AN ENHANCED TOLERANCE TO PERMETHRIN IN ANOPHELES STEPHENSI WITH PERMETHRIN AND OTHER ENZYME INDUCERS

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Abstract - Pretreatment of adults of IRAQ strain of An.stephensi with a sub-lethal dose of permethrin resulted in a 65% enhancement of tolerance to this insecticide. Similarly, pre-exposure of DUB-LPR larvae to a sub-lethal dose of permethrin resulted in increased tolerance to permethrin. For the sensitive IND-S strain, the inductive effect was smaller. The results of bioassays of resistant and susceptible An.stephensi females following pretreatment with sodium phenobarbital and subsequent exposure to permethrin indicated that sodium phenobarbital increases the tolerance level of the IND-S strain to a greater extent than the DUB-APR strain. There was some evidence of enhancement of permethrin tolerance in larvae of DUB-LPR and IND-S strains when they were treated with sodium phenobarbital. Larvae of DUB-LPR and IND-S strains pretreated with menthol-related compounds exhibited a significantly enhanced tolerance to permethrin. Treatment with a menthol - related compound induced a significant level of enhanced tolerance, as high as 3-fold in the case of larvae exposed to breeding water containing peppermint leaves in which menthol is probably the main active compound.

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Key Words: Malaria, Pyrethroids, Resistance, Enzyme Induction

INTRODUCTION

An.stephensi is an important malaria vector in the Persian Gulf, the Middle-East and the Indian subcontinent. The larvae and eggs of this species share their habitats with diverse flora and fauna. The presence of such flora and fauna may have a positive or negative effect on larval and adult development as well as on the insecticide toxicity. For example, decomposition of some vegetation in the breeding places may release plant allelochemicals which are capable of inducing detoxification enzymes in the mosquito and this may be sufficient to increase the tolerance of the insect to various insecticides. Alternatively some of these compounds may inhibit rather than induce the action of insect enzymes.

Taking a blood meal by female mosquitoes is, in most species, a necessary prerequisite for egg production. During oocyte development, hormonal regulation occurring in the body requires enzyme activation. Bladridge and Feyereisen showed that total cytochrome P-450 activity in blood-fed females of *Cx.pipiens* is different from that in non-blood-fed insects (1). It is conceivable that enhanced P-450s in blood-fed mosquitoes may detoxify insecticides. Apart from the attraction of female mosquitoes to blood sources, both sexes visit flowers for nectar and respond to floral extracts (2).

Development of resistance in the field is multidimensional. It depends on the interaction of several important factors such as potentiating (genetic, reproductive, behavioural, ecological) and operational factors. Therefore investigation on the development of resistance should ideally take into account all of these factors.

A wide variety of chemicals have been shown to elicit induction of detoxification enzymes. Those which are of interest in entomology include the allelochemicals of plants, insecticides, insect hormones and their analogues, as well as the experimentally useful barbiturate, phenobarbital.

We have established that the DUB-APR strain of *An.stephensi* is resistant to pyrethroid insecticides and this resistance is Moltifactorial. Synergist tests suggest that MFO and carboxylesterase enzymes are involved in the resistance but the target site in sensivitiy (kdr) plays a major role (Vatandoost et al., in press). The role of MFO as a mechanism of resistance to permethrin in the larvae of *An.stephensi* was developed by selection with permethrin. Synergist tests with piperonyl butoxide clearly suggest that permethrin resistance in the larvae of DUB-LPR strain is mainly due to detoxification of permethrin by MFO.

The persent study was performed to assess the possible effects of induction on the development of resistance of insects to chemical pesticides. Inducers can "turn on" the detoxyfying enzymes, enhance the existing detoxification machinery, speed the development of resistance and cause cross-tolerance to other pesticides under field conditions. The question of whether exposure of *An.stephensi* in nature, to compounds which induce detoxification enzymes, could lead to

enhanced tolerance to insecticides led to the experiments on enzyme induction reported here.

MATERIALS AND METHODS

Insecticide

Permethrin; 3-phenoxybenzyl-(1R)-cis, trans-3(2), 2-dichlorovinyl)2, 2, dimethyl cyclopropanecarboxylate, technical grade 96.2%, density = 1.2 and cis/trans ratio 40/60.

Other materials

Menthol; (5-methyl-2-[1-methlethyl] cyclohexanel), Sigma Ltd sodium phenobarbital; (5-ethyl-5-phenyl-2,4, 6-trioxohexa-hydtopyrimidine) Sigma Ltd dried leaves of peppermint (provided by the Ministry of Agriculture, Iran)

Mosquito strains

The main *An.stephensi* strains used throughout this work are as follows:

DUB-S : a wild strain based on larvae, collected from Dubai (United Arab Emirates).

DUB-LPR : a strain with larval permethrin resistance.

DUB-APR : a sub-strain from DUB-S, selected with permethrin at the adult stage.

IND-S : a strain susceptible to permethrin, and a standard laboratory colony originating from New Delhi in 1947.

IRAQ; It is susceptible to pyrethroids.

Pretreatment of mosquito with sub-lethal dose of insecticide

Adults : Females were treated with the sub-lethal dose of permethrin and after 24 hour they were exposed to permethrin at the LT50.

Larvae : The late 3rd and 4th instar larvae were treated with a sub-lethal dose of permethrin for 24 hours, followed by insecticide test (sub-lethal dose was estimated from the probit regression line of each strain, which gives approximately 5-10% mortality).

Pretreatment of mosquito with inducers to test for evidence of insecticide tolerance

Adults : It was found that 10^{-4} molar of sodium phenobarbital was not toxic for adults, hence adult female mosquitoes were given access to food from a cotton wool soaked with water solution of sodium phenobarbital, plus 10% sucrose, plus a blue dye (blue food colouring), to enable a visual check that the compound was ingested 24 h before exposure to insecticide in a bioassy.

Larvae : 10^{-8} molar sodium phenobarbital, 10^{-10} molar menthol, and 250 mg/l peppermint were applied into larval rearing trays, so that 1st, 2nd and 3rd instar of larvae were treated with inducers prior to insecticides. Standard bioassays were used to determine probit regression lines.

Statistical methods

Dosage mortality regression lines were determined by the probit analysis (3), using the Probit 75 programme on an IBM computer. Goodness of fit of the points to a straight line were tested by Chi-square (χ^2) analysis.

RESULTS

Effect of sub-lethal dose of permethrin on the tolerance level of mosquito adults to subsequent exposure

When insecticides are applied to the control of mosquitoes in their environment, it is inevitable that the target population will be in contact with varying amounts, over the whole range from negligible (usually weathered or decomposed) to freshly deposited highly toxic levels. In the field, the importance of sub-lethal effects depend on the frequency of application and the persistence of the insecticide. Sub-lethal doses of insecticides have been shown to cause latent toxicity, enzyme induction, stimulatory or inhibitory effects on reproduction, altered behavioural and insect physiology (4). These sub-lethal effects must occur in a proportion of insect populations during exposure to chemical control agents, hence such phenomena must be considered when the overall efficacy of control measures is assessed. To investigate the effect of sub-lethal doses of permethrin on tolerance levels of *An.stephensi* to the same insecticide, the IRAQ strain (permethrin susceptible) was used. One group of females were pretreated with permethrin at a sub-lethal dose (<10% mortality), and subsequently were held for 24 hour. The mortality rate was scored after 24 hours. The expected mortality having been obtained (5-10%), the remaining adults were exposed at the LT50 of permethrin and then held for another 24 hours. A second control group were not pretreated with permethrin, hence they were exposed to the LT50 of permethrin after a 24-hour holding period. The mortality rates were then scored after a further 24-hours holding period. The results of pretreatment with a sub-lethal dose of permethrin are illustrated in Figure 1. There is a significant difference between the two groups. Adults of the permethrin sensitive strain (IRAQ) that were pre-exposed to a sub-lethal dose of permethrin showed a 28% mortality (n=377) on subsequent exposure to a dose of permethrin close to the LT50, compared to a mortality of 47% in the control group (n=398); a 65% enhancement of permethrin tolerance resulted from pre-treatment

($\chi^2_{(1)}$ = 27, P< 0.0001).

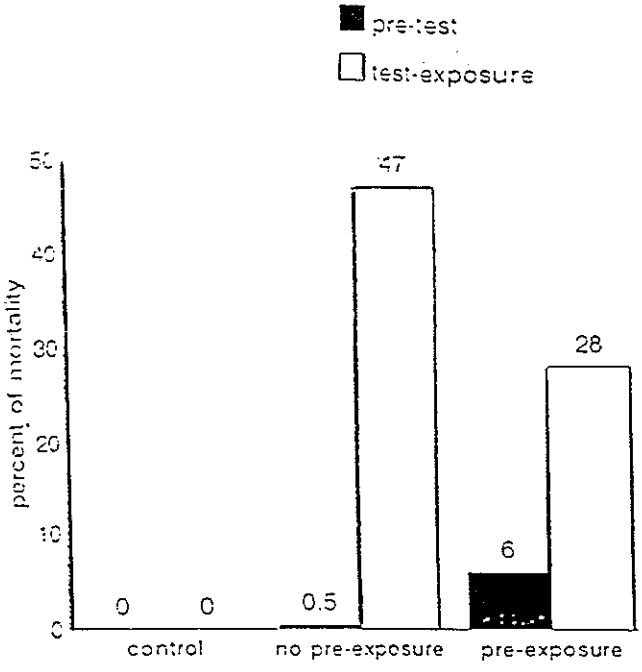

Fig. 1- Induction of enhanced tolerance in adults of IRAQ An.stephensi by pre-exposure to sublethal dose of permethrin

Effect of sub-lethal dose of permethrin on the tolerance level of mosquito larvae to subsequent exposure

In this study the DUB-LPR and IND-S strains were pretreated with sub-lethal doses of permethrin before the 4th and at the early fourth instar stage were subjected to permethrin. Pre-exposure of DUB-LPR larvae to a sub-lethal dose of permethrin resulted in a 50% increase in the LC_{50} (P<0.05) and a two fold increase in the LC_{90} (P<0.05) in a bioassay with permethrin. For the sensitive IND-S strain there appeared to be an inductive effect but it was not significant (Table 1 and Fig. 2).

Sub-lethal doses of permethrin have been found to induce enhanced tolerance in both adults (IRAQ strain) and larvae of IND-S and DUB-LPR strains. The results showed increased tolerance to permethrin in pretreated mosquitoes. Since the induced mosquito becomes more tolerant to subsequent exposure to permethrin, there are two possiblities to explain these observations. One is that pre-exposure to permethrin might result in selecting the more resistant individual within the strains. However, since permethrin was used at a concentration that killed less than 10% of exposed mosquitoes, election is most unlikely to explain the results. The other possibility is that sub-lethal doses of permethrin turn on the detoxification machinery in the mosquito and give them some protection against subsequent exposure. Vulule and co-workers (5) reported a rise in level of pyrethroid tolerance in *An.gambiae* population during a village scale trial of impregnanted nets in Kenya. However, recently they reported that long-term use of permethrin - impregnanted nets did not further increase permethrin tolerance in this population (6). The low level permethrin tolerance (2.5 - fold) in adults of this species collected from Kenyan villages could be

Table 1. The influence of pre-exposure of larave of An.stephensi to a sub-lethal dose of permethrin following by bioassay with permethrin (IR = induction ratio)

Strains	Pre-exposure with permethrin	LC_{50} (mg/l)	LC_{90} (mg/l)
DUB-LPR	+	18.1	75.5
—	13.06	36.9	
IR		1.39	2.04
P value		< 0.05	< 0.05
IND-S	+	0.16	0.59
	—	0.13	0.44
OR		1.23	1.35
P value		N.S.	N.S.

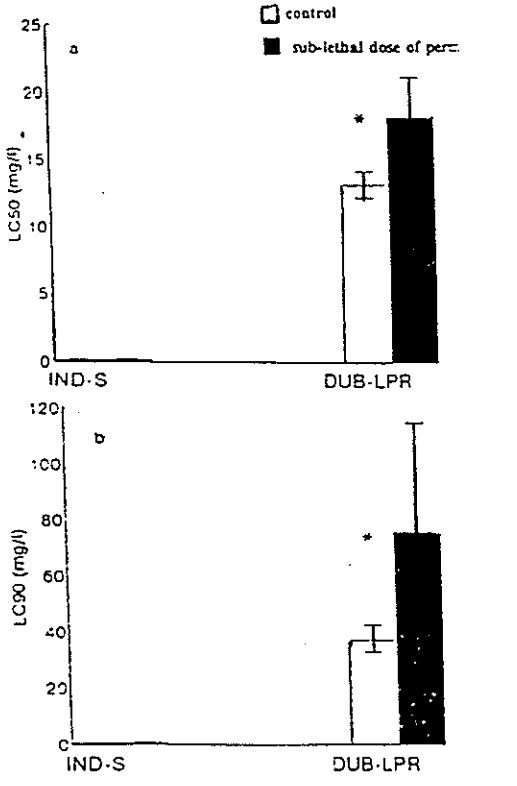

Fig. 2. The influence of sub-lethal dose of permethrin on tolerance of An.stephensi larvae to permethrin a) at the LC_{50}; b) at the LC_{90}. Asterisks indicate significant difference between control and treatment (P<0.05) Vertical bars = 95% C.I.

due to earlier contact with impregnated nets in the village causing enzyme induction from sub-lethal doses of permethrin which gave them some protection to subsequent exposure. Apart from this possible nongenetic form of permethrin tolerance, Vulule and co-workers (5) subsequently produced a strain with genetic tolerance to permethrin by laboratory selection.

Pretreatment of mosquito adults with sodium phenobarbital

Many substances have been shown to be inducers, such as phenobarbitone which induces particular P-450 isozymes. Phenobarbital induces glutathione S-transferases (7) and MFO (8) in *M.domestica.*. These two reactions are responsible for detoxification and activation and are increased in the phenobarbital pretreated housefly.

The results of bioassays of resistant and susceptible *An.stephensi* females following pretreatment with 10^{-4} molar sodium phenobarbital and subsequent exposure to permethrin in Tables 2 and Figure 3 indicate that sodium phenobarbital increases the pyrethroid tolerance level of the IND-S strain to a greater extent than the DUB-APR strain. We reasoned that the reduced effect of inducers on the resistant strain is the consequence of the initially higher enzyme activity in this strain. The phenomenon of a pyrethroid resistant strain being less sensitive to phenobarbital induction compared to susceptible files has been noted (9). They found that pretreatment of pyrethroid - resistant house flies with phenobarbital caused no, or only a small increase in cytochrome $P\text{-}450_{lpr}$, total cytochrome P-450s and P-450 monooxygenase activities. In contrast, phenobarbital increased the cytochrome P-450 content in tissues of a susceptible strains to levels similar to those found in tissues of a resistant strain. The low responsiveness to phenobarbital in tissues from resistant strains suggests that production of cytochrome P-450 may be constitutively expressed in them at near maximal level.

Table 2. The influence of pretreatment of females of *An.stephensi* to 10^{-4} M sodium phenobarbital followed y exposure to 10 $\mu g/cm^2$ permethrin

Strains	Pretreatment with sodium phenobarbital	LT_{50} (minutes)	LT_{90} (minutes)
DUB-APR	+	62.3	110.2
	–	82.0	139.2
IR		0.76	0.79
P value		N.S.	N.S.
IND-S	+	16.0	53.5
	–	10.6	23.4
IR		1.5	2.29
P value		< 0.05	< 0.01

Pretreatment of mosquito larvae with sodium phenobarbital

The MFO system is known to be inducible in the house fly by phenobarbital (7). Terriere and co-workers (8) reported that the effect of increased MFO activity on phenobarbital induction was greater in a house fly strain initially high in oxidase activity than in a strain low in this respect. The induction of MFO by phenobrabital in the house fly was affected by age, sex, strain and dose of inducer (10).

In this study there was some evidence of enhancement of permethrin tolerance in the larvae of DUB-LPR and IND-S strains when they were pretreated with 10^{-8} M sodium phenobarbital (Table 3 and Fig. 4). Pretreatment of house flies with phenobarbital provides some protection against methyl parathion, methyl paraoxon, azinphosmethyl, and methidathion toxicity. The LD_{50} value of these insecticides increased 1.86, 2.8, 1.33 and 1.95-fold, respectively in pretreated house flies (10). Our findings indicate that the detoxification reaction was accelerated by sodium phenobarbital and exceeded the activation reaction. Data obtained (11) suggested that phenobarbital induced several P-450's in the blow fly, *Phormia regina.*

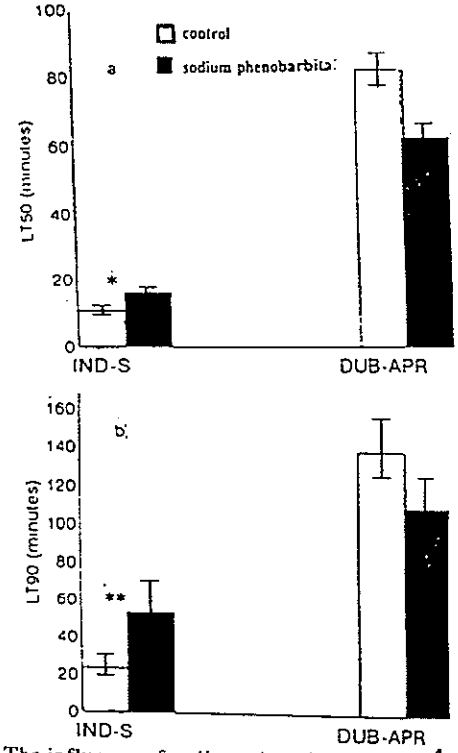

Fig. 3. The influence of sodium phenobarbital (10^{-4} M) on tolerance of *An.stephensi* females to permethrin a) at the LT_{50}; b) at the LT_{90}

Asterisks indicate significance between control and treatment ($P<0.05$, $P<0.01$)

Vertical bars = 95% C.I.

Table 3. The influence of pre-exposure of larvae of *An.stephensi* to 10^{-8} M sodium phenobarbital on subsequent tolerance to permethrin (IR = induction ratio)

Strains	Pre-exposure with sodium phenobarbital	LC_{50} (mg/l)	LC_{90} (mg/l)
DUB-LPR	+	16.3	64.8
	–	13.06	36.9
	IR		1.251.80
P value		< 0.05	< 0.05
IND-S	+	0.11	0.57
	–	0.13	0.44
IR		.88	1.30
P value		N.S.	< 0.05

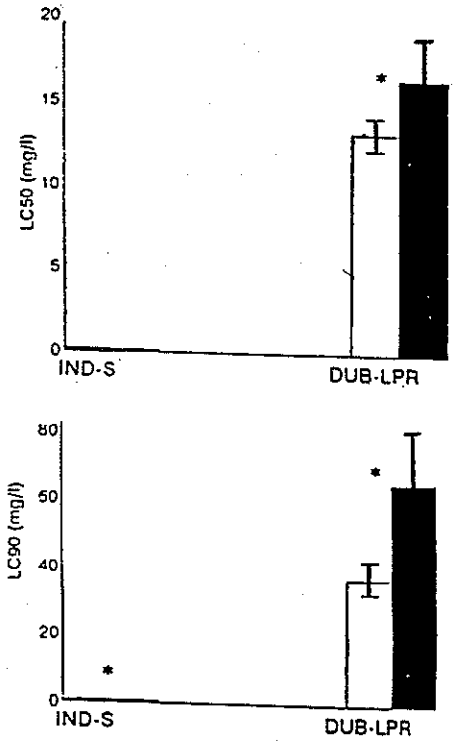

Fig. 4. The influence of sodium phenobarbital (10^{-8}M) on tolerance of *An.stepehnsi* larvae to permethrin a) at the LC_{50}; b) at the LC_{90}. Asterisks indicate significant difference between control and treatement ($P<0.05$) Vertical bars = 95% C.I.

Pretreatment of mosquito with menthol-related compounds

A wide variety of allelochemicals of plants such as menthol and menthone, are potent and effective at dietary doses in eliciting induction. The first evidence that plant allelochemicals could induce enzyme systems was presented (12); the treated larvae of *Spodoptera eridania* were shown to be less susceptible to nicotine. Tolerance to malathion was greater in larvae of *Peridrona saucia* that were fed peppermint leaves. Similar results were obtained with last instar larvae of *Trichoplusia ni* fed peppermint. Peppermint was active in stimulating aldrin epoxidase and P-450. Bioassays of larvae indicated that tolerance to carbaryl and methonyl was greater than with control larvae. This study was directed to detemine whether monoterpens such as menthol which occur in peppermint leaves enhances the tolerance of *An.stephensi* larvae to permethrin. Larvae of DUB-LPR and IND-S strains pretreated with 10^{-10} M menthol exhibited a small but significant enhancemen of tolerance to permethrin (Table 4). Pre-exposure of larvae of a sensitive strain (IND-S) to 10^{-10} M menthol produced a 1.7 - fold increase ($P<0.05$) in the LC_{50} and a 2.5 - fold increase ($P<0.05$) in the LC_{90}. For the resistant strains (DUB-LPR) the inductive effect was less pronounced (Figs. 5, 6).

According to results summarized in table 5, more enhanced tolerance was observed in larvae pretreated with 250 mg/l of peppermint leaves. When peppermint leaves were used as the inducer, the LC_{50} of the sensitive strain (IND-S) increased 2-fold ($P<0.01$) and LC_{90} 2.6 - fold ($P<0.01$). For the resistant strain (DUB-LPR) the LC_{50} increased 2.9 - fold ($P<0.01$) and the LC_{90} 3.3 - fold ($P<0.01$) (Figs. 7, 8).

Table 4. The influence of pre-exposure of larvae of *An.stephensi* to 10^{-10} M menthol on subsequent tolerance to permethrin (IR = induction ratio)

Strains	Pre-exposure with menthol	LC_{50} (mg/l)	LC_{90} (mg/l)
DUB-LPR	+	14.40	67.73
	–	13.06	36.90
IR		1.1	1.84
P value		N.S.	P< 0.05
IND-S	+	0.22	1.08
–	0.13	0.44	
IR		1.70	2.5
P value		P< .05	P < 0.05

In an experiment (13), larvae of alfalfa looper (California autographica) and cabbage looper (Trichoplusia ni) reared on peppermint indicated that stimulation of microsomal oxidase activity by the peppermint constituents provided increased tolerance for carbaryl and methomyl but not the organophosphate, acephate. The difference in insecticide susceptibility in cimparison with control larvae was attributed to increased microsomal oxidase activity as indicated by a nearly 10- fold increase in aldrin epioxidation by the midgut microsome of mint-fed larvae. Some allelochemicals of plants inhibit, rather than induce, detoxication enzymes.

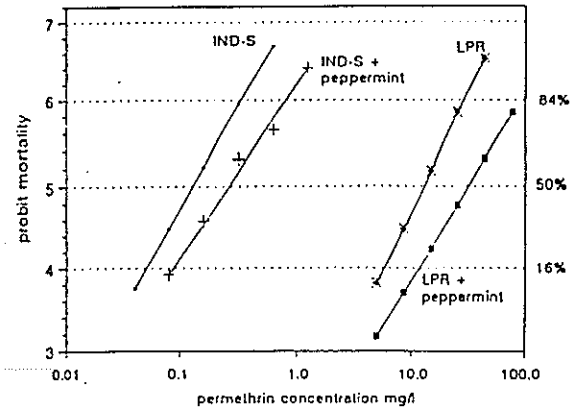

Fig. 5. Effect of pre-exposure to menthol (10^{-10} M) on the mortality of larvae subsequently exposed to permethrin

Table 5. The influence of pre-exposure of larvae of *An.stephensi* to peppermint (250 mg/l) on subsequent tolerance to permethrin (IR = induction ratio)

Strains	Pre-exposure with peppermint	LC_{50} (mg/l)	LC_{90} (mg/l)
DUB-LPR	+	37.75	122.10
	—	13.06	36.90
IR		2.9	3.3
P value		P< 0.01	P< 0.01
IND-S	+	0.27	1.16
	—	0.13	0.44
IR		2.0	2.6
P value		P< 0.01	P< 0.01

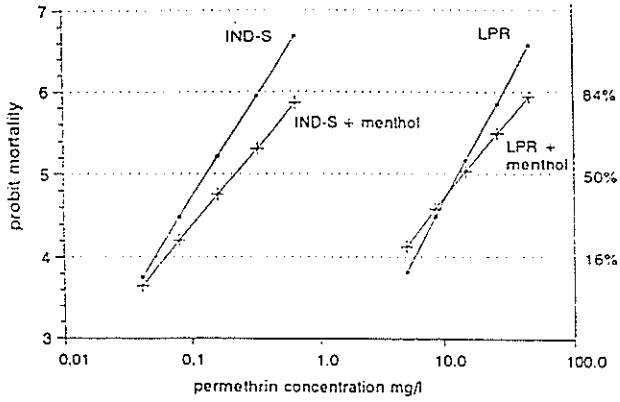

Fig. 7. Effect of pre-exposure to peppermint (250 mg/l) on the mortality of larvae subsequently exposed to permethrin

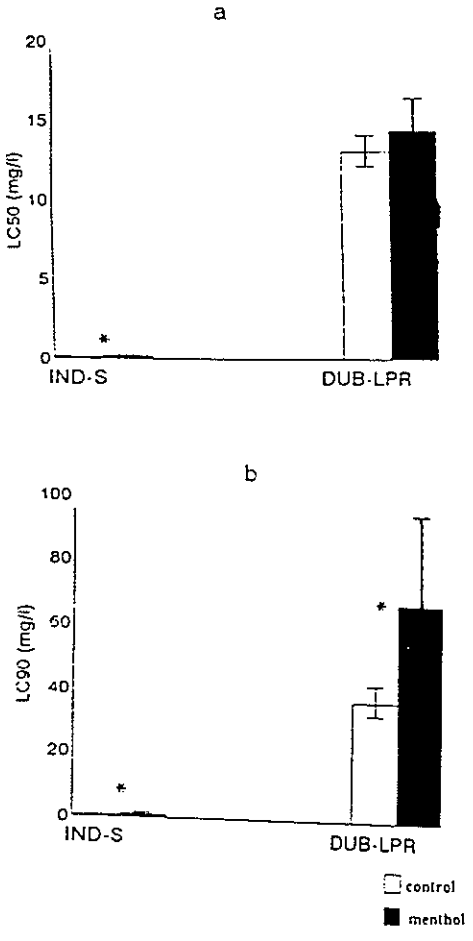

Fig. 6. The influence of menthol (10^{10} M) on tolerance of An.stephensi larvae to permethrin a) at the LC_{50}; b) at the LC_{90}. Asterisks indicate significant difference between control and treatment (P<0.05). Vertical bars = 95% C.I.

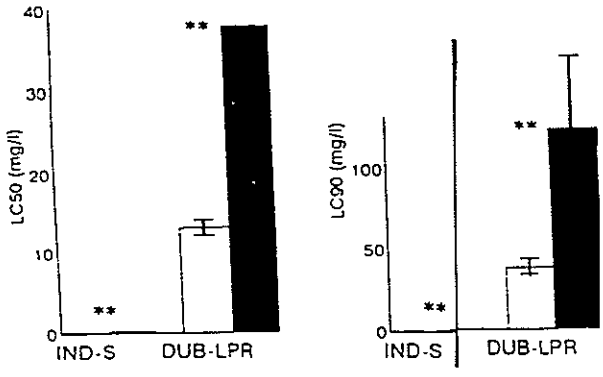

Fig. 8. The influence of peppermint (250 mg/l) on tolerance of An.stephensi larvae to permethrin a) at the LC_{50}; b) at the LC_{90}. Asterisks indicate difference between control and treatment (P<0.05, P< 0.01). Vertical bars = 95% C.I.

DISCUSSION

The treatments described here were able to induce a significant level of enhanced tolerance, as high as 3-fold in the case of larvae of DUB-LPR exposed to breeding water containing peppermint leaves. Siegfried & Young (14) found that different detoxification enzymes including general esterases, permethrin hydrolysis, total cytochrome P-450, aldrin epoxidase and glutathione S-transferases were present in aquatic insects such as black fly, damsel fly and dragon fly. They concluded that these enzyme activites in the aquatic insects were influenced by the presence of environmental contaminants in the aquatic habitats. Such influences (e.g. induction) might explain the high activites of detoxification enzymes in aquatic insects.

Some insecticides instead of being inactivated by detoxification, are made more toxic, at least temporarily, it would seem that enhancement of such metabolism could be advantageous (15). Some inducers are more potent than others. Correspondingly, we would expect differences in the amount of a pesticide required to control a given insect in different habitats. It should be noted that enzyme induction depends on species and life stage. Since a number of enzyme systems involved in insecticide resistance are known to be inducible (16), it cannot be concluded with certainty that the increased tolerance is mediated solely through enhanced P-450 activity. Further immunological and synergistic assays suggest enzyme differences between induced and non-induced mosquitoes. Overall, based on our studies, we suggest that mosquitoes possess a battery of detoxification enzymes of differing substance specificities and that these may be expressed differentially in different strains of *An.stephensi*. The ability to detoxify a given substance by reactions mediated by the detoxification enzymes may thus represent the expression of a number of different genes.

Knowledge gained in the area of induction has the potential for exploitation in vectore control programmes. The inductive effect of some inducers which are present in habitats of mosquitoes may turn on the detoxification mechanisms in insects and results in a higher pesticide degradation rate in induced insects than the non-induced populations. Enzyme induction, by enhancing the breakdown of pyrethroids in the mosquito, may have a practical effect on the response of mosquitoes to pyrethroids, particularly in aquatic environments. Although induction may only be temporary, it is conceivable that enhanced inducibility is a heritable trait and hence of selective advantage in the field. Further studies are required to clarify this phenomenon.

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