 6.3 permethrin selection on adult females of the DUB-APR strain.

Generation of selection	LT50 95% C.L (min)	LT90 95% C.L (min)	Slope ± S.E	X^2 (df)	P	Resistance ratio (RR)
P	33.33 42.98 51.16	67.97 84.53 135.40	4.36 ± 0.38	7.76 (3)	0.051	-
F1	66.20 69.27 72.28	99.11 105.58 114.55	7.01 ± 0.56	3.29 (3)	0.351	1.61
F2	99.27 102.68 106.28	133.80 141.05 150.86	9.29 ± 0.69	3.14 (3)	0.370	2.37
F4	259.92 276.88 295.45	492.62 557.58 661.48	4.22 ± 0.38	2.71 (4)	0.607	6.44
F5*	-	-	-	-	-	-
F6	298.92 313.05 328.59	463.79 506.51 571.54	6.13 ± 0.55	1.54 (3)	0.308	7.28
F8	268.47 →299.12 330.98	367.54 415.42 531.76	8.98 ± 0.69	8.45 (3)	0.038	7.0

* At the F5 generation, 80.4% and 87.1% mortality was recorded for adult females and males at 420 and 360 minutes exposure time respectively.

Table 6. 4 Summary of permethrin selection on adult females and males of the DUB-APR strain.

	♂				♀			
Gen	Exposure time (min)	No, of individuals exposed	No, of survivors	Selection pressure % mortalities	Exposure time (min)	No, of individuals exposed	No, of survivors	Selection pressure % mortalities
P	60	697	95	86,37	70	686	108	84,23
F1	90	1213	146	87,96	100	1224	201	83,58
F2	120	1360	190	85,03	130	1316	231	82,45
F4	360	599	122	79,63	420	632	116	78,80
F5	360	660	85	87,12	420	592	116	80,41
F6	420	718	108	84,96	420	731	150	79,48
F8	360	1197	175	85,38	420	1094	333	69,56

X Due to oviposition difficulty, the strain was released from insecticide pressure at the F3 and the F7 generations,

Table 6.5 Response of larvae of An. stephensi following adult

selection with permethrin.

Strains	LC50 95% C,L (mg/l)	LC90 95% C,L (mg/l)	Slope ± S,E	X^2 (d,f)	P	Resistance ratios (RR) *
IND-S	0,0403 0,0431 0,0461	0,075 0,083 0,095	4,505 ± 0,385	1,761 (3)	0,624	-
DUB-S	0,312 0,438 0,581	6,928 10,18 16,82	0,938 ± 0,0784	4,316 (5)	0,505	-
DUB-APR	0,410 1,585 2,800	9,08 20,15 273,93	1,160 ± 0,145	6,427 (3)	0,093	3,62
DUB-LPR	36,09 44,29 58,80	180,93 306,80 723,45	1,525 ± 0,192	2,174 (3)	0,537	101,13

* RR= Ratio of LC50 of the DUB-APR to LC50 of the DUB-S strain,

Table 6.6 Permethrin and lambdacyhalothrin tests on different strains of <u>An. stephensi</u>.

Insecticides tested	Strains used	LT50 95% C.L (min)	LT90 95% C.L (min)	Slope ± S.E	X² (d.f)	P	Resistance ratios RR*
Permethrin	IND-S	28.36 30.11 31.85	44.26 47.95 53.28	6.342 ± 0.569	2.725 (3)	0.436	-
	DUB-S	33.34 42.98 51.16	67.97 84.53 135.40	4.363 ± 0.379	7.757 (3)	0.051	1.43
	DUB-LPR	123.94 130.04 136.23	187.21 200.46 218.70	6.818 ± 0.525	4.127 (3)	0.248	4.32
	DUB-APR	268.50 +299.12 330.98	367.54 415.40 531.76	8.98 ± 0.687	8.542 (3)	0.038	9.93
lamb*	IND-S	19.76 21.18 22.64	35.70 39.20 44.02	4.793 ± 0.342	2.230 (3)	0.526	-
	DUB-S	21.70 23.27 24.91	40.07 44.26 50.15	4.588 ± 0.332	4.718 (3)	0.194	1.10
	DUB-LPR	58.75 62.38 66.30	98.57 107.75 120.38	5.4 ± 0.38	2.350 (3)	0.503	2.95
	DUB-APR	74.51 81.36 88.12	126.70 141.39 164.84	5.34 ± 0.577	4.324 (3)	0.229	3.84

* RR = Ratio of LT50 of the DUB-APR to LT50 of the IND-S strain.
* lam = lambdacyhalothrin.

Table 6.7 Effect of synergist (PB) on adult females of

different strains of <u>An. stephensi.</u>

Insecticide/ synergist	strains	KT50 95% C.L (min)	KT50 95% C.L (min)	Slope ± S.E	X^2 (df)	P	Resistance ratio RR *	Synergist ratio SR *
*PR alone		40.82	60.73	6.99 ±	14.680	0.04		
		43.26	65.99	0.40	(7)			
	IND-S	45.75	74.02				-	1.13
PR+PB		36.95	57.77	6.40 ±	6.009	0.539		
		38.37	60.86	0.38	(7)			
		39.75	64.80					
PR		49.95	91.33	4.65 ±	7.938	0.440		
		52.28	98.64	0.32	(8)			
	DUB-S	54.54	108.79				1.21	1.08
PR+PB		45.99	89.16	4.25 ±	8.14	0.520		
		48.23	96.51	0.28	(9)			
		50.49	106.57					
PR		326.02	494.19	6.86 ±	3.203	0.921		
		336.75	517.82	0.38	(8)			
	DUB-APR	347.32	547.43				7.78	1.94
PR+PB		168.46	247.93	7.412 ±	11.029	0.198		
		173.72	258.67	0.41	(8)			
		178.85	272.01					

* SR = LT50 of insecticide alone to LT50 of the selected strain,
* RR = Ratio of LT50 of the selected strain to LT50 of the IND-S strain,
* PR = permethrin,

Table 6.8   Effect of synergists DMC, a dehydrochlorinase
inhibitor, and PB, a mixed function oxidase
inhibitor, on different strains of <u>An. stephensi</u>.

Strains	synergist/DDT	Exposure time (min)	% Mortality
IND-S	DMC*	45	0 (84)*
	DDT 4%	60	36,6 (101)*
	DDT + DMC	60	34 (101)*
	PB*	45	0
	DDT + PB	60	38 (100)*
DUB-S	DMC	45	0 (81)*
	PB	45	0 (97)*
	DDT	60	0 (82)
	DDT + DMC	60	0 (98)*
	DDT + PB	60	0 (100)*

* DMC and PB impregnated papers were prepared at 3,6μg/cm².
* The numbers in the brackets present the total tested.

Table 6,9 Permethrin tests on the adult females of the F1 progeny

from crosses between the DUB-APR and the IND-S strains,

the F2 generations and back-cross progeny (F1 x DUB-APR).

Crosses	LT50 95% C.L (min)	LT90 95% C.L (min)	Slope ± S.E	X^2	P	Resistance ratios RR*
F1 I♂ X D♀	63,49 71,11 79,98	93,81 109,08 145,05	6,89 ± 0,52	6,906 (3)	0,075	2,36
F1 D♂ X I♀	68,64 71,86 75,27	104,68 112,77 124,14	6,55 ± 0,50	0,520 (3)	0,914	2,39
F2 (I♂XD♀) X (I♂XD♀)	58,62 63,40 68,41	126,42 141,56 162,85	3,67 ± 0,25	2,88 (4)	0,579	2,11
F2 (D♂XI♀) X (D♂XI♀)	55,91 60,80 65,91	128,78 145,49 169,45	3,38 ± 0,34	3,72 (4)	0,446	2,02
BC *F1♂ X D♀	86,48 91,64 97,12	144,65 157,83 176,20	5,43 ± 0,40	2,72 (3)	0,437	3,04
BC D♂ X *F1♀	93,27 98,59 104,26	150,65 163,68 181,69	5,82 ± 0,42	0,45 (3)	0,93	3,27

I = IND-S, D = DUB-APR
*F1 = I♂ X D♀

Table 6,10 Comparison between mortality and knock-down on exposure

of adult females of the F1, F2 and back-cross

progeny to permethrin ,

Crosses	Mortality			knock-down (KD		
	LT50 (min)	LT90 (min)	Slope± S,E,	LT50 (min)	LT90 (min)	Slope ± S,E
F1	63,49	93,81	6,89 ±	79,92	117,24	7,38 ±
(I♂ X D♀)	71,11	109,08	0,52	81,76	121,95	0,36
	79,98	145,05		83,60	127,73	
F1	68,64	104,68	6,55 ±	83,65	126,50	6,71 ±
(D♂ X I♀)	71,86	112,77	0,50	85,76	133,16	0,37
	75,27	124,14		88,00	141,69	
F2	58,62	126,42	3,67 ±	68,12	174,83	2,97 ±
(I♂ X D♀) X	63,40	141,56	0,25	73,10	197,61	0,174
(I♂ X D♀)	68,41	162,85		78,34	229,46	
BC	86,48	144,65	5,43 ±	79,89	148,82	4,53 ±
(I♂ X D♀) ♂	91,64	157,83	0,40	83,24	159,67	0,25
X D♀	97,12	176,20		86,71	173,73	

I= IND-S strain, D= DUB-APR strain,

Table 6.11 Comparison between mortality and knock-down (KD) in adult

females of the F1 progeny from crosses between adult   males

of the IND-S and adult females of the DUB-APR   strains.

Exposure time (min)	KD/M	Mortalities after 24hr holding period knock-down after exposure time											
		REP1		REP2		REP3		REP4		TOTAL			
		A	KD/M	A	KD/M	A	KD/M	A	KD/M	A	KD/M	T	%M/KD
45	KD	25	0	25	0	24	1	24	1	98	2	100	2
	M	24	1	24	1	25	0	25	0	98	2	100	2
55	KD	23	3	20	5	22	3	19	6	84	17	101	16,8
	M	24	2	22	3	23	2	22	3	91	10	101	10
70	KD	18	7	15	10	15	9	16	9	64	35	99	35,4
	M	19	6	17	8	17	7	18	8	70	29	99	29,3
90	KD	10	15	8	17	11	14	6	19	35	65	100	65
	M	8	17	6	19	7	18	5	20	26	74	100	74
120	KD	5	20	3	22	5	20	2	23	15	85	100	85
	M	3	22	1	24	3	22	0	25	7	93	100	93

M =mortalities after 24 hrs holding period, A =alive, KD = knock-down mosquitoes at the end of exposure time.

Table 6.12 Irritability levels of different strains of

An. stephensi to permethrin.

Strains	No.of take offs /20 females/ 15 min	No.of take offs /female/15 min	No.of take offs /female/min ± SD
IND-S	945 (19)	49,74	3,32 ± 1,90
DUB-S	745 (20)	37,25	2,48 ± 0,79
DUB-APR	120 (20)	6	0,4 ± 0,23
DUB-LPR	372 (20)	18,6	1,24 ± 0,45

The number in brackets represents the number tested.

Table 6.13 Comparison of irritability levels of

different strains of <u>An. stephensi</u>

to permethrin.

STRAINS	DUB-S	DUB-APR	DUB-LPR
IND-S	t=1,787 *	t=6,651	t=4,653
DUB-S		t=11,366	t=6,108
DUB-APR			t=7,434

* P>0,05

Fig 6.1 Comparison of susceptibility of the IND-S strain to permethrin (0.25% WHO impregnated paper) held in vertical and horizontal positions of the testing tubes.
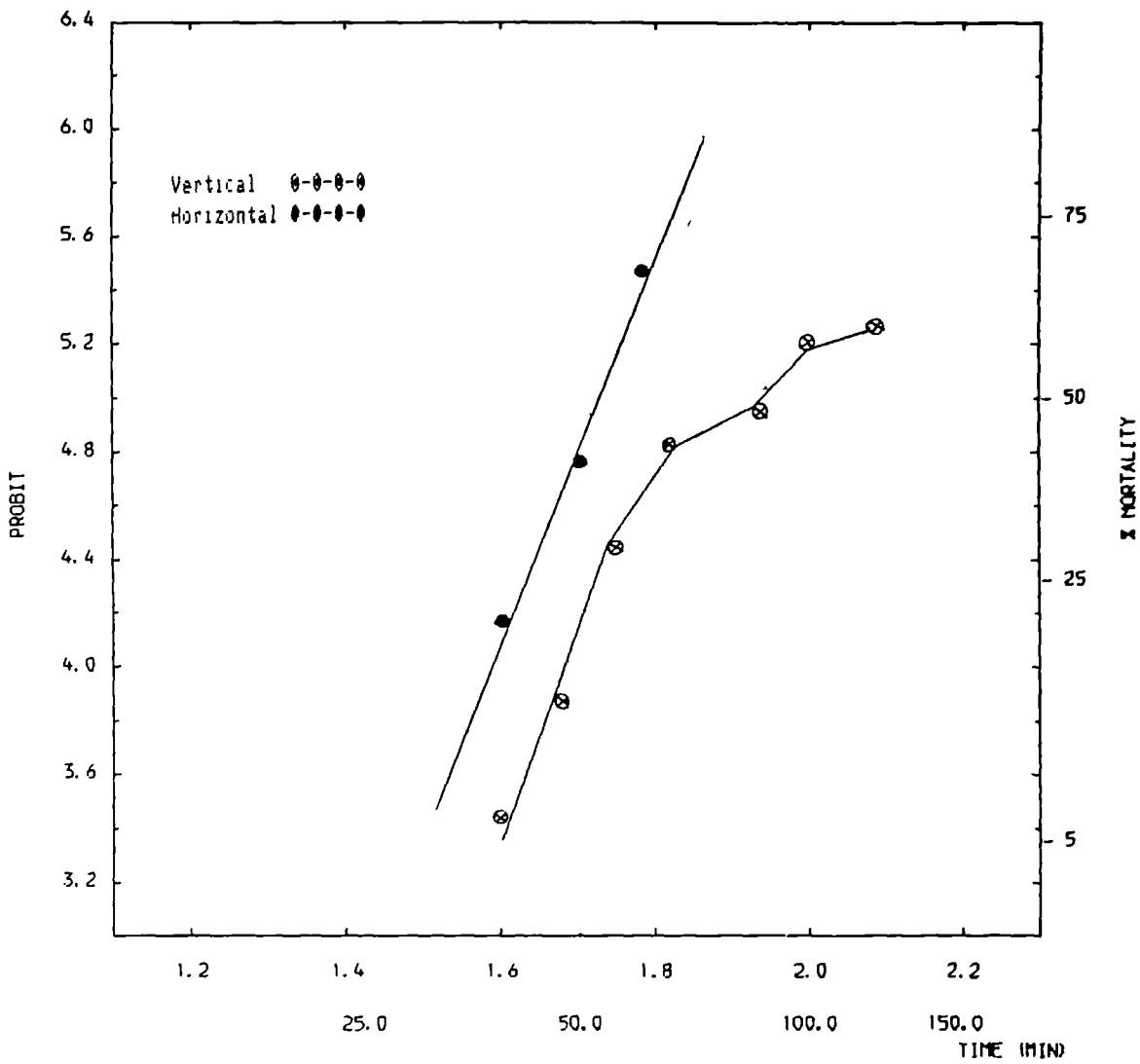
Fig 6.2 Comparison of susceptibility of the DUB-S strain to permethrin (0.25% WHO impregnated paper) held in vertical and horizontal positions of the testing tubes.
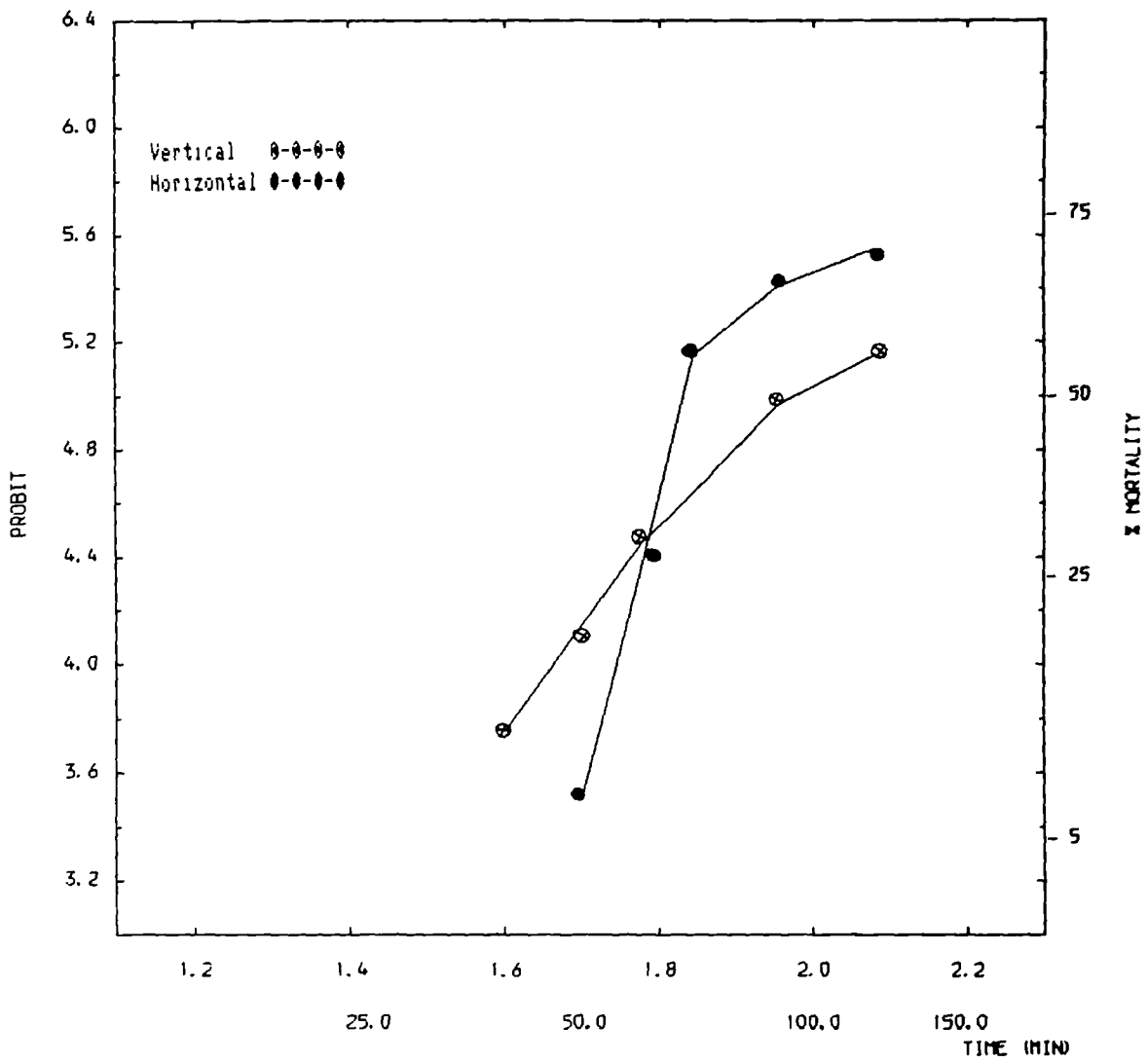
Fig 6.3 Susceptiblity of adults of the IND-S, DUB-S and DUB-APR

strains to lambdacyhalothrin (horizontal test).
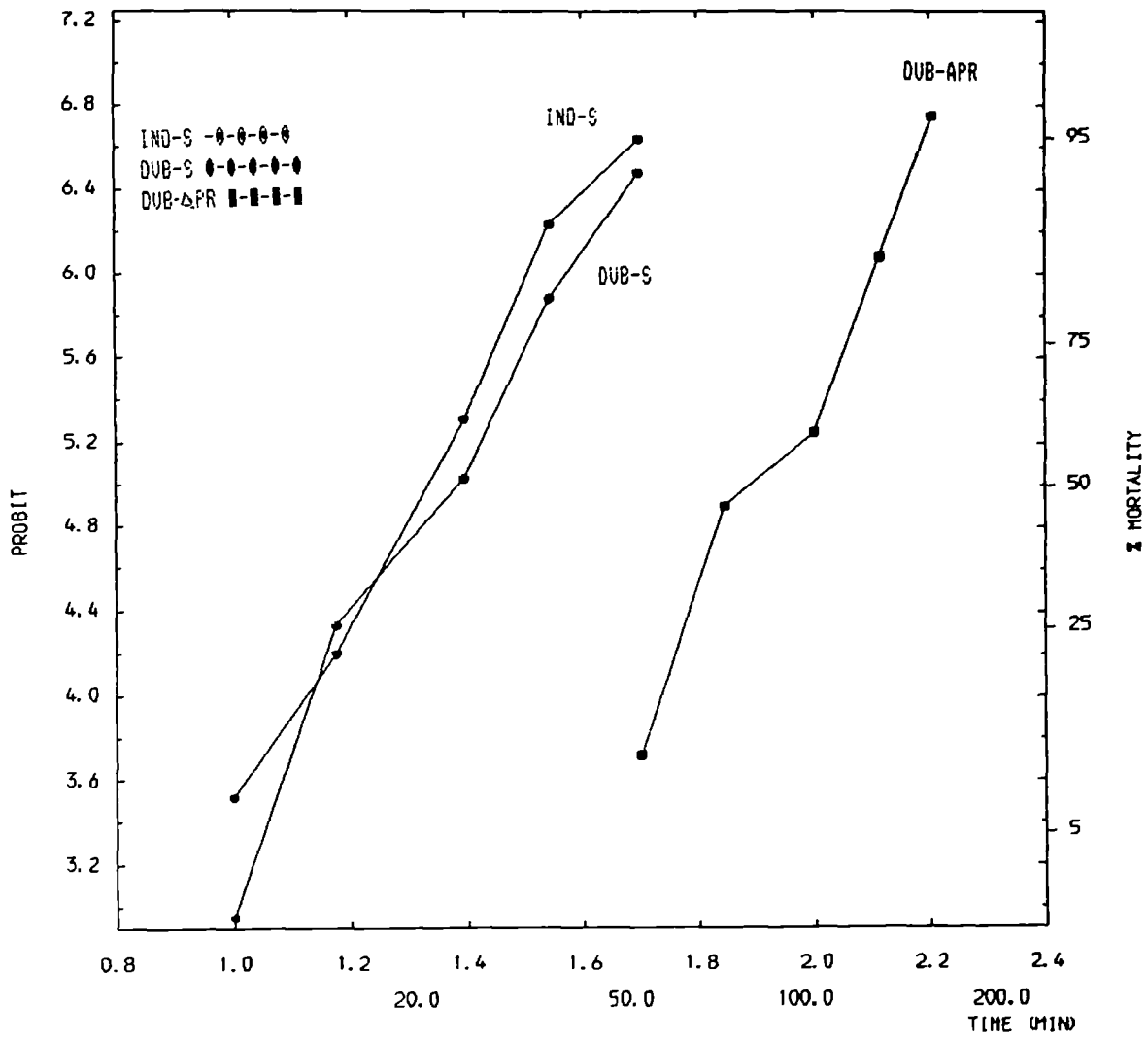
Fig 6.4 Effect of permethrin selection on adults of the

DUB-APR strain (horizontal test).
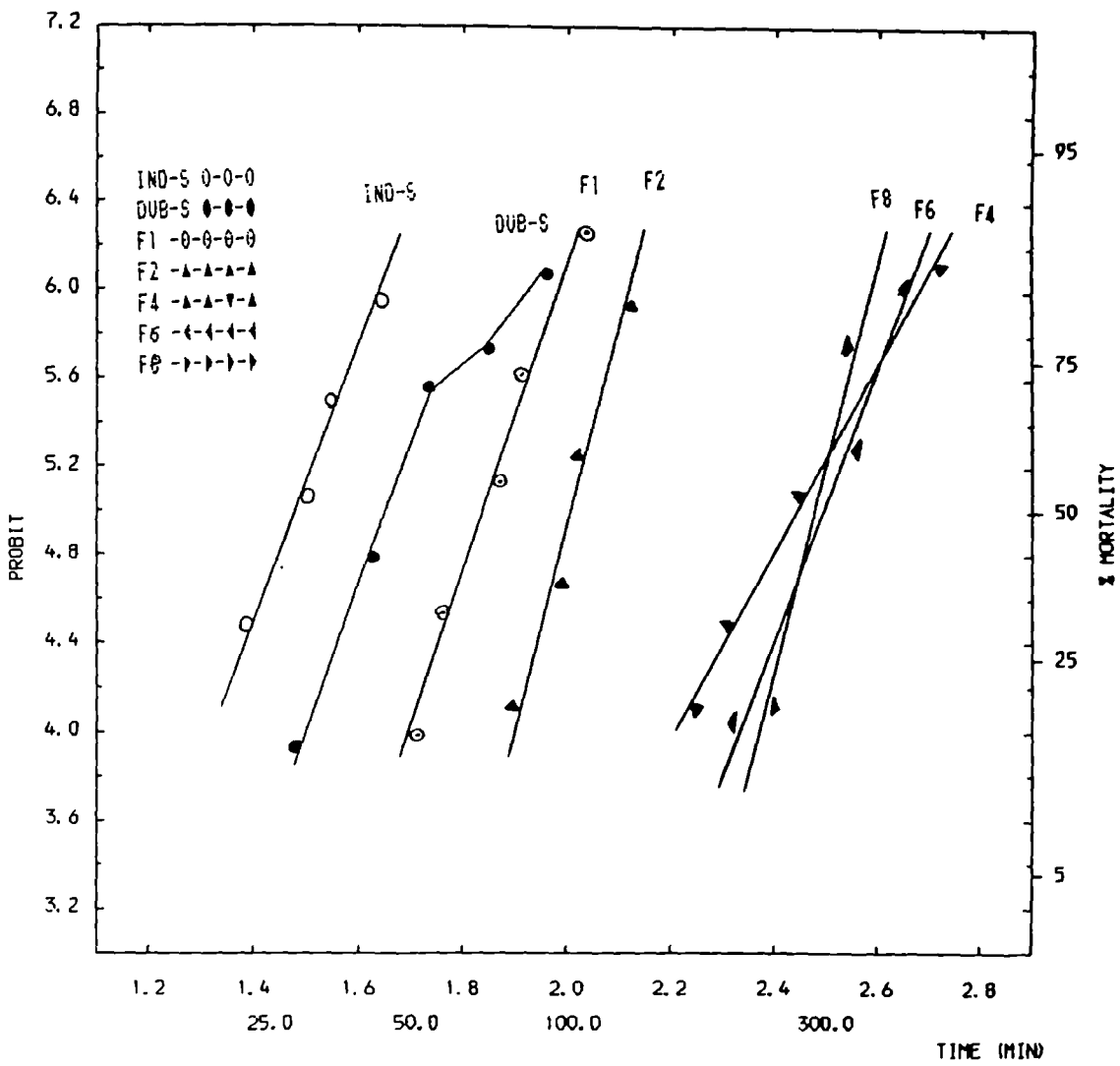
Fig 6.5 Response of adult females of the DUB-LPR strain

Following larval selection with permethrein.

TIME (MIN)
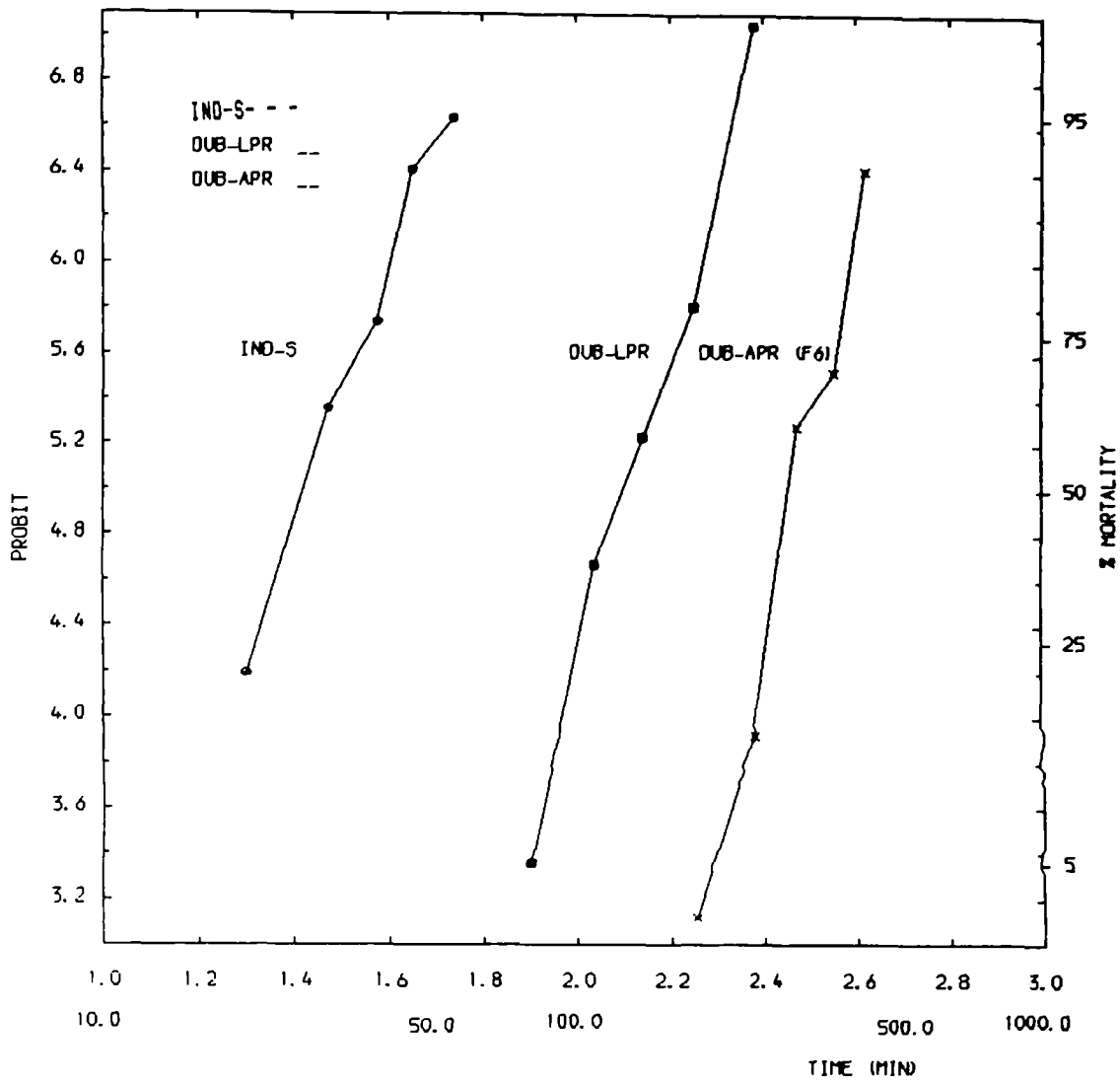
Fig 6.6 Genetics of permethrin resistance in adult females

of the DUB-Apr strain.
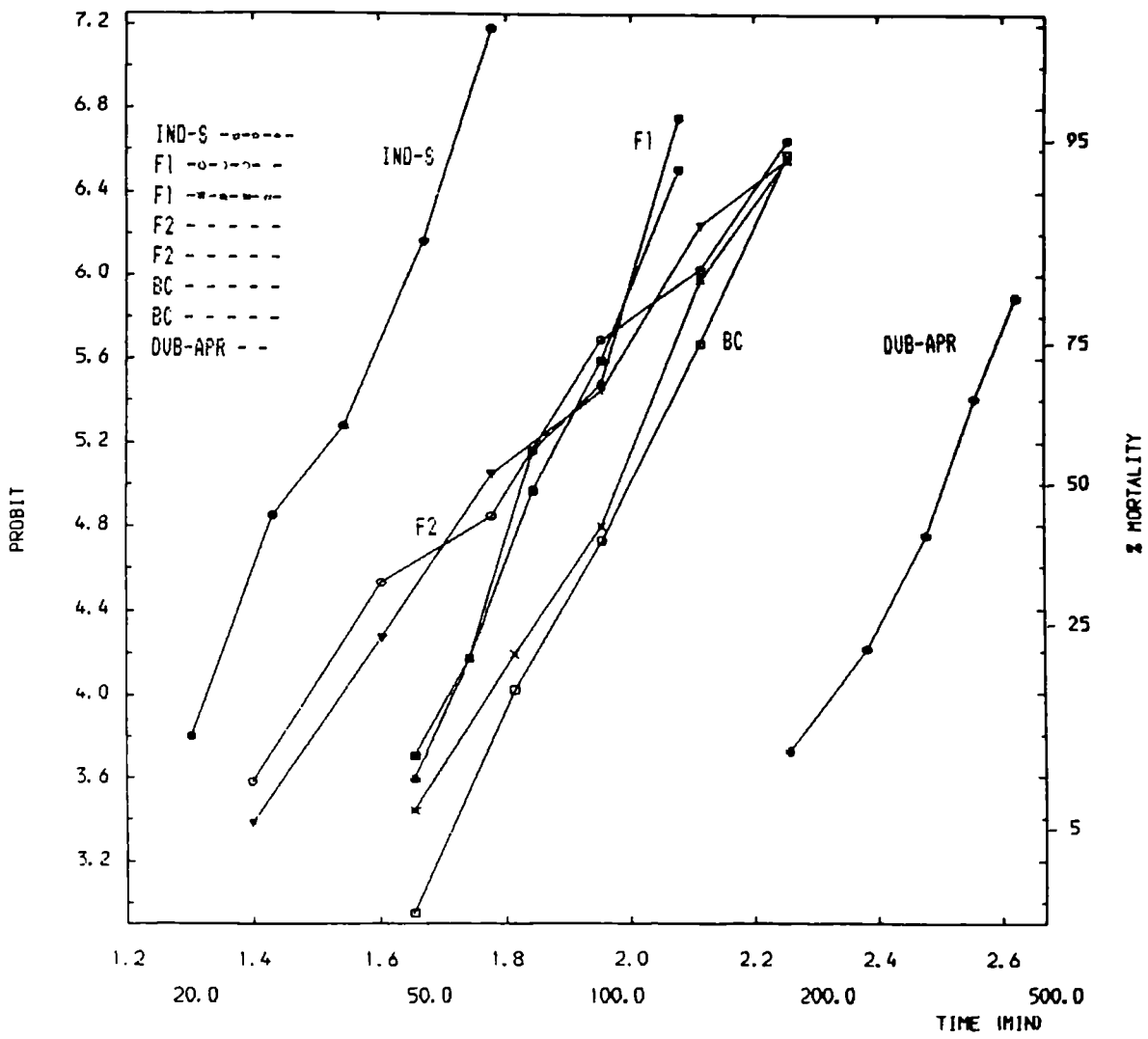
Fig 6.7 Irritability of adult females of stock strains and permethrin selected lines to permethrin.
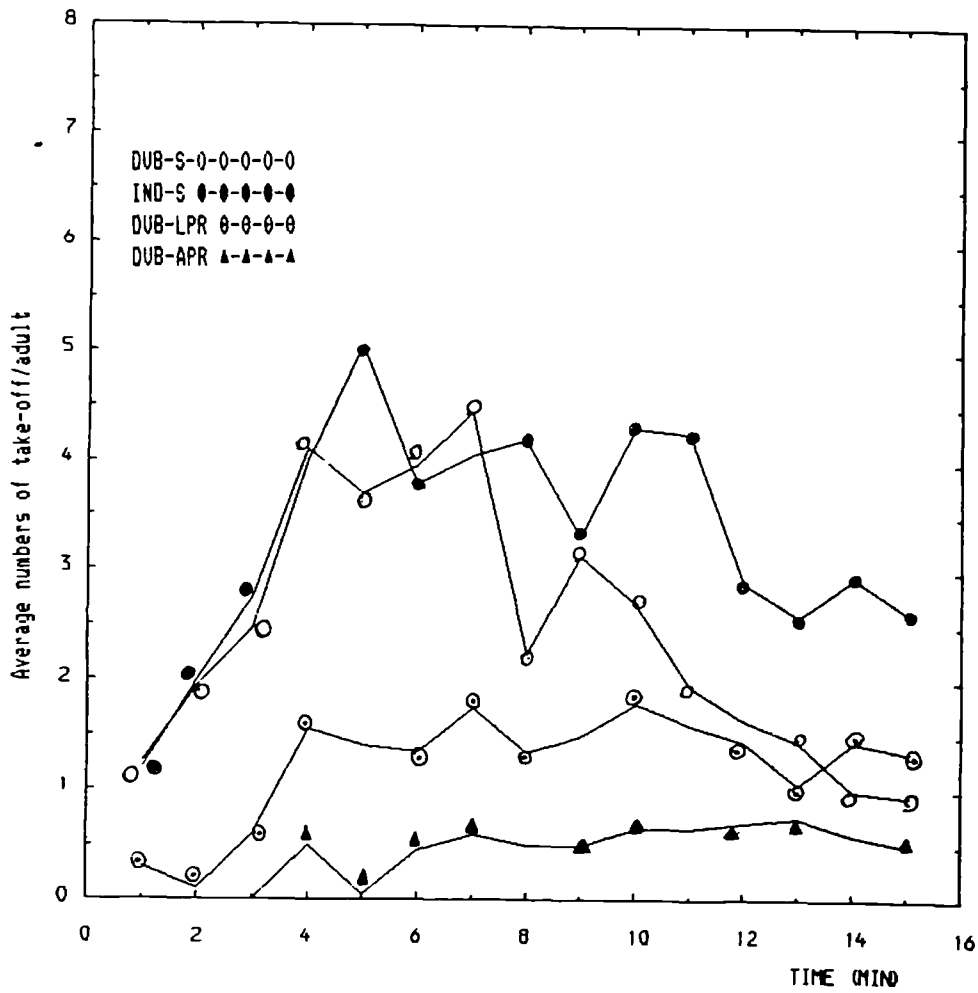
Chapter 7: Genetics and mechanisms of permethrin resistance in larvae of <u>An. stephensi</u>

7.1 Introduction

In chapter 6 the relationship between resistance to DDT and pyrethroids, and the role of the <u>kdr</u> gene in adults of <u>An. stephensi</u> and other mosquito species, has been discussed. Knock-down behaviour and recovery from knock-down were also studied.

In this chapter a similar study has been performed to investigate the mode of inheritance and mechanism of permethrin resistance, and the possible role of a <u>kdr</u> type gene in permethrin resistance in the larvae of <u>An. stephensi</u> from Dubai.

7.2 Permethrin selection on larvae of the DUB-LPR strain.

Since larvae of the IND-S strain showed susceptibility to four main groups of insecticides, it was used in this study as a susceptible stock.

The IND-S strain showed an LC50 of 0.043 mg/l compared with 0.022, 0.011 and 0.029 mg/l, for the other permethrin susceptible strains from India, Pakistan and India respectively (see table 9.4).

The DUB-S strain, which has recently been colonised in the laboratory, showed moderate to low levels of tolerance to a number of organophosphorus insecticides, high resistance to DDT, and moderate levels of tolerance to lambdacyhalothrin (see section 5.3 and table 5.2). Permethrin tests on the larvae also showed that this strain is highly heterogeneous for permethrin resistance, with a flat dosage mortality regression line of b= 0.840±.085 compared with b= 4.51 ± 0.35

for the IND-S strain. This suggests that the gene(s) for permethrin resistance is present in this population at a high frequency (see table 7.1 and Fig 7.1).

Selection was carried out on the larvae of the DUB-LPR strain, a sub-strain derived from the DUB-S strain. The results of successive selection are presented in table 7.2 and Fig 7.1.

Due to the genetic heterogeneity of the parental stock and in order to avoid rapid elimination of background genetic material leading to reduced genetic variation, selection began at a moderate level (62-65% mortality) for the first two generations of selection. From the 3rd generation, onwards, a high level of resistance was detected in the larvae, and despite the use of high insecticide concentrations, high levels of mortality could not be achieved. In consequence, the selection pressure had to be maintained at the lower level of 40-47%. The results of selection are shown in the table 7.3.

The parental stock with an initial LC50 of 0.32 mg/l was subjected to selection. Successive generations of selection resulted in a steady increase in LC50. At the F3 generation of selection, the LC50 increased 128-fold relative to the unselected DUB-S and resistance was fully developed in the larvae. Selection was continued for a further 7 generations, but from the F4 generation selection had only a slight effect on the LC50's or slope of selected lines. At the F7 generation, the LC50 of the selected strain had increased 138-fold relative to the DUB-S strain and 1030-fold that of the IND-S strain (table 7.2).

Recovery from knock-down to pyrethroid and DDT insecticides has been observed in a number of adult mosquitoes (Georghiou, 1962; Hitchen & Wood, 1974; Malcolm, 1988), but in the larvae it is rather

complicated. There is little literature referring to the knock-down phenomenon in larvae, although observation suggest that it does occur. Thus in the present study, the treated larvae showed a quick response to permethrin within the first 2 hours of exposure time. The larvae accumulated on the surface of the water, behavioural activities decreased and they showed no positive response to environmental stimuli, unless they were agitated by means of a pipette or water disturbance. In this case the live larvae quickly swam to the bottom and then tried to swim back to the surface.

Clearly in natural conditions larvae subjected to knock-down will, if susceptible, inevitably be killed by pyrethroids whereas adults may by virtue of knock-down be removed from contact before acquiring a lethal dose. In selection studies, normally a group of 200 larvae were exposed to an appropriate concentration of permethrin for a period of 24 hours. In order to score the mortalities, larvae were transferred into clean water and maintained for a further 24-48 hours to allow the live larvae to recover and also to record the delayed mortality. No recovery from the knock-down was recorded after the exposure period, whereas an additional mortality of between 4 to 8% was recorded during the first 24 hours of the holding period (delayed mortality).

In a study by Omer et al. (1980), DDT susceptible and resistant · larvae of An. stephensi from Pakistan were exposed to 10 and 100 ppm of DDT respectively for 6 hours, followed by 18 hours in clean water. The treated larvae which were unable to swim to the surface were considered as knocked-down and no recovery was observed from knocked-down larvae. In another study by Malcolm (1988 a), the knock-down activity of the larvae of An. stephensi from Pakistan to permethrin was studied. The

larvae were exposed to permethrin for 0.5, 1, 2, 4, and 24 hrs. In order to score the knock-down phenomenon, the larvae were gently pushed below the surface of water. Those larvae which were not able to swim back to the surface, were recorded as killed or knocked-down. At 2 and 4 hrs exposure followed by a 24 hr recovery period, a number of larvae were shown to recover from knock-down.

7.3 Cross-resistance

The cross-resistance spectrum of DUB-LPR, the permethrin resistant larvae, was determined with lambdacyhalothrin, deltamethrin and DDT respectively.

Initially the larvae of the DUB-S strain showed 2, 7.5, 1.43, and 133-fold greater LC50's than the IND-S strain to lambdacyhalothrin, permethrin, deltamethrin and DDT respectively (see table 7.1 and Figs 7.2, and 7.4).

At the F7 generation of permethrin selection, larvae of the DUB-LPR strain showed 95, 27.2 and 0.9 fold cross-resistance to lambdacyhalothrin, deltamethrin and DDT respectively, compared with the DUB-S strain, and 192, 39 and 133-fold compared with the IND-S strain, indicating that cross-resistance was fully developed to lambdacyhalothrin and permethrin, but not to DDT (table 7.1).

7.4 Response of larval selection in adults

In order to determine whether larval selection could produce adult resistance, the adults of the DUB-LPR strain (permethrin selected larvae) were tested with permethrin and lambdacyhalothrin at 10 and 1.2 $\mu g/cm^2$ respectively. The results are shown in table 7.4.

Permethrin selection on the larvae increased tolerance in adults to lambdacyhalothrin and permethrin to levels 2.7 and 3-fold that of the DUB-S strain, and 3 and 4.3-fold that of the IND-S strain.

7.5 Synergist effects

The effects of piperonyl butoxide (PB), a mixed function oxidase inhibitor, in the presence and absence of DDT, lambdacyhalothrin and permethrin were tested on the IND-S, DUB-S and DUB-LPR strains respectively. The results are presented in table 7.5 and Figs 7.3 and 7.4.

PB had no significant synergistic effect on DDT in the IND-S (DDT susceptible larvae) and DUB-S (DDT resistant larvae) (synergist ratio = 1.2 and 1.24 respectively), and this indicates that microsomal oxidase systems are not involved in the DDT resistance.

Chlorofenethol (DMC), a dehydrochlorinase inhibitor, was then tested with DDT in DDT susceptible and resistant larvae. These results also indicate no evidence of a dehydrochlorinase mechanism in DDT resistant larvae (synergistic ratio = 1.12, 1.11 respectively) see section 5.3 and table 5.7. This suggests that in DDT resistant larvae (DUB-S) neither mixed function oxidases nor dehydrochlorinase are involved.

PB in the presence and absence of permethrin and lambdacyhalothrin produced in the DUB-S strain synergistic ratios of 4.7 and 1.3 compared with resistance ratio's of 7.5 and 2 respectively. Strong synergistic ratios of 426 and 72.6 , compared with resistance ratios of 1030 and 192.5, were obtained when the DUB-LPR strain was tested with permethrin and lambdacyhalothrin in presence and absence of PB respectively (see

tables 7.1 and 7.5). Synergist results clearly indicated the involvement of an oxidase-based resistance mechanism in permethrin resistant larvae.

7.6 Mode of inheritance of permethrin resistance

In order to determine the mode of inheritance of permethrin resistance in the larvae of DUB-LPR strain, the IND-S (permethrin susceptible) and DUB-LPR (permethrin selected larvae) were reciprocally crossed by mass mating about 150 virgin adults of each sex, the sexes being separated at the pupal stage. The F1 progeny of the two reciprocal crosses were tested with permethrin and lambdacyhalothrin (see table 7.6 and Fig 7.5). The F1's showed no significant difference in their responses to permethrin (D_1=-0.34, D_2= -0.36, t=0.285, and P> 0.05) and to lambdacyhalothrin (D_1= -0.33, D_2 = -0.38, t = 0.585, P> 0.05), indicating that permethrin and lambdacyhalothrin resistance are autosomally inherited. The F1 results also indicate that resistance is inherited as a semidominant character. The single gene hypothesis was tested by reciprocal crosses of the F1 to the resistant and susceptible strains (back-crosses), and the F2 generations. If permethrin resistance is due to a major autosomal gene which is semi dominant, 1:1 ratios in each back-cross and 1:2:1 ratios in the F2 generations would be expected.

The expected mortalities for back-cross progeny and F2 generations, based on a single gene hypothesis, were calculated. The agreement of the observed response to expected in back-crosses and F2 generations were calculated by χ^2 method. These tests indicated no significant deviation at the 5% level from those expected on the single gene

hypothesis, when a semidominant genetic factor is operating (see tables 7.7 and 7.8 and Fig 7.5).

In another method to determine whether permethrin resistance was inherited in a monofactorial or polyfactorial manner, the offspring of crosses between the F1 generation and resistant strain (back-cross) were exposed to a discriminating dose to eliminate the heterozygous resistant larvae. This process was repeated through 3 further generations (repeated back-cross with selection). The results are shown in table 7.9. χ^2 tests indicated that the results of 3 back-crosses with selection did not differ significantly from those expected on the single gene hypothesis, suggesting that permethrin resistance is monofactorially inherited (see tables 7.7 and 7.8)

7.7 Discussion

Initially the DUB-S strain appeared highly heterogeneous for permethrin resistance. After only three generations of selection at a moderate level of selection pressure (40-65% mortality), permethrin resistance was fully developed in the larvae with a resistance ratio 138 that of the parental stock and 1030 compared with the IND-S strain. The resistant larvae also showed cross-resistance to lambdacyhalothrin, a relatively new synthesised pyrethroid insecticide, and deltamethrin.

Permethrin resistance following selection, and cross-resistance to other pyrethroids, has been reported in a number of mosquito species, including Culex pipiens quinquefasciatus (Priester & Georghiou, 1978) and An.stephensi from Pakistan (Omer et al., 1980).

The larvae of An.stephensi from Pakistan were subjected to DDT selection for 4 generations followed by a further 2 generations of

selection with DDT in conjunction with synergists (DMC + PB). Selection resulted in 187-fold resistance to DDT and 23-fold cross-resistance to permethrin. Selected larvae have also conferred 11-fold cross-resistance in adults. Synergist studies provided no evidence for enhanced metabolism due to dehydrochlorinase, or oxidases. This suggested that the resistance mechanism was of the kdr type (Omer et al, 1980).

Selection for permethrin resistance in larvae has been shown to confer adult resistance in a number of mosquito species including An. stephensi from Pakistan (Omer et al., 1980) and larvae of C. pipiens qinquefasciatus (Priester & Georghiou, 1978). In the present study, permethrin selection on the larvae of the DUB-LPR produced tolerance in the adults.

Piperonyl butoxide (PB), a mixed function oxidase inhibitor, and DMC, a dehydrochlorinase inhibitor had no synergistic effect on DDT in DDT resistant larvae, suggesting that microsomal oxidases and dehydrochlorinase are not the important detoxication mechanism in DDT resistant larvae. PB had a strong synergistic effect on permethrin and lambdacyhalothrin in the DUB-LPR strain, but it could not eliminate the resistance completely, suggesting that apart from microsomal oxidases as a primary detoxication mechanism, probably another genetic factor (s) with minor effect is involved in permethrin resistance in larvae of the selected strain.

As in An. stephensi from Pakistan (Omer et al, 1980), if a kdr type mechanism (site insensitivity) is expected to be involved in permethrin resistant larvae, synergists would not alter the resistance levels, and selection would confer or produce an increase in cross-

resistance to DDT. In the present study, the results clearly indicate the involvement of an oxidase-based mechanism rather than a _kdr_ type mechanism in permethrin resistant larvae.

The crossing experiments indicated that permethrin resistance is inherited as a monofactorial semidominant character with no indication of sex linkage. No comparative study on the mode of inheritance of permethrin resistance in either larvae or adults of _An. stephensi_ has been published. The mode of inheritance of permethrin resistance has been studied in adults of _Ae. aegypti_ from Bangkok. Permethrin resistance was found to be inherited as a monofactorial intermediate character (Malcolm & Wood, 1982), whereas a polyfactorial intermediate character was postulated for permethrin resistance in the larvae of _C. pipiens quinquefaciatus_ (Priester & Georghiou, 1978).

Table 7.1   Permethrin, lambdacyhalothrin, deltamethrin and DDT tests on different strains of *An. stephensi*.

Insecticide tested	Strains used	LC50 95% C.L mg/l	LC90 95% C.L mg/l	Slope± S.E	X² (df)	P	Resistance ratios (RR)*
permethrin	IND-S	0.040 0.043 0.046	0.075 0.083 0.095	4.505± 0.350	1.761 (3)	0.624	-
	DUB-S	0.200 0.321 0.468	6.587 10.760 21.170	0.840± 0.085	0.574 (3)	0.902	7.45
	DUB-LPR	36.09 44.29 58.80	180.93 306.80 732.45	1.525± 0.192	2.174 (3)	0.537	1030
lambdacyh-lothrin	IND-S	0.035 0.040 0.043	0.100 0.117 0.143	2.686± 0.198	0.254 (4)	0.993	-
	DUB-S	0.071 0.081 0.093	0.237 0.308 0.442	2.197± 0.189	3.102 (4)	0.541	2.03
	DUB-LPR	5.71 7.70 9.70	14.39 19.43 36.43	3.187± 0.274	7.049 (3)	0.07	192.5
deltamethrin	IND-S	0.443 0.555 0.690	1.90 2.70 4.60	1.864± 0.227	2.174 (3)	0.537	-
	DUB-S	0.577 0.795 1.052	4.16 6.51 13.15	1.403± 0.183	1.401 (3)	0.705	1.43
	DUB-LPR	18.868 21.651 25.491	38.42 49.04 73.57	3.609± 0.510	3.061 (2)	0.216	39.01
DDT	IND-S	0.026 0.0285 0.031	0.063 0.072 0.085	3.189± 0.215	2.170 (4)	0.705	-
	DUB-S	3.48 3.87 4.31	8.17 9.62 11.89	3.204± 0.264	2.002 (2)	0.367	133.4
	DUB-LPR	3.10 3.41 3.75	6.23 7.11 8.45	4.01± 0.324	0.728 (2)	0.695	117.6

*RR = Ratio of LC50 of the selected strain to LC50 of the IND-S strain.

Table 7.2　　　　　Results of permethrin selection on larvae of

An. stephensi, DUB-LPR strain.

Generation	LC50(mg/l) 95% C.L (min)	LC90 95% C.L (min)	Slope± (b)	X² (df)	P	Resistance ratio(RR)*
DUB-S	0.20 0.32 0.47	6.59 10.76 21.17	0.840± 0.085	0.574 (3)	0.902	-
F1	0.73 0.96 1.23	9.38 14.61 27.05	1.083± 0.103	3.978 (3)	0.264	3.0
F2	- 3.39 -	- 100.40 -	0.910± 0.091	9.341 (2)	0.009	10.6
F3	30.42 40.87 72.48	154.69 361.38 2364.3	1.354± 0.265	0.352 (2)	0.839	127.7
F4	31.44 41.64 88.87	126.35 321.78 5897.5	1.443± 0.381	1.366 (2)	0.505	130.1
F7	36.09 44.29 58.80	180.93 306.80 723.45	1.525± 0.192	2.174 (3)	0.537	138.4

*RR = Ratio of LC50 of the selected strain to LC50 of the DUB-S strain,

Table 7.3   Summary of permethrin selection on larvae of

the DUB-LPR strain.

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Generation	Insecticide conc, mg/l	No, individuals exposed	No, survivors	Selection pressure % mortalities
DUB-S	0,54	1315	456	65,3
F1	2	1556	580	62,72
F2	8	2055	754	63,31
F3	18	1563	908	41,91
F4	18	1395	833	40,29
F5	24	1905	1140	40,16
F6	24	3096	1646	46,83
F7	24	3731	2179	41,6

Table 7.4 Response of adult females of <u>An.stephensi</u> following larval

selection with permethrin.

Insecticide tested	Strains	LT50 95% C.L (min)	LT90 95% C.L (min)	Slope± (b)	X² (df)	P	Resistance ratio RR*
permethrin	IND-S	28.36 30.11 31.85	44.26 47.95 53.28	6.342± 0.563	2.725 (3)	0.436	-
	DUB-S	33.34 42.98 51.16	67.97 84.53 135.40	4.363± 0.379	7.757 (3)	0.051	1.43
	DUB-LPR	123.99 130.04 136.23	187.21 200.46 218.70	6.818± 0.525	4.127 (3)	0.248	4.32
lamb*	IND-S	19.76 21.18 22.64	35.70 39.20 44.02	4.793± 0.342	2.230 (3)	0.526	-
	DUB-S	21.70 23.27 24.91	40.07 44.26 50.15	4.588± 0.332	4.178 (3)	0.194	1.1
	DUB-LPR	58.75 62.38 66.30	98.57 107.75 120.38	5.4± 0.38	2.350 (3)	0.503	2.95

* RR = Ratio of LT50 of the selected strain to LT50 of the IND-S strain,
*lamb = lambdacyhalothrin,

Table 7.5 Effect of synergist (PB) on larvae of different strains of

An. stephensi.

Insecticide/ PB	Strains	LC50 95%C.L mg/l	LC90 95%C.L mg/l	Slope± S.E	X2 (df)	P	Resistance ratio RR‡	Synergist ratio SR‡
DDT		0.026 0.029	0.063 0.072	3.189± 0.215	2.170 (4)	0.705		
	IND-S	0.031	0.085				-	1.16
DDT+PB		0.027 0.025	0.0526 0.060	3.348± 0.231	2.601 (4)	0.627		
		0.027	0.070					
DDT		3.10 3.41	6.23 7.11	4.01± 0.324	0.728 (2)	0.695		
	DUB-S	3.74	8.43				119.47	1.24
DDT+PB		0.667 2.754	4.11 7.45	2.97± 0.257	10.527 (2)	0.005		
		6.582	17.79					
‡PR		0.040 0.0431	0.075 0.089	4.505± 0.350	1.761 (3)	0.624		
	IND-S	0.046	0.095				-	0.9
PR+PB		0.046 0.048	0.074 0.079	5.809± 0.398	5.598 (4)	0.231		
		0.051	0.088					
PR		0.200 0.321	6.59 10.76	0.840± 0.085	0.574 (3)	0.902		
	DUB-S	0.468	21.17				7.45	4.72
PR+PB		0.062 0.068	0.130 0.147	3.803± 0.308	4.861 (3)	0.182		
		0.074	0.173					
PR		36.09 44.29	180.93 306.80	1.525± 0.192	2.174 (3)	0.537		
	DUB-LPR	58.8	723.45				1027.7	425.9
PR+PB		0.094 0.104	0.308 0.362	2.367± 0.164	4.115 (6)	0.661		
		0.115	0.445					
lambd‡		0.071 0.081	0.237 0.308	2.197± 0.189	3.102 (4)	0.541		
	DUB-S	0.093	0.442				2.03	1.27
lambd‡+PB		0.058 0.064	0.139 0.159	3.226± 0.259	1.888 (3)	0.596		
		0.070	0.191					
lambd‡		5.71 7.70	14.386 19.432	3.187± 0.274	7.049 (3)	0.070		
	DUB-LPR	9.7	36.434				192.5	72.64
lambd‡+PB		0.086 0.106	0.193 0.251	3.44± 0.23	5.529 (3)	0.137		
		0.129	0.400					

‡RR = Ratio of LC50 of the selected strain to LC50 of the IND-S strain,

‡SR = LC50 of insecticide alone to LC50 of insecticide in the presence of synergist

‡lambd = lambdacyhalothrin, ‡PR = permethrin,

Table 7.6 Permethrin and lambdacyhalothrin tests on larvae of the F1

progeny from crosses between permethrin susceptible

(IND-S) and permethrin resistant larvae, (DUB-LPR).

Insecticide used	Crosses	LC50 95%C,L mg/l	LC90 95%C,L mg/l	Slope± S,E	X^2 (df)	P	Resistance ratio(RR)
permethrin	F1 *Idx*D♀	0.391 0.432 0.474	0.946 1.086 1.3	3.198± 0.259	1.9 (3)	0.593	10.04
	F1 *Ddx*I♀	0.358 0.395 0.432	0.822 0.934 1.10	3.430± 0.281	2.287 (3)	0.515	9.19
	F2*	0.534 0.746 1.05	19.38 37.14 89.45	0.755± 0.044	18.827 (11)	0.064	
	BC*	1.223 1.939 3.052	51.06 21.76 235.5	3.430± 0.281	18.479 (7)	0.01	
lamb*	F1 *Idx*D♀	0.209 0.230 0.253	0.484 0.556 0.661	3.353± 0.256	2.344 (3)	0.504	5.75
	F1 *Ddx*I♀	0.185 0.205 0.225	0.430 0.492 0.585	3.360± 0.265	3.220 (3)	0.359	5.13

*I = IND-S *D = DUB-S, *F2 = (Idx D♀) x (Idx D♀), BC = (Idx D♀)♂ x DUB-LPR♀* Lambdacyhalothrin

Table 7.7 Results of permethrin tests on back-cross progeny, from crosses between the F1 and permethrin resistant larvae (DUB-LPR) and the F2 generation.

Doses ng/l	*F2=(F1xF1) obs	1:2:1	4 reciprocal BC of F1 x RR* observed mortality X²	*AdxD♀	*A♀xDd	*BdxD♀	*B♀xDd	Exp 1:1	X² df=1
0,02	3 (100)	1,5	1,523						
0,036	13 (98)	8,75	2,468						
0,063	22 (101)	18,75	0,625						
0,11	28 (100)	25,75	0,264						
0,2	37 (99)	32,0	1,303	5 (100)	8 (100)	7 (99)	9 (100)	7,5	0,901
0,36	47 (103)	45,0	0,0142	22 (102)	22 (100)	27 (101)	24 (101)	20,5	0,098
0,64	51 (101)	61,0	4,676*	36 (100)	42 (101)	43 (100)	39 (99)	37	0,043
1,14	61 (99)	71,25	4,447	42 (98)	46 (102)	49 (102)	47 (98)	46	0,370
2	70 (100)	75,0	1,333	56 (102)	51 (100)	54 (101)	53 (102)	50	0,980
3,6	71 (100)	75,75	1,244	61 (103)	54 (98)	57 (99)	55 (100)	51,5	2,488
6,4	73 (101)	77,5	1,596	68 (98)	62 (99)	65 (98)	64 (100)	54,5	8,772*
11,4	75 (99)	78,25	0,372	75 (99)	74 (100)	72 (100)	71 (101)	61,5	8,483*
20	82 (101)	85,5	1,550	82 (100)	81 (101)	82 (101)	83 (99)	71	5,876*

*A = F1 =(IND-S d x DUB-LPR ♀), *B = F1 =(IND-S ♀ x DUB-LPR d) *D = DUB-LPR
* F2 = A x A ,
* The numbers in brackets represent the total tested,
* P< 0,05
* RR= permethrin resistant strain,

Table 7.8 Results of permethrin tests on back-cross progeny from

crosses between the F1 and permethrin

susceptible larvae (IND-S).

Doses ng/l	4 reciprocal BC of F1 x *SS				EXP. mortality 1:1	X^2 df=1
	observed mortality					
	I A♂xI♀	II A♀xI♂	III B♂xI♀	IV B♀xI♂		
0.02	4 (100)	5 (102)	5 (100)	6 (103)	3	0.34
0.027	13 (101)	11 (101)	14 (98)	15 (100)	8.5	2.46
0.036	29 (99)	33 (99)	35 (100)	33 (98)	17.5	5.78
0.047	44 (102)	39 (100)	44 (97)	40 (101)	27.5	*12.60
0.063	50 (101)	53 (100)	47 (101)	45 (99)	38.0	*5.65
0.08	55 (102)	54 (103)	52 (100)	54 (97)	44.5	*3.66
0.11	57 (100)	55 (98)	54 (99)	57 (103)	50	1.96
0.15	61 (100)	67 (101)	59 (98)	64 (101)	53.5	2.26
0.2	64 (99)	65 (100)	63 (102)	66 (100)	57	2.38
0.27	67 (101)	68 (100)	65 (100)	69 (100)	63	0.49
0.36	72 (100)	71 (101)	70 (101)	73 (101)	70.5	0.14
0.48	81 (103)	87 (99)	77 (99)	75 (98)	79	0.01
0.64	89 (102)	88 (100)	85 (101)	85 (99)	86	0.14
0.85	93 (100)	93 (101)	94 (100)	91 (100)	92.5	0.04
1.14	96 (100)	95 (99)	97 (100)	95 (101)	95	0.21
X^2	38.86	40.907	42.34	28.250		
df	13	13	13	13		

A = (IND-S ♂ x DUB-LPR ♀), B = (IND-S ♀ x DUB-LPR ♂), I = IND-S

* P< 0.05

* SS= permethrin susceptible

Table 7.9   Results of back-crosses with selection (F1 x DUB-LPR)

to distinguish between monofactorial and

polyfactorial inheritance.

Back-crosses	No. tested	No. dying	X^2 1:1 Expection	P
1st	599	316	1.818	NS*
2nd	580	297	0.338	NS*
3rd	677	363	3.547	NS*

* $P > 0.05$
Larvae were exposed to a concentration of 2 mg/l permethrin for 24 hr and the number of dying recorded.

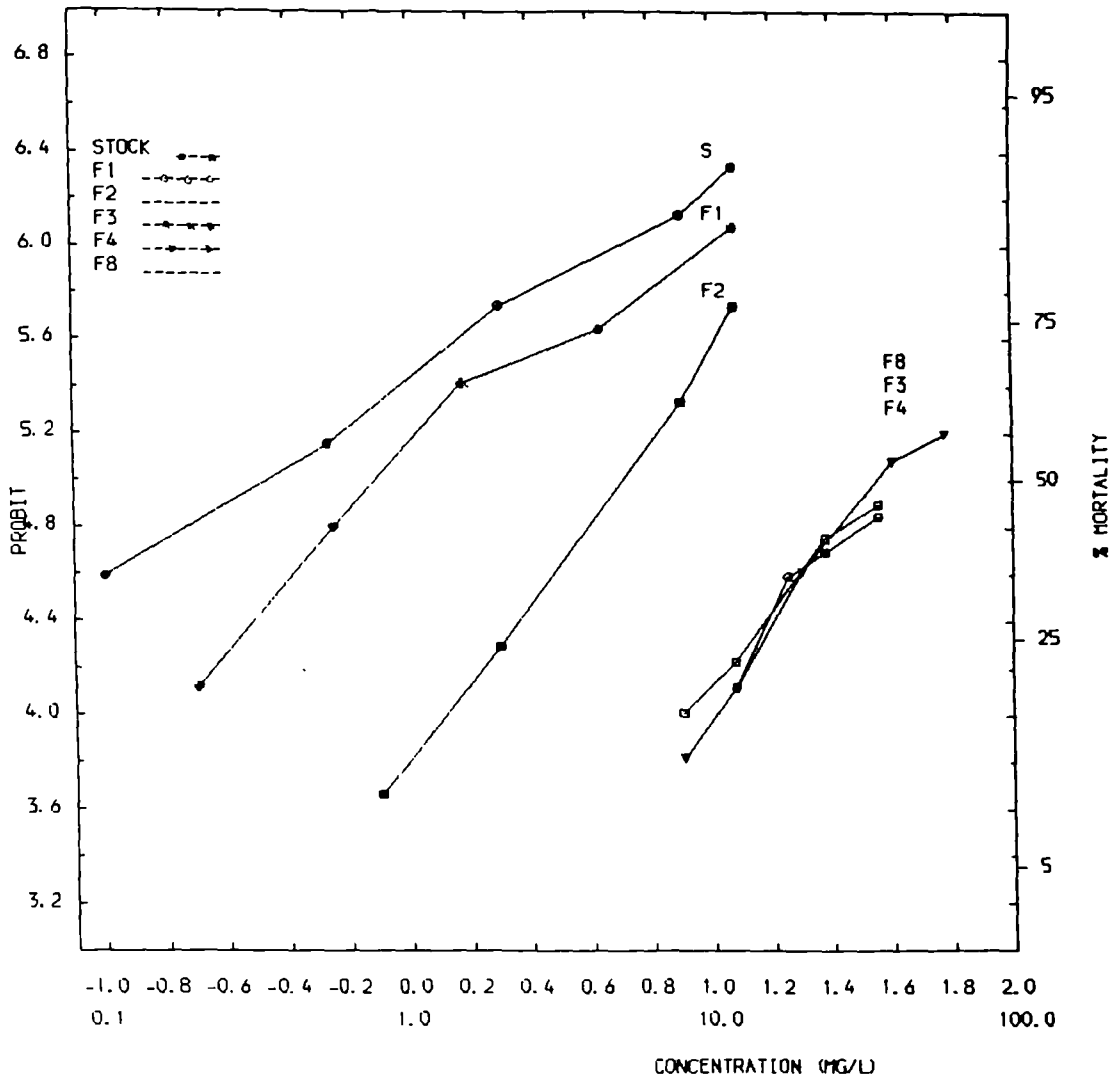# Fig 7.1 Effect of permethrin selection on larvae of the DUB-LPR strain.

Fig 7.2 Susceptiblity of larvae of the IND-S and DUB-LPR
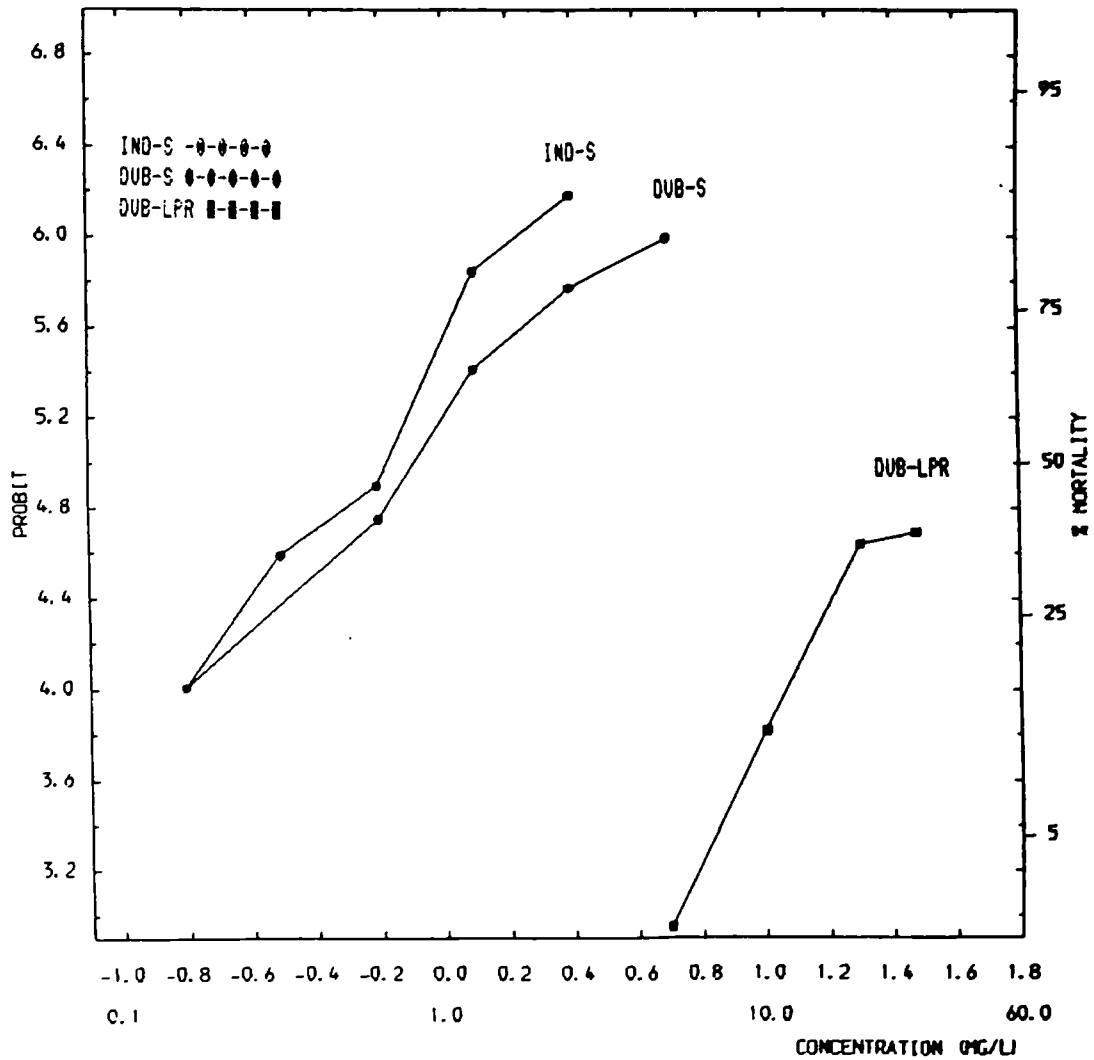
Strains to deltamethrin.

Fig 7.3 Effect of synergist (PB) on permethrin resistant larvae of theDUB-LPR strain.
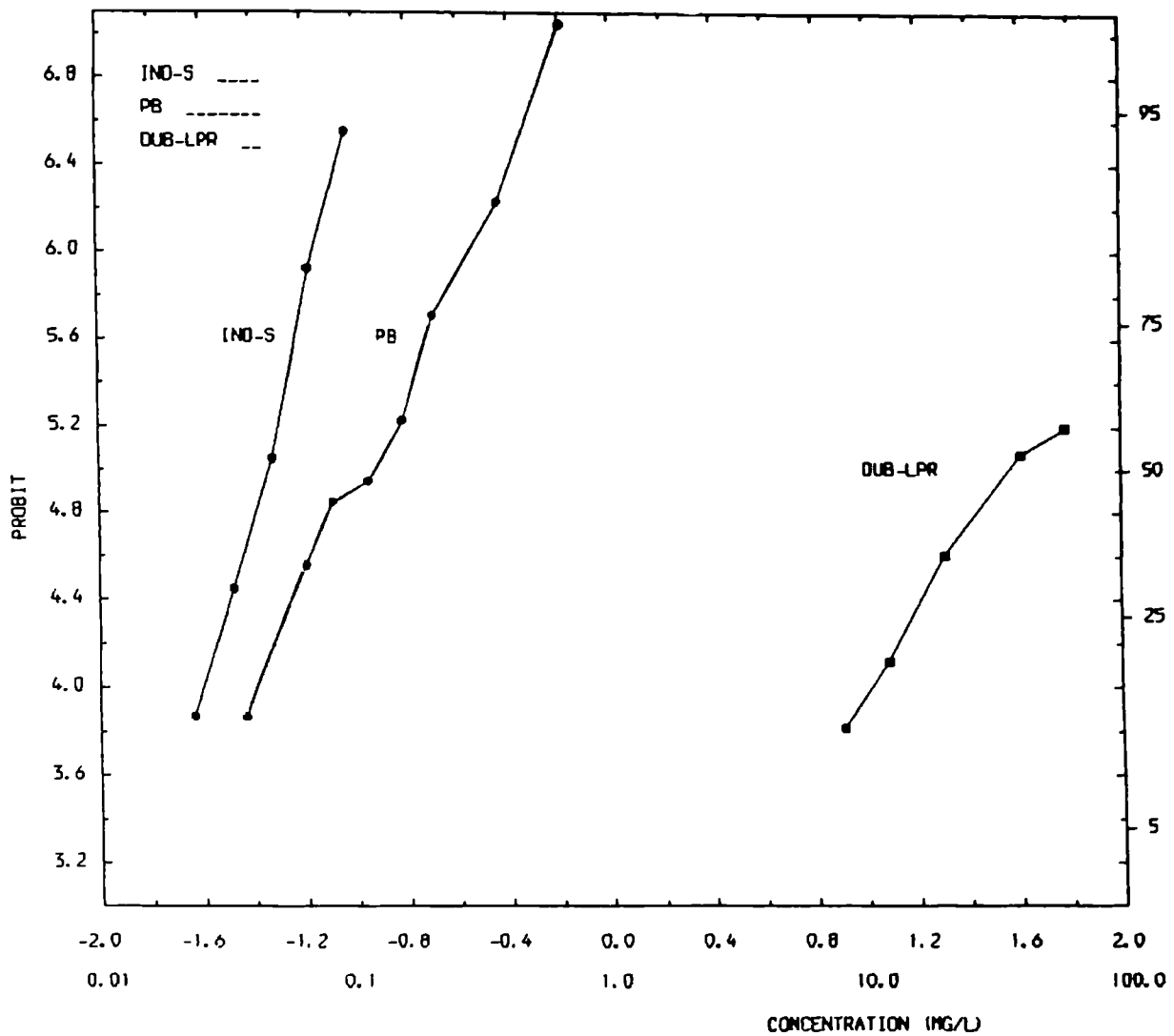
Fig 7.4 Effect of synergist (PB) on lambdacyhalothrin resistant

larvae of the DUB-LPR strain.
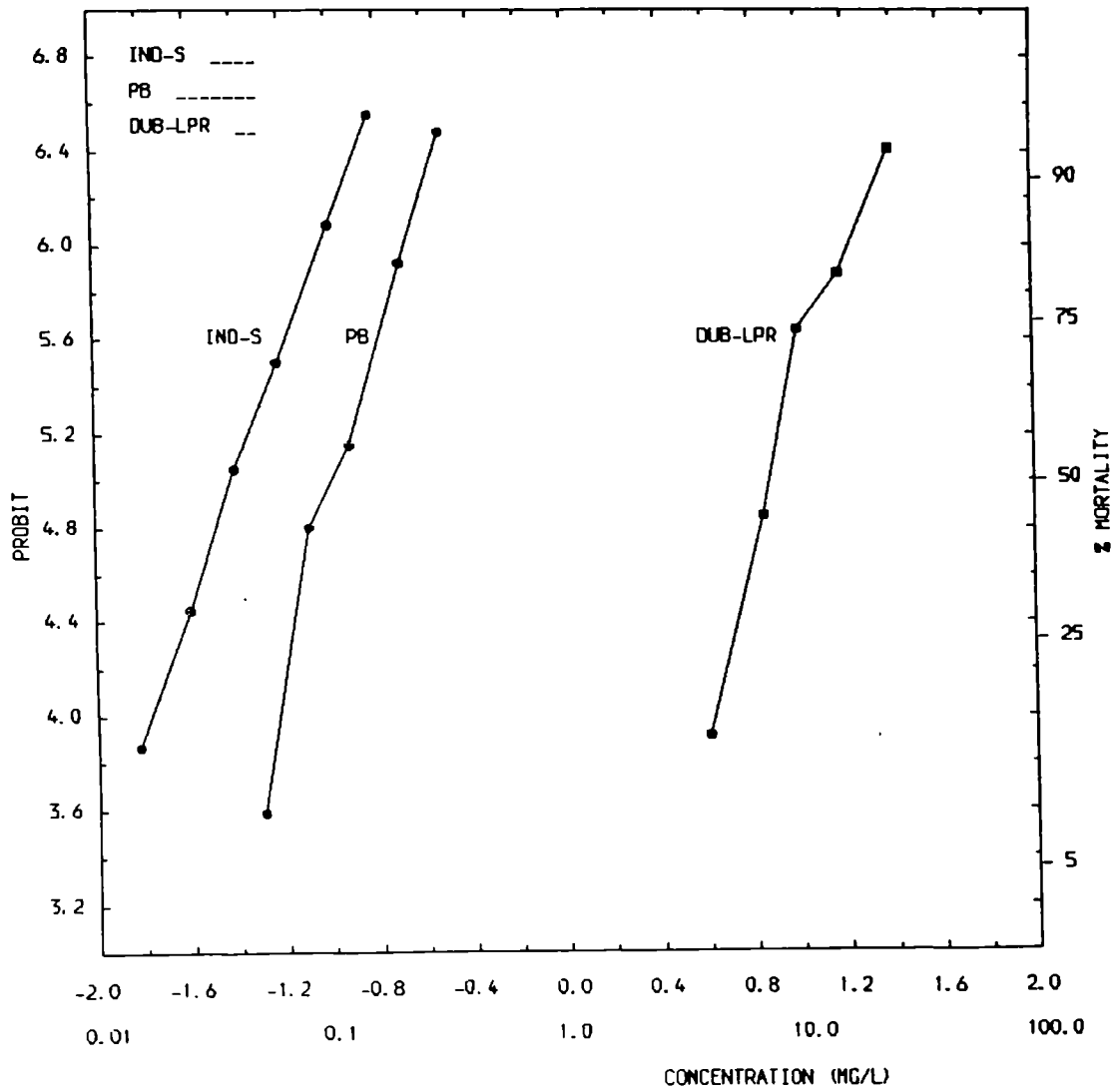
Fig 7. 5 Genetics of permethrin resistance in larvae of

the DUB-LPR strain.
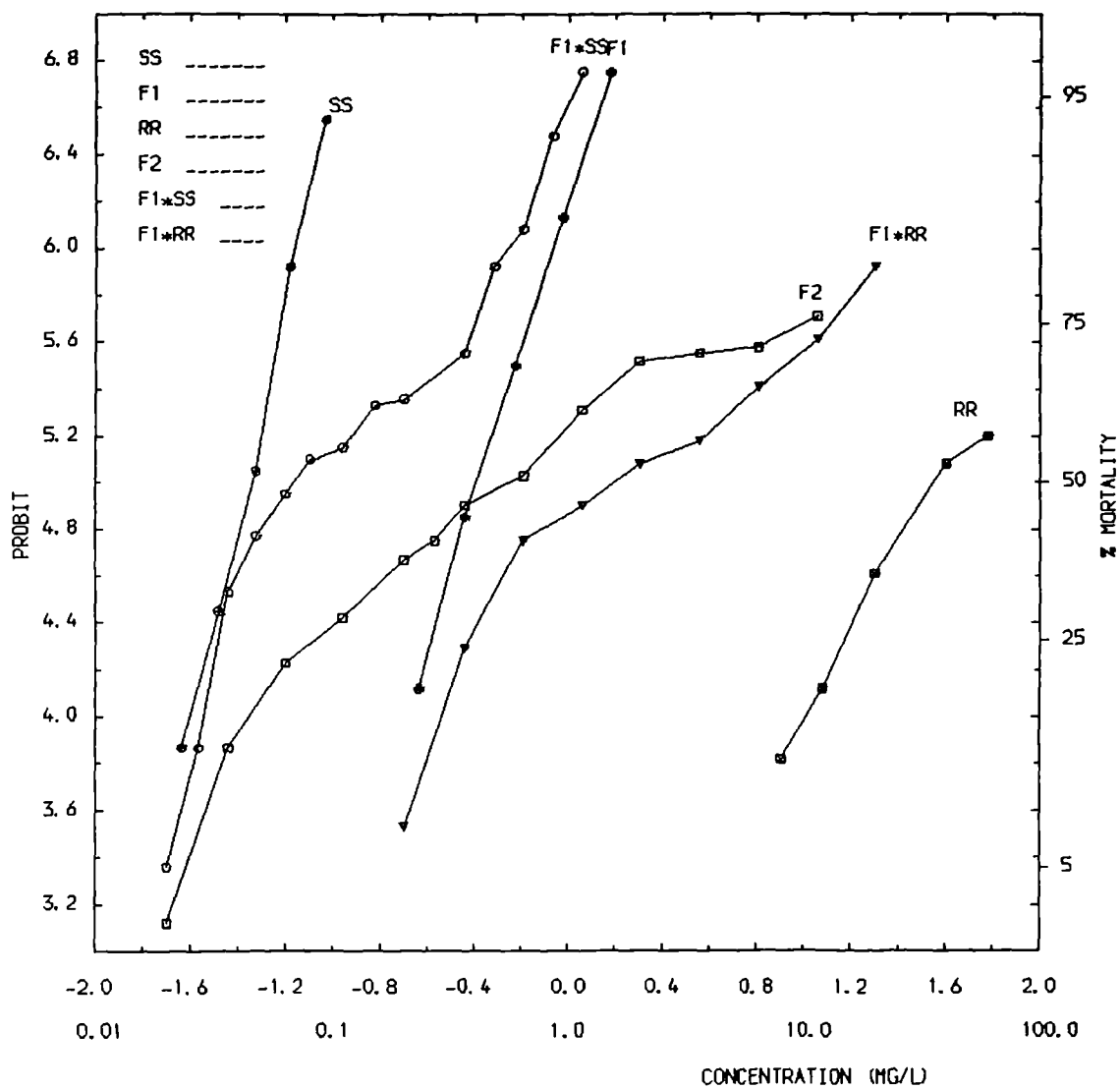
8. GENERAL DISCUSSION

8.1 Base-line susceptibility and genetics of DDT resistance in the larvae.

The base-line susceptibility of stock strains to different insecticides was determined. The IND-S strain, a laboratory stock which has been maintained in LSTM for some 10 years, showed resistance to DDT and dieldrin at the adult stage, and susceptibility to four groups of insecticides at larval stage. The DUB-S has been used in this study as a wild strain. It was obtained from a natural breeding place in Dubai (170 larvae) and tested after three generations of colonization.

The insecticides currently used in malaria control programmes in the U.A.E are mainly DDT and pyrethroids. as residual insecticides, temephos as a larvicide and pirimiphos-methyl as both an adulticide and larvicide (Malaria Control in U.A.E, 1981-1983; Farid, 1981). Due to the long history of insecticide application and the variety of insecticides used as larvicides or adulticides in mosquito control as well as in agricultural pest control programmes in U.A.E, the adults of the DUB-S strain showed a quite different pattern of susceptibility compared to the IND-S strain. It was resistant to DDT and dieldrin, tolerant to permethrin at the adult stage, and susceptible to the other insecticides tested. Susceptibility of the IND-S, DUB-S, and permethrin selected lines has been compared with the other susceptible and resistant strains of An. stephensi. The results are shown in tables 8.1 - 8.6.

DDT resistance with cross-resistance to pyrethroids has been reported in several strains of mosquitoes. The observed tolerance is unlikely to be the result of cross-tolerance induced by DDT, because synergist studies have indicated the involvement of different mechanism for resistance to DDT and resistance to permethrin (absence of a kdr gene). On the other hand there is evidence of the use of pyrethroid insecticides in malaria control programmes in the U. A. E .

In spite of the development of malathion resistance in An. stephensi in a number of countries in the Persian Gulf and Indian subcontinent, such as Iran, Pakistan, Iraq (WHO, 1985), the adults and larvae of the DUB-S strain showed susceptibility to malathion. Resistance to propoxur has not been reported in this species.

At the larval stage the DUB-S strain showed high resistance to DDT with an LC50 of 3.4 mg/l which is similar to the values of 4.6 and 3 in other DDT resistant strains from Iraq and India respectively (see table 8.4) and was highly heterogeneous for permethrin resistance. The larvae of this strain showed 1.6 and 1.8-fold tolerance to temephos and pirimiphos-methyl respectively, probably because of the use of these insecticides as potent insecticides in the U. A. E.

8.2 Genetics and mechanisms of DDT resistance

The mechanism of DDT resistance has been studied in different strains of An. stephensi. In a DDT-tolerant strain, originating from India, more than one-third of the absorbed DDT was converted to DDE in 24 hrs (Perry. 1960). In another DDT resistant strain from Iraq, piperonyl butoxide (PB), a mixed function oxidase inhibitor, and WARF, a dehydrochlorinase inhibitor, did not make the resistant strain much

more susceptible (see Brown & Pal, 1971).

In the present study, synergist studies suggested that neither dehydrochlorination nor hydroxilation mechanisms were involved in DDT resistant larvae, and also suggest that mixed function oxidases and dehydrochlorinase are not the important DDT resistance mechanisms in An. stephensi. Piperonyl butoxide (PB) had some synergistic effects on permethrin in permethrin tolerant larvae, indicating that mixed function oxidases are probably involved in the detoxication of permethrin in larvae of the DUB-S strain. These results suggest that different genetic factors are responsible for DDT and permethrin resistance in the larvae.

Inheritance of DDT resistance has been studied in other species of anopheline mosquito as well as in An. stephensi. In most cases, resistance has been found to be due to a single gene, varying in behaviour from incompletely dominant through to recessive. The expression of ancillary genes and influence of other factors on the genetic background have also been reported (Davidson & Mason, 1964; Davidson & Jackson, 1961, 1962).

In a study by Hemingway (1981), three DDT resistant species, An. atroparvus, An. gambiae and An. arabiensis were tested with the synergist, F-DMC, a dehydrochlorinase inhibitor. The F-DMC had a strong synergistic effect on DDT in An. atroparvus, indicating that dehydrochlorinase was the major resistance mechanism in this strain. In the case of An. gambiae and An. arabiensis, the synergist tests also suggested that dehydrochlorinase is involved in DDT resistant strains. However further selection with F-DMC/DDT produced a significant decrease in mortality, suggesting the involvement of an additional

genetic factor(s) for DDT resistance in both of these strains.

In the present study, crossing experiments indicated that the DDT resistance is inherited as an autosomal recessive character. The results from back-crosses, the F2 generations and repeated back-crosses with selection suggest the involvement of more than one genetic factor in DDT resistant larvae.

8.3 Pirimiphos-methyl selection

Pirimiphos-methyl selection was carried out on the larvae and adults of the IND-LPM and IND-APM strains respectively, two sub-strains derived from the IND-S, and larvae and adults of the DUB-LPM and DUB-APM strain respectively, two sub-strains derived from the DUB-S.

Pirimiphos-methyl selection on larvae of the IND-LPM and DUB-LPM strains resulted in increases in tolerance of 3.2 and 3.7-fold. Similarly, adults of the IND-APM and DUB-APM showed increases in tolerance of 2.8 and 3.8-fold that of the parental strains.

The IND-S strain has been used in this study as a laboratory stock. Inbred strains normally tend to lose fitness and genetic variability and this eventually leads to uniformity. Failure of selection for pirimiphos-methyl resistance in this strain could be mainly due to the loss of genetic variability as the result of inbreeding in the laboratory.

The DUB-S strain was used in this study as a wild strain. After initial colonization in the laboratory and two generations to increase numbers in the breeding stock, selection was commenced in the 3rd generation. In spite of the use of pirimiphos-methyl in the U.A.E as an adulticide and larvicide, pirimiphos-methyl selection on the adults and

larvae of this strain resulted in only a modest increase in tolerance. Failure of selection for pirimiphos-methyl resistance in this strain could be due to a number of factors such as: the effective population size is small, the genes concerned are subjected to very little selection, the sub-population has been isolated (restriction of gene pool), and the gene(s) for resistance are rare. The reduction in susceptibility of the selected strains might be due to interaction of multiple or ancillary genes, each of which only has a slight effect on tolerance.

Reversion of tolerance was studied when the selected strains were released from insecticide selection pressure. The tolerant strains reverted to susceptibility within a few generations of withdrawing the insecticide.

The larvae of selected strains showed a moderate level of cross-tolerance to malathion, temephos and fenthion (between 1.4 - 2.3-fold). A greater level of tolerance was observed when the DUB-LPM strain was tested with chloropyrifos (resistance ratio = 4.4), higher than pirimiphos-methyl itself (3.7 fold). No cross-tolerance was observed when the selected larvae were tested with DDT and propoxur.

The cross-tolerance spectrum of adults of the DUB-APM strains was tested with different insecticides. The adults showed a moderate level of tolerance to malathion and propoxur.

The enzymatic activity of different malathion resistant strains of An. stephensi has been studied by Herath & Davidson, (1981). In a strain from Iran, synergist tests suggested the involvement of carboxylesterase in malathion resistance and mixed function oxidases were involved only in the activation of malathion to toxic malaoxon .

In another strain from Pakistan carboxylesterase was found to be the basis of the malathion resistance mechanism.

In the present study, PB produced a continuous antagonism at all dosages tested. This could be attributed to the inhibition of those oxidases which are involved in the oxidative conversion of P=S to P=O during the activation of pirimiphos-methyl to a more toxic compound.

The non-specific esterase activity of pirimiphos-methyl selected and unselected strains was compared. There was no obvious increase in esterase levels of selected strain compared with unselected strains.

The AchE activity of the unselected stocks was compared with the selected strains. The results indicated that there was no correlation between enzyme activity and resistance. The observed differences in enzyme activity might be due to differences in the genetic background of strains rather than to the selection with pirimiphos-methyl.

8.4 Method of adult testing and selection studies with pyrethroids

Residual pyrethroids have been known to produce a permanent or temporary knock-down (kd) in mosquito species. Recovery from knock-down normally occurs, probably because some mosquitoes pick up insufficient amounts of insecticide to cause mortality. Consequently, in the WHO standard susceptibility test, increases in exposure time are not always related to increases in mortality, so that it is necessary to measure resistance to knock-down as well as resistance to kill.

Recovery from knock-down has produced problems during selection studies. Hemingway (1981) selected adults of An. gambiae from Nigeria for permethrin resistance, but 12 successive generations of selection failed to produce permethrin resistance. Rapid knock-down action of

permethrin did not make it possible to increase exposure time for higher selection pressure. Insecticide concentration had to be increased from 0.2% to 0.8%, but this also had little effect on selection. An attempt by Chadwick *et al* (cited in Malcolm, 1982) to increase the resistance level in Ae. aegypti, by increasing selection pressure with higher concentrations of insecticide and tubes used in the standard WHO manner, also failed, probably because the recovery of a number of mosquitoes made it difficult to attain homozygosity for permethrin resistance.

Hemingway (1980, 1981), in her selection studies on four species of anopheline mosquitoes with permethrin, segregated the mosquitoes which recovered from early knockdown (0-20 minutes), and late knock-down (20-60 minutes), in a modified WHO adult testing method. The recovered mosquitoes from the two distinct exposure times were allowed to breed, and selection was continued for a number of generations as parental stock with constant exposure time. Successive generations of selection failed to produce permethrin resistance, and this could have been due to the low level of selection pressure (2.2-7.7% mortality) applied to the late knock-down group (20-60).

In a study by Malcolm & Wood, (1982), a strain of Ae. aegypti was subjected to mass selection with permethrin, using the WHO standard susceptibility test. Selection resulted in a 20-fold increase in 10 generations of selection. From the F10 generation, selection procedures were changed and survivors were selected at the end of exposure time rather than after the 24 hrs recovery period. This resulted in about 30 fold increase in resistance by the F12 generation. The increase in resistance level could be due to the increase in selection pressure and

also to the elimination of knocked-down mosquitoes at the exposure time to reduce the chance of recovery from knock-down.

In the present study, due to the rapid knock-down action of permethrin and recovery of knocked-down mosquitoes during the holding period, the observed mortality line for IND-S, a permethrin susceptible strain, was curved mainly at the higher exposure times. In order to eliminate the early knocked-down adults during the exposure time, the WHO adult susceptibility tests were carried out with some modification. Testing and holding tubes were held in the horizontal rather than the normal vertical position, so that the knocked-down mosquitoes were obliged to stay on the insecticide impregnated paper during the exposure time, thus reducing the chance of recovery from knock-down (for more detail see section 6.2).

Following this method, permethrin selection was carried out for 8 generations. A high level of resistance was recorded at the F6 generation of selection with an increase in resistance of about 10-fold and delay to knock-down of 7.8-fold compared with IND-S, a permethrin susceptible strain.

Pyrethroid resistance in the field has so far been reported in only two species of anopheline mosquitoes, An. albimanus and An. sacharovi (see Malcolm, 1988). No additional information on the mode of inheritance and resistance level of these two strains has been published. Low level pyrethroid tolerance has been reported in 6 anopheline species; of which only An. gambiae from Burkina Faso showed comparatively high tolerance (5.8 fold) to bioallethrin (Rongsriyam & Busvine, 1975).

In culicine mosquitoes, a high level of permethrin resistance (30-

fold) has been reported in a field strain of Ae. aegypti from East Coast Demerara as the result of cross-resistance developed by DDT (Prasittisuk & Busvine, 1977).

In selection studies, a high level of permethrin resistance (30 fold) has been reported in the adults of Ae. aegypti from Bangkok (Malcolm, 1982). In An. stephensi, larvae of a strain from Pakistan were selected with DDT alone, DDT plus synergist (DMC+PB) and permethrin. The adults of selected larvae showed 9.4, 11 and 10-fold cross-resistance to permethrin respectively.

The mechanisms and relationship between resistance to DDT and permethrin in adults of Ae. aegypti have been studied by Malcolm & Wood (1982). Two major DDT resistance genes were identified; R^{DDT}, which is located on chromosome 2, controls the dehydrochlorination resistance mechanism, but confers no cross-resistance to permethrin; R^{DDT2} located on chromosome 2, is allelic to R^{PY} (permethrin resistance gene), confers 3-4 times less resistance to DDT than the former, but does confer cross-resistance to permethrin. In Ae. aegypti from East Coast Demerara, synergist tests indicated that a considerable part of DDT resistance was due to a dehydrochlorination mechanism. Further tests with PB suggested that microsomal oxidase systems were also involved in permethrin and DDT resistance (Prasittisuk & Busvine, 1977).

In the present study, piperonyl butoxide (PB), a mixed function oxidase inhibitor, and chlorofenethol (DMC), a dehydrochlorinase inhibitor, had no synergistic effect on DDT in DDT resistant adults. A synergistic ratio of 1.9 with PB, compared with a resistance ratio of 10, was recorded for permethrin in the selected adults. This indicates

that oxidative detoxication of permethrin by mixed function oxidases is not the major resistance mechanism in the adults of <u>An. stephensi</u>. These results raise the possibility that reduced sensitivity at the target site is the primary mechanism for permethrin resistance.

The inheritance of permethrin resistance in the selected strains was also investigated. Reciprocal crosses between the DUB-LPR, the permethrin selected adults, and IND-S, a permethrin susceptible strain, revealed that resistance is inherited as a partially recessive character. Tests on the back-cross progeny, and the F2 generations also suggest that permethrin resistance is an autosomal polyfactorial character. The knock-down effect of permethrin on the F1 generation, the F2, and back-cross progeny was also determined. A similar pattern of inheritance as above was obtained. This suggests that resistance to knock-down is genetically inherited and probably the genes for resistance to kill and resistance to knock-down are identical, further implicating target site insensitivity as the primary mechanism.

Inheritance of permethrin resistance in adults of <u>Ae. aegypti</u> resistant to permethrin has also been studied by Malcolm (1982). Resistance was found to be inherited as monofactorial intermediate character.

Cross-tolerance and delayed knock-down of the permethrin resistant strain was determined to lambdacyhalothrin. An increase in tolerance of 3.8-fold and delay to knock-down of 4-fold was recorded for adult females, compared to the IND-S strain. Adult selection also increased the cross-tolerance in larvae 36.6-fold that of the IND-S and 3.6-fold that of the parental stock.

Irritability of adult females of the IND-S and DUB-S, (stock

strains), DUB-APR, permethrin selected adults, and DUB-LPR, permethrin selected larvae, were determined to permethrin. The stock strains showed no significant differences in their irritability to permethrin, but the selected strains showed significantly reduced irritability to permethrin compared with stock strains (P<0.05). Reduced irritability of the selected strain compared with stock strains could be caused partly by oxidative detoxication of permethrin by mixed function oxidases as a minor factor and reduced sensitivity of active site (kdr like mechanism) as a primary factor in adults.

8.5 Genetics and mechanisms of permethrin resistance in larvae

Pyrethroid resistance in the larvae of mosquitoes has been relatively more studied than the adults, almost always as the result of selection studies in the laboratory.

95 species of mosquitoes (56 anopheline, 39 culicine) have been recorded as resistant to DDT in the field (Brown, 1986). Resistance to pyrethroids as the result of cross-resistance developed by DDT resistance has been reported in Culex tarsalis by Hoyer & Plapp, (1968). In other mosquitoes, larvae of Ae. aegypti and An. gambiae showed an increase in tolerance 2.9-3 to allethrin and 4.2-5.8 to bioallethrin respectively (Rongsriyam & Busvine, 1975). The presence of synergistic action by piperonyl butoxide mainly in An. gambiae suggested that the tolerance depended on a microsomal oxidation system.

The DDT resistant larvae of Culex tarsalis showed 10-fold cross-resistance to pyrethrins. A kdr-like mechanism was postulated for the observed resistance. Breeding experiments demonstrated that resistance to DDT and pyrethrins was genetically linked, and possibly controlled

by a similar mechanism which was not metabolic.

In selection studies, larvae of An.stephensi from Pakistan initially showed low levels of resistance to DDT, but susceptibility to pyrethroids. The larvae were subjected to selection with DDT for 4 generations. Selection increased resistance to DDT about 98-fold and cross-resistance to permethrin about 12-fold. A further 2 generations of selection with DDT in conjunction with synergists (DMC+PB) resulted in even higher levels of DDT resistance (187-fold) and cross-resistance to permethrin (23-fold). Synergist studies provided no evidence for enhanced metabolism due to dehydrochlorinase, or oxidases. This suggested that the resistance mechanism was of the kdr type (Omer et al, 1980).

In a study by Priester and Georghiou (1978, 1979), larvae of Culex pipiens quinquefasciatus were subjected to selection with d-trans permethrin. Selection resulted in a more than 4000-fold increase in resistance and above 2000-fold increase in cross-resistance to DDT. Piperonyl butoxide had little effect on DDT resistance, although it had some synergistic effect on permethrin. A kdr type resistance mechanism (non-metabolic mechanism) was postulated for the major part of permethrin resistance.

In the present study, larvae of the DUB-S strain were initially resistant to DDT and highly heterogeneous for permethrin resistance . After only 3 generations of permethrin selection , resistance was fully developed in the larvae 1028-fold compared with the IND-S strain, and 138-fold that of the parental stock. The selected strain showed cross-resistance to lambdacyhalothrin and deltamethrin, but not to DDT, probably because this strain was already highly resistant to DDT.

Permethrin selection also produced adult tolerance. This has also been observed in a number of pyrethroid resistant mosquitoes, including Culex quinquefasciatus (Priester & Georghiou, 1978) and An. stephensi (Omer *et al*, 1980).

In the present study the relationship between DDT and permethrin resistance was examined in the selected strain. Piperonyl butoxide and DMC had no significant synergistic effects on DDT in both the parental stock and permethrin selected larvae. However, PB produced a strong synergistic effect on permethrin and lambdacyhalothrin in the selected larvae. This suggests an oxidase-based resistance mechanism for the observed resistance in the larvae.

Inheritance of permethrin resistance has been studied in the larvae of Culex quinquefasciatus (Priester & Georghiou 1978). Permethrin resistance was found to be inherited as an intermediate polyfactorial character. In the present study the crossing experiments suggest that permethrin resistance is inherited as a monofactorial semidominant character with no indication of sex linkage.

Mosquito control programmes have been faced with a number of serious problems. Inadequate operational methods, poor quality spray application, low rate of coverage of insecticide and, most importantly, the exophilic habits of mosquito vectors are probably the main reasons for the failure of current mosquito control programmes.

Ways of improving operational methods and increasing the efficacy of compounds have always been investigated.

In malaria control programmes, emphasis has recently been placed on the use of bednets impregnated with pyrethroid insecticides for personal protection (WHO, 1983). The effectiveness of bednets

impregnated with permethrin in the laboratory followed by testing on a village scale has been reported by Lines et al. (1987) and Charlwood and Graves (1987). In China, in three experimental villages, mosquito nets were treated with 0.5 g/m² permethrin. During 5 months of post-treatment evaluation in the villages, permethrin-impregnated mosquito nets reduced the number of mosquitoes found inside nets by 75-99.3% (Jinjiang et al., 1988). There are several other reports on the effectivness of impregnated bednets in North America, Africa, Malaysia, Papua New Guinea and Suriname (see Lines et al., 1987).

This method of personal protection appears to be extremely effective and hence of considerable promise for malaria control. It is therefore of some concern that the pyrethroid resistance observed in the DUB-APR strain may well seriously effect the efficacy of impregnated nets. This is a topic urgently requiring investigation. If such resistance were to become a more widespread phenomenon, then there would have to be changes in the ways in which impregnated nets were used.

Dr C.F. Curtis (pers comm) has suggested the use of insecticide mixtures such as OP compounds in permethrin impregnated bednets. Residual and side effects of mixtures, particularly OP compounds, require attention. The other possibility might be to use synergists combined with pyrethroids. However such synergists are normally more expensive than the insecticide itself, and the synergists are normally unstable in light. Therefore attention must be given to cost and stability. The other subject requiring further investigation is the nature of the kdr type resistance mechanism (target site insensivity) as opposed to the metabolic breakdown of insecticide, because in the

<u>kdr</u> type resistance, synergists would have little or no effect on pyrethroids. Further investigations in the laboratory and in the field, could probably answer these questions.

Table 8.1 Susceptibility of adults of different strains of
Anopheles stephensi to different insecticides.

Strains	Origin	type of insecticides	Exposure time(min)	% Mort	References
ST Susceptible	India lab stock	dieldrin 0.4%	1hr	91.3	Hemingway, 1981,
ST-Pond	Field strain Pondicherry India	dieldrin 0.4%	1hr	100	
ST-Iraq	Field strain Basrah Iraq	dieldrin 4%	2hr	24.1	
ST-Iran	Field strain Bandarabbas	dieldrin 4%	2hrs	49.8	
ST-Lahore	Field strain Lahore	dieldrin 4%	2hrs	100	
ST Iran	Iran lab stock	dieldrin 4%	1hr	70	Present study
IND-S	India lab stock	dieldrin 4%	1hr	39.6	
DUB-S	Field strain Dubai U.A.E.	dieldrin 4%	1hr	0	
ST-Pond	Field strain Pondicherry India	DDT 4%	1hr	18.2	Hemingway, 1981,
ST-Iraq	Field strain Basrah Iraq	DDT 4%	-	1hr	
ST Iran	Field strain Bandarabbas	DDT 4%	1hr	2.2	Present study

Table 8.2 Susceptibility of adults of different strains of

Anopheles stephensi to different insecticides.

Strains	Origin	type of insecticides	LT50 (min)	Exposure time(min)	% Mort	References
ST Lahore	Field strain Lahore	DDT 4%	-	1hr	36.1	Hemingway, 1981.
ST Iran	Iran lab stock	DDT 4%	-	1hr	5	Present study
IND-S	India lab stock	DDT 4%	-	1hr	36.6	
DUB-S	Field strain Dubai U.A.E.	DDT 4%	-	1hr	0	
Walter reed	Susceptible strain India	permethrin	44	-	-	Omer et al, 1980.
Kasur-P	Field strain Pakistan	permethrin	48	-	-	
DDT/Syn RF2	DDT resistant strain with DDT+DMC+PB	permethrin	477	-	-	
IND-S	India lab stock	permethrin	30.1	-	-	Present study
DUB-S	Field strain Dubai U.A.E.	permethrin	43	-	-	
DUB-APR	permethrin selected line Dubai U.A.E.	permethrin	299.1	-	-	

Table 8.3 Susceptibility of adults of different strains of
Anopheles stephensi to different insecticides.

Strains	Origin	type of insecticides	LT50 (min)	exposure time(min)	% Mort	References
ST Iraq	Field strain Basrah	Malathion 5%	-	1hr	36,2	Hemingway, 1981
ST Iran	Field strain Bandarabbas	Malathion 5%	-	1hr	49,6	
ST Lahore	Field strain Lahore	Malathion 5%	-	1hr	0	
IND-S	India Lab stock	Malathion 5%	19,2	-	-	Present study
DUB-S	Field strain Dubai U.A.E.	Malathion 5%	27,3	-	-	

Table 8.4 Susceptibility of different strains of __Anopheles stephensi__

larvae to different insecticides.

Strains	Origin	type of insecticides	LC50 mg/l	References (mg/l)
ST,m	Monlaha Iraq 1966 colony	DDT	4,6	Rongsriyam and Busvine 1975,
ST susceptible	India lab stock	DDT	0,08	
ST-IND	Field strain India	DDT	2,98	Verma & Rahman, 1984,
IND-S	India lab stock	DDT	0,029	Present study
DUB-S	Field strain Dubai U.A.E.	DDT	3,41	-
Walter Reed	India lab stock	permethrin	0,022	Scott and Georghiou 1986,
Mal,R	Field strain Pakistan	permethrin	0,011	
BARR	Bangalore originally	permethrin	0,029	Malcolm, 1988,
Walter Reed	originated from India susceptible	trans-permethrin	0,013	Omer et al, 1980,
		cis-permethrin	0,0035	
DDT/Syn	DDT resistant strain selected with DDT+DMC+PB	trans-permethrin	0,25	-
		cis-permethrin	0,82	

Table 8.5   Susceptibility of different strains of <u>Anopheles stephensi</u>

larvae to different insecticides.

Strains	Origin	Type of insecticides	LC50 mg/l	Conc. (mg/l)	% Mort	References
IND-S	India lab stock	permethrin	0,043	-	-	Present study
DUB-S	Field strain Dubai U.A.E.	permethrin	0,321	-	-	
DUB-LPR	permethrin selected larvae	permethrin	21,65	-	-	
ST	India lab stock	Malathion	-	0,125	71,6	Hemmingway, 1986 1981,
ST Lahore	Field strain Lahore	Malathion	-	0,125	39,1	
ST Iraq	Field strain Iraq	Malathion	-	0,5	38	
ST Pond	Field strain Pondicherry	Malathion	-	0,5	34,2	
ST Iran	Field strain Iran	Malathion	-	0,5	35,8	
ST,m	Moalaha Iraq 1966 colony	Malathion	0,032	-	-	Rongsriyam & Busvine 1975,
ST Pond	Pondicherry India	Malathion	0,18	-	-	Das and Rajagopalan, 1981,
Walter Reed	India	Malathion	0,18	-	-	Scott and Georghiou 1986,
Mal-R	Khano Harni Pakistan	Malathion	1,6	-	-	

Table 8.6   Susceptibility of different strains of _Anopheles stephensi_

larvae to different insecticides.

Strains	Origin	type of insecticides	LC50 mg/l	Conc, (mg/l)	% Mort	References
IND-S	India lab stock	Malathion	0,086	-	-	Present study
DUB-S	Field strain Dubai U.A.E.	Malathion	0,131	-	-	
ST Lahore	Field strain Lahore	temephos	-	0,0075	30	Hemmingway 1981
ST Pond	Field strain Pondicherry	temephos	0,0015	-	-	das and Rajagopalan 1981.
IND-S	India lab stock	temephos	0,0016	-	-	Present Study
DUB-S	Field strain Dubai U.A.E.	temephos	0,0026	-	-	
ST	India lab stock	fenthion	0,0033	-	-	Rongsriyam and Busvine 1975
ST,M	Field strain Momlaha Iraq	fenthion	0,0139	-	-	
ST Pond	Field strain Pondicherry	fenthion	0,016	-	-	Das and Rajagopalan, 1981.
IND-S	India lab stock	fenthion	0,0056	-	-	Present study
DUB-S	Field strain Dubai U.A.E.	fenthion	0,0073	-	-	

Chapter 9. Summary

9.1 Base-line susceptibility and genetics of DDT resistance in the larvae of An. stephensi

The IND-S strain, a laboratory stock of An.stephensi which has been maintained in LSTM for some 10 years, showed resistance to DDT and dieldrin at the adult stage and susceptibility to four main groups of insecticides at the larval stage. The DUB-S strain has been used in this study as a wild strain. It was obtained from a natural breeding place in Dubai, and tested after three generations of colonisation.

Adults of the DUB-S strain were resistant to DDT and dieldrin tolerant to permethrin and susceptible to other insecticides tested such as malathion, pirimiphos-methyl and propoxur.

At the larval stage, the DUB-S strain showed a different pattern of susceptibility compared to the IND-S. It was extremely highly resistant to DDT (LC50 = 3.41 mg/l) with a resistance ratio of 119.5 that of the IND-S strain, and was highly heterogeneous for permethrin resistance. The larvae of DUB-S showed 1.6 and 1.8-fold tolerance to temephos and pirimiphos-methyl respectively.

Inheritance of DDT resistance in larvae of the DUB-S strain was studied. Crossing experiments indicated that the DDT resistance is inherited as an autosomal recessive character. The results from back-crosses and the F2 generation, and repeated back-crosses with selection, suggest the involvement more than one genetic factor in DDT resistant larvae. The effects of synergists, piperonyl butoxide (PB), a mixed function oxidase inhibitor and chlorofenethol (DMC), a

dehydrochlorinase inhibitor, in the presence or absence of DDT were determined on DDT resistant larvae. PB and DMC had no significant synergistic effects on DDT in the larvae of this strain. This suggests that neither mixed function oxidase nor dehydrochlorinase are involved in DDT resistance in the larvae. The effect of PB was also studied on permethrin in permethrin tolerant larvae. PB had some synergistic effects on permethrin in permethrin tolerant larvae. This suggests that mixed function oxidases are probably involved in detoxication of permethrin in the DUB-S strain.

9.2 Pirimiphos-methyl selection of the adults and larvae of two strains of An. stephensi

Pirimiphos-methyl selection was carried out on the larvae and adults of the IND-LPM and IND-APM strains, respectively, two sub-strains derived from IND-S, and larvae and adults of the DUB-LPM and DUB-APM respectively, two sub-strains derived from DUB-S strain.

Pirimiphos-methyl selection on larvae of the IND-LPM and DUB-LPM strains resulted in increases in tolerance of ,3.2 and 3.7-fold respectively. Similarly, adults of the IND-APM and DUB-APM strains showed increases in tolerance of 2.8 and 3.8-fold that of the parental strains. Failure of selection for pirimiphos-methyl resistance in the adults and larvae of the IND strain could be mainly due to loss of genetic variability as the result of inbreeding of the strain in the laboratory.

In spite of the use of pirimiphos-methyl in the U.A.E as an adulticide and larvicide, selection on the adults and larvae of the DUB strain resulted in only a modest increase in tolerance. Reversion of

tolerance was studied when the selected strains were released from insecticide selection. The tolerant strains reverted to susceptibility after a few generations of withdrawing the insecticide.

The larvae of selected strains showed a moderate level of cross-tolerance to malathion, temephos, and fenthion (1.4 - 2.3-fold). A greater level of tolerance was observed when the DUB-LPM strain was tested with chloropyrifos (resistance = 4.4), which was higher than pirimiphos-methyl itself (3.7). No cross-tolerance was observed when the selected larvae were tested with DDT, propoxur and permethrin. The adults of the selected strains also showed a moderate level of tolerance to malathion (1.2 - 2.1-fold). No cross-tolerance was observed when the selected strains were tested with propoxur.

The activity of mixed function oxidases in the selected and unselected strains was assessed by means of piperonyl butoxide (PB), a mixed function oxidase inhibitor. Pretreatment of the adults and larvae of selected and unselected strains with PB produced a continuous antagonism at all the doses tested. This could be attributed to the inhibition of those oxidases which are involved in the oxidative conversion of $P=S$ bond to $P=O$ during the activation of pirimiphos-methyl to a more toxic compound. There was no evidence to suggest any MFO involvement in pirimiphos-methyl detoxication. The esterase activity of selected and unselected strains was assessed using TPP, a carboxylesterase inhibitor. Only the DUB-LPM and DUB-APM strains showed a slight synergistic effect. This suggests the possibility of low esterase activity in these strains. Non-specific esterase activity of selected and unselected strains was compared. There was no obvious increase in esterase levels of selected strains compared with

unselected strains. The AchE activity of adults of selected strains was compared with the stock strain. The results indicate that there was no correlation between enzyme activity and resistance.

9.3 Genetics and mechanisms of permethrin resistance in the adults

Due to rapid knock-down action of pyrethroids and recovery of a number of knocked-down mosquitoes during the holding period in WHO standard susceptibility tests, the WHO adult susceptibility tests were carried out with some modifications. Testing and holding tubes were held in the horizontal rather than vertical position and insecticide impregnated papers such as permethrin and lambdacyhalothrin were prepared in our laboratory at 10 and 1.2 $\mu g/cm^2$.

Adult females of the DUB-S strain showed LT50's of 43 and 23.3 minutes to permethrin and lambdacyhalothrin respectively, compared with 30.6 and 18.5 minutes for the IND-S strain. The knock-down behaviour and rate of recovery of adult females were measured using permethrin and lambdacyhalothrin when the testing tubes were held in the vertical position. Knock-down was scored during the exposure time and recovery from knock-down after a period of 24 hr. Adult females of the DUB-S strain showed KT50's of 52.3 and 31.5 minutes with permethrin and lambdacyhalothrin respectively, compared with 43.3 and 24.7 minutes for the IND-S strain. Recovery from knock-down of the stock strains was determined. 7-14% recovery from knock-down was recorded when the stock strains were tested with permethrin and lambdacyhalothrin.

Permethrin selection was carried out on adult females and males of DUB-APR strain for 8 generations at a selection pressure of 70-80% mortality. Selection resulted in an increase in resistance of about 10-

fold and an increase in resistance to knock-down of 7.8-fold, compared with IND-S, a permethrin susceptible strain. Cross-tolerance and delayed knock-down of the permethrin resistant strain was determined to lambdacyhalothrin. An increase in tolerance of 3.8-fold and a delay to knock-down of 4-fold were recorded for adult females, compared with the IND-S strain. Adult selection also increased the cross-tolerance of larvae, 36.6-fold that of the IND-S strain and 3.6-fold that of the parental stock.

Inheritance of permethrin resistance in the selected strain was determined. Crossing experiments suggested that permethrin resistance is inherited as a polyfactorial partially recessive character with no indication of sex linkage.

The relationship between DDT and permethrin resistance and the role of kdr type resistance mechanism was studied in the DUB-APR strain. Piperonyl butoxide (PB), a mixed function oxidase inhibitor, and chlorofenethol (DMC), a dehydrochlorinase inhibitor, had no significant synergistic effect on DDT in DDT resistant strains. A synergistic ratio of 1.9 with PB, compared with a resistance ratio of 10, was recorded for permethrin in adult females of DUB-APR strain. This indicates that oxidative detoxication of permethrin by mixed function oxidases is not the major resistance mechanism in the adults of An. stephensi. These results raise the possibility of involvement of a kdr type resistance mechanism (target site insensitivity) as the primary mechanism for permethrin resistance.

Irritability of adult females of the IND-S and DUB-S (stock strains), DUB-APR (permethrin selected adults) and DUB-LPR (permethrin selected larvae) was determined with permethrin. The stock strains

showed no significant differences in their irritability to permethrin, but the selected strain showed significantly reduced irritability to permethrin compared with stock strains.

9.4 Genetics and mechanisms of permethrin resistance in the larvae

The larvae of the DUB-S strain were resistant to DDT and highly heterogeneous for permethrin resistance. Permethrin selection was carried out on the larvae of this strain, and the selected sub-strain was designated DUB-LPR. After only 3 generations of permethrin selection, resistance was fully developed in the larvae, 1028-fold that of the IND-S strain and 138-fold that of the parental stock. The selected strain showed cross-resistance to lambdacyhalothrin and deltamethrin, but not to DDT, because this strain was already highly resistant to DDT. Permethrin selection also produced adult tolerance.

The relationship between DDT and permethrin resistance was examined in the selected strain. Piperonyl butoxide and DMC had no significant synergistic effects on DDT resistance in the parental stock. However PB produced a strong synergistic effect on permethrin and lambdacyhalothrin in larvae of the selected strain. This suggests an oxidase-based resistance mechanism is responsible for resistance in the larvae.

Inheritance of permethrin resistance in resistant larvae was studied. Crossing experiments suggests that permethrin resistance is inherited as a monofactorial semidominant character with no indication of sex linkage.

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